LÍVIA BORDALO TONUCCI

EVALUATION OF A PROBIOTIC FERMENTED GOAT MILK AND ITS CLINICAL APPLICATION IN TYPE 2 DIABETES MELLITUS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de Doctor Scientiae.

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APROVADA: 11 de dezembro de 2014.

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À Deus, aos diabéticos e a minha família.

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CONTEÚDO

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RESUMO

BORDALO, Lívia Tonucci, D.Sc., Universidade Federal de Viçosa, Dezembro de 2014. **Caracterização de leite fermentado caprino contendo probióticos e sua aplicação clínica no diabetes mellitus tipo 2.** Orientadora: Hércia Stampini Duarte. Coorientadores: Karina Maria Olbrich do Santos, Sônia Machado Rocha Ribeiro e Leandro Licursi de Oliveira.

A ingestão de probióticos tem sido relatada como sendo um dos métodos mais amplamente utilizados para modular a microbiota intestinal. Curiosamente, o diabetes mellitus tipo 2 tem sido associado à disbiose e uma das possíveis vias de reestabelecer a microbiota intestinal saudável é por meio da ingestão regular de probióticos, os quais vem se destacando na indústria alimentícia. Assim, o objetivo deste estudo foi, primeiramente, desenvolver um leite fermentado de origem caprina, saborizado com suco de uva, contendo probióticos e, posteriormente, avaliar o seu efeito metabólico em diabéticos. Leites fermentados contendo ou não bactérias probióticas (Lactobacillus acidophilus La-5 e Bifidobacterium animalis subsp. lactis BB-12) foram submetidos a analises físico-químicas, microbiológicas, sensoriais, além da caracterização nutricional do produto, incluindo atividade antioxidante. O teor de compostos fenólicos totais e atividade antioxidante do leite fermentado contendo probióticos foi maior ($p < 0,01$) do que o leite fermentado convencional. Observou-se uma maior perda da viabilidade celular para L. acidophilus do que para o B. animalis. No entanto, a viabilidade de todas as bactérias foi adequada ($> 10^6$ UFC/ mL) até o 28º dia de armazenamento a 4 ºC. Ambos os leites fermentados analisados apresentaram boas características sensoriais, não havendo diferença ($p > 0,05$) entre os mesmos. Um estudo duplo-cego, randomizado e placebo-controlado, incluindo 50 indivíduos diabéticos, foi desenvolvido posteriormente. Os diabéticos foram divididos em dois grupos, recebendo 120 mL/dia de uma das bebidas durante 6 semanas. Medidas antropométricas, de composição corporal, coleta de sangue e amostras fecais foram obtidos no início e ao final do estudo. A ingestão de leite fermentado contendo probióticos promoveu uma redução ($p \leq 0,05$) nos níveis de frutosamina e uma tendência à redução ($p = 0,07$) nos níveis de hemoglobina glicada. Em ambos os grupos foram observados reduções significativas nos níveis de TNF-α e resistina e a concentração fecal de ácido acético aumentou ao final do estudo, enquanto os níveis de IL-10 foi reduzida ($p < 0,001$) apenas no grupo controle. Houve diferença significativa entre os grupos em relação às alterações de

HbA_{1c}, colesterol total e lipoproteína de baixa densidade. Não houve alterações ($p > 0,05$) na capacidade antioxidante total e F2-isoprostano. Este estudo desenvolveu uma bebida funcional com boa qualidade em termos de sobrevivência de bactérias e características sensoriais e nutricionais. A ingestão regular da bebida contendo probióticos melhorou o controle glicêmico em diabéticos, no entanto, a ingestão de leite fermentado caprino saborizado com suco de uva, esteve envolvido com outras alterações metabólicas.

ABSTRACT

BORDALO, Lívia Tonucci, D.Sc., Universidade Federal de Viçosa, December, 2014. **Evaluation of a probiotic fermented goat milk and its clinical application in type 2 diabetes mellitus.** Advisor: Hércia Stampini Duarte. Co-Advisor: Karina Maria Olbrich do Santos, Sônia Machado Rocha Ribeiro and Leandro Licursi de Oliveira.

The administration of probiotics and prebiotics has been reported to be one of the most widely used approaches to modulate intestinal microbiota. Interestingly, type 2 diabetes has been associated with dysbiosis and one of the possible routes for restore a healthy gut microbiota is by the regular ingestion of probiotics, which has been highlighted in the food industry. The present study aimed, first, to develop a flavored fermented goat milk containing probiotics and assess their metabolic effect in diabetics. Fermented milk with or without probiotic bacteria (Lactobacillus acidophilus La-5 and Bifidobacterium animalis subsp. lactis BB-12) were subjected to physicochemical, microbiological and sensory analysis, besides the nutritional characterization of the product, including antioxidant activity. Total phenolic contents and antioxidant activity of probiotic flavored fermented milk were significantly higher ($p < 0.01$) than conventional fermented milk. A higher loss in cell viability was observed for L. acidophilus than for the B. animalis. However, the viability of all bacteria was adequate ($> 10^6$ cfu/mL) until day 28 of storage. The fermented milk showed good sensory characteristics and no significant sensory preference among the fermented milks was found. A double-blind, randomized, placebo-controlled study including 50 diabetic patients, was developed later. The subjects were divided into two groups, receiving 120 mL/ day one of the fermented milks for 6 weeks. Anthropometric measurements, body composition, fasting blood and faecal samples were taken at baseline and after 6 weeks of intervention. The study demonstrated a significant decrease in fructosamine levels ($p \leq 0.05$) and haemoglobin A_1c tended to be lower ($p = 0.07$) in probiotic group. TNF- α and resistin were significantly reduced and faecal acetic acid was increased in both groups after the end of trial, while IL-10 was reduced ($p < 0.001$) only in the control group. There was a significant difference between groups concerning mean changes of HbA1c, total cholesterol and low-density lipoprotein. No significant changes ($p > 0.05$) from baseline were detected in plasma total antioxidant status and F2-isoprostane. This study developed a beverage of good quality, in terms of survival of bacteria and sensory

and nutritional characteristics. Probiotic flavored fermented milk consumption improved the glycemic control in diabetic subjetcs, however, the intake of flavored fermented goat milk was involved with others metabolic changes.

EVALUATION OF A PROBIOTIC FERMENTED GOAT MILK AND ITS CLINICAL APPLICATION IN TYPE 2 DIABETES MELLITUS

GENERAL INTRODUCTION

Currently**,** there has been a progressive increase in the global prevalence of type 2 diabetes mellitus (T2DM) and its complications. According to the International Diabetes Federation, 382 million people worldwide have diabetes, with 80% of the total number affected living in low- and middle-income countries, where the epidemic is gathering pace at alarming rates. Nowadays, China and India lead the world rankings of the numbers of people with T2DM and Brazil is at the fourth place [1].

The mechanisms and factors that trigger T2DM have been subjected to intense discussion. Genetic factors, high caloric intake, and physical inactivity are well established as major risk factors for the T2DM [2]. Currently, studies aimed at investigating the importance of other factors such as gut microbiota and inflammatory and oxidative stress markers [3].

Many external factors influence the composition of the gut microbiota, especially the diet, antibiotic use, hygiene conditions and it appears that organic disease and other drugs can modulate microbiota composition [4].

The administration of probiotics and prebiotics has been reported to be one of the most widely used approaches to modulate intestinal microbiota [5]. Interestingly, among the foods whose alleged health claims, the ones with probiotic strains stand out in the food industry [6]. The dairy sector, which is strongly linked to probiotics, is the largest functional food market accounting for nearly 33% of the broad market [7].

Additionally, the use of non-bovine milk as an alternative milk product has increased lately. However, goat milk is not well accepted by many consumers, due to its typical flavor derived from caprylic, capric, and caproic acids present in this milk [8].

The supplementation of probiotic fermented milks with functional ingredients, such as fruit pulp or juice, has been proposed as a key factor in the higher consumer acceptability of goat's milk beverages [9-10], besides enhancing the functional properties of probiotic fermented milks [11]. In this way, purple grape juice contains rich flavonoids, specifically anthocyanidins and resveratrol [12-13] and evidences

indicate that these compounds have a wide range of biological functions and health benefits [14-15]. However, only a few publications on these dairy products are available in literature, and data regarding the possible impact of these fruits on the viability of the probiotic microorganism in the food product are scarce [16], as well as the action of probiotics in antioxidant activity of drinks [17].

Although the effectiveness of different probiotic strains and their formulations has been demonstrated in various intestinal disorders, such as, diarrhea, peptic ulcers, inflammatory bowel disease and colorectal cancer [18], few studies have investigated the role of probiotics in patients with diabetes [19-20].

With the increasing annual growth of industrial production of food-containing probiotics around the world [7], the interest to find out how the changes in the gut microbiota as a result of probiotics ingestion could serve as a new way of regulating metabolism in subjects with chronic diseases is increasing.

In the context of T2DM, we hypothesized that the daily intake of flavored fermented milk containing probiotics can improve glycemic control, the gut production of short chain fatty acids, and inflammatory and oxidative stress parameters, providing a generalized metabolic improvement in diabetics and contributing to greater life expectancy.

The present study aimed, first, discuss the main links between metabolic control and the gut microbiota in T2DM, and then develop a flavored fermented goat milk containing probiotics and assess their metabolic effect in diabetic subjects.

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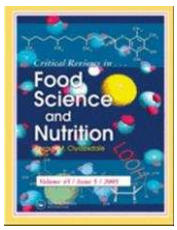
AIMS OF THE STUDY

General aim

Investigate the efficacy of the intake of a flavored fermented milk containing Lactobacillus acidophilus La-5 and Bifidobacterium animalis BB-12 on glycemic control, lipid profile, inflammation, oxidative stress and faecal short chain fatty acids in T2D subjects.

Specific aims

- Develop two flavored fermented goat milks: a conventional and other containing probiotic;
- Analyze the physicochemical, microbiological and nutritional profile of the fermented milks;
- Evaluate the acceptability of the fermented milks;
- Characterize the food consumption and nutritional status of the study participants;
- Assess the impact of the fermented milks in plasma levels of total cholesterol, LDL-C, HDL-C and triglycerides;
- Evaluate the effect of the fermented milks in glycemic control;
- Assess the baseline and pos-intervention oxidative stress by determining the levels of specific plasma biomarkers: total antioxidant capacity and the F2-isoprostane;
- Evaluate the baseline and pos-intervention inflammatory profile by determining the plasma levels of cytokines: tumor necrosis factor α, resistin, adiponectin and interleukin 6 and 10;
- Analyze the impact of the fermented milk intake on the concentration of faecal short chain fatty acids.



Article 1 – GUT MICROBIOTA AND PROBIOTICS: FOCUS ON DIABETES MELLITUS

Article accept for publication (10-june-14) in Critical Reviews in Food Science and Nutrition (Impact Factor: 5.548) (Appendex I).

ABSTRACT

TONUCCI, Lívia Bordalo, D.Sc., Universidade Federal de Viçosa, December, 2014. **Gut microbiota and probiotics: focus on diabetes mellitus**. Advisor: Dra. Hércia Stampini Duarte. Co-Advisor: Dra. Karina Maria Olbrich do Santos, Dra. Sônia Machado Rocha Ribeiro and Dr. Leandro Licursi de Oliveira.

The characterization of gut microbiota has become an important area of research in several clinical conditions, including type 2 diabetes. Changes in the composition and/or metabolic activity of the gut microbiota can contribute to human health. Thus, this review discusses the effects of probiotics and gut microbiota on metabolic control in these individuals. Relevant studies were obtained from electronic databases such as PubMed/Medline and ISI Web of Science. The main probiotics used in these studies belonged to the genera Lactobacillus and Bifidobacterium. We found seven randomized placebo-controlled clinical trials and thirteen experimental studies directly related to the effect of probiotics on metabolic control in the context of type 2 diabetes mellitus. The hypothesis that gut microbiota plays a role in the development of diabetes indicates an important beginning, and the potential of probiotics to prevent and reduce the complications of diabetes was better observed in animal studies. In clinical trials, the use of probiotics in glycemic control presented conflicting results, and only few studies have attempted to evaluate factors that justify metabolic changes, such as markers of oxidative stress, inflammation, and incretins. Thus, further research is needed to assess the effects of probiotics in the metabolism of diabetic individuals, as well as the main mechanisms involved in this complex relationship.

Keywords: Gut Microbiota, type 2 diabetes, probiotics, oxidative stress, inflammation.

Introduction

Currently, there has been a progressive increase in the global prevalence of type 2 diabetes mellitus (T2DM) and its complications. According to the World Health Organization (WHO), 346 million people worldwide have diabetes (World Health Organization, 2012). On an average, 8% of adults living in developed cities and more than 10% living in developing countries are diagnosed with T2DM. Nowadays, China and India lead the world rankings of the numbers of people with T2DM and Brazil is at the fifth place (Scully, 2012).

The mechanisms and factors that trigger T2DM have been subjected to intense discussion. Genetic factors, high caloric intake, and physical inactivity are well established as major risk factors for the T2DM (Lyssenko et al., 2008). Currently, studies aimed at investigating the importance of other factors such as gut microbiota, and inflammatory and oxidative stress markers (Andersson et al., 2010; Lin et al., 2014).

Gut microbial composition among healthy humans is complex and the distribution of microorganisms throughout the gastrointestinal tract is not homogenous. The colon provides optimal conditions for the growth of microorganisms due to absence of digestive secretions, slow peristalsis and abundant nutritional supply (Neish, 2009; Qin et al., 2010).

Many external factors influence the composition of the gut microbiota, especially the diet, antibiotics, hygiene conditions and it appears that organic disease and other drugs can modulate microbiota composition (Claesson et al., 2012).

The unbalance gut microbiota, known as dysbiosis, seems to be able to influence the metabolism of the host promoting susceptibility to metabolic disorders such as insulin resistance (Cani et al., 2007b) and others components of metabolic syndrome (Petruzzelli and Moschetta, 2010; Vijay-Kumar M et al., 2010). Differences in the composition of the adult intestinal microbiota among those with diabetes mellitus and control subjects (Larsen et al., 2010; Qin et al., 2012) suggest that the composition of intestinal microbiota may influence the energy extraction of ingested foods, mucosal immunity, permeability and transit-time intestinal and systemic inflammation (Backhed et al., 2004; Cani and Delzenne, 2007; Gravitz, 2012). These factors have also been highlighted as triggers in the development and progression of T2DM and its complications (Ceriello and Motz, 2004; Dandona et al., 2004; Larsen et al., 2010; Stephens et al., 2009).

Interestingly, administration of probiotics and prebiotics has been reported to be one of the most widely used approaches to modulate intestinal microbiota and may subsequently prevent or delay diabetes incidence (Jacobsen et al., 1999; Cani et al., 2009a; Cani et al., 2009b). This possibility was first demonstrated in a study in mice supplemented with prebiotics. These mice exhibited increased levels of Gram-positive Bifidobacterium spp., which were associated with improved glucose tolerance and decrease in inflammation (Cani et al., 2007a). Additionally, these investigators demonstrated that intestinal Gram-negative bacteria produced lipopolysaccharide (LPS), which is a well-known proinflammatory molecule, can translocate to the bloodstream from a leaky gut, and causes metabolic endotoxemia (Cani et al., 2007b; Cani et al., 2008).

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defined probiotics as "live micro-organisms", which when administered in adequate amounts confer a health benefit on the host (FAO and WHO, 2002). The bacterial genera most commonly used in probiotic preparations are Lactobacillus and Bifidobacterium.

Many clinical trials examining the effects of different probiotic strains and their formulations on various intestinal disorders such as diarrhea, peptic ulcers, inflammatory bowel disease, colorectal cancer, atopic dermatitis, and allergies have shown a positive influence (Ritchie and Romanuk, 2012). However, few studies have investigated the role of probiotics in patients with diabetes.

With the increasing annual growth of industrial production of food-containing probiotics around the world, the interest to find out how the changes in the intestinal microbiota as a result of probiotics ingestion could serve as a new way of regulating metabolism in subjects with chronic diseases is increasing.

The present review discusses the effects of probiotics on metabolic control in T2DM subjects as well as the main mechanisms involved, with an emphasis on the involvement of gut microbiota, to better understand its clinical application, effectiveness, and safety.

Gut microbiota and diabetes mellitus

The human gut houses trillions of bacteria representing more than 500 species belonging to four major phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Among them, ~ 60% of the total gut bacteria belong to the phylum

Firmicutes, with more than 250 genera of Gram-positive bacteria, including Lactobacillus, Streptococcus, Staphylococcus, Clostridium, and Mycoplasma. Among the phylum Actinobacteria (Gram-positive bacteria), the major genus found is Bifidobacterium and related to Gram-negative bacteria, Bacteroidetes and Proteobacteria represent 15 and 1%, respectively, and highlight the genera Bacteroides and Prevotella (Eckburg et al., 2005; Gill et al., 2006).

Recently, the presence of three main enterotypes has been reported to characterize the gut microbiome according to their co-occurrence in healthy adult European, North American, and Japanese subjects. The changes in the levels of Bacteroides (phylum Bacteroidetes, enterotype 1), Prevotella (phylum Bacteroidetes, enterotype 2), and Ruminococcus (phylum Firmicutes, enterotype 3), and the respective classes of microorganisms that use different routes for energy generation have been suggested to affect their synergistic relationship with the human host. However, the three enterotypes were not found to be significantly correlated with age, sex, body mass index (BMI), or nationality, except enterotype 1, which were more in the Japanese subjects (Arumugam et al., 2011).

In 2004, Fredrik Bäckhed et al. found that germ-free mice had lower body weight, when compared with conventional mice. However, transplantation of feces of the conventional mice into the germ-free ones induced weight gain and decreased the glycemic control in germ-free mice (Backhed et al., 2004). These results were also observed in obese humans and patients with metabolic syndrome, who, after 6 weeks of allogeneic or autologous fecal microbiota transplant from normal individuals, exhibited an improvement in insulin sensitivity (Vrieze et al., 2012). Since then, the study of gut microbiota in models of diabetes and obesity, either in animals or humans, has attracted the interest of many researchers (Gravitz, 2012).

Studies that characterize the gut microbiota of diabetics and evaluate the possible correlations between the abundance of certain groups and metabolic aspects are fundamental to clarify and strengthen the role of microbiota in this clinical condition. Some studies have reported that patients with T2DM have a high number of opportunistic pathogenic bacteria (Clostridium clostridioforme, Bacteroides caccae, Clostridium hathewayi, Clostridium ramosum, Clostridium symbiosum, Eggerthella sp., and Escherichia coli) and a low number of butyrate-producing bacteria (Clostridiales spp. SS3/4, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, and Roseburia inulinivorans) (Karlsson et al., 2013; Larsen et al., 2010; Qin et al.,

2012). The increase in Roseburia spp. and butyrate levels have been reported to be associated with improved insulin sensitivity (Vrieze et al., 2012). These evidences suggest the importance of butyrate-producing bacteria in glycemia regulation. Moreover, these bacteria potentially stimulate bacterial defense mechanisms against oxidative stress (Qin et al., 2012).

Larsen et al. (2010) found a low number of bacteria belonging to the phylum Firmicutes and a high number of Betaproteobacteria in diabetic patients, when compared with those in non-diabetics. The Betaproteobacteria levels were positively correlated with the plasma glucose levels. In addition, the ratios between Firmicutes and Bacteroidetes, Bacteroidetes and Prevotella spp., and Clostridium coccoides and E. rectale were observed to be higher in diabetic patients and positively and significantly correlated with plasma glucose, but not with the BMI. Thus, these bacteria were more specific to T2DM than obesity. A previous study showed that the decrease in the number of Prevotella spp. was significantly associated with the decrease in metabolic endotoxemia and inflammation in ob/ob mice with glucose intolerance (Cani et al., 2008). On the other hand, the diabetes progression was observed to increase in association with the decrease in the number of Firmicutes and Bacteroidetes over time (Giongo et al., 2011).

Recently, gut dysbiosis and possible blood bacterial translocation were reported in patients with type 2 diabetes and high rate of gut bacteria in the circulation suggested translocation of bacteria from the gut to the bloodstream. In this study, the counts of the Clostridium coccoides group, Atopobium cluster, and Prevotella were significantly lower, while the counts of total Lactobacillus were significantly higher in fecal samples of diabetic patients than in those of control subjects. Especially, the counts of Lactobacillus reuteri and Lactobacillus plantarum subgroups were significantly higher (Sato et al., 2014).

Different bacterial phyla exert different effects on carbohydrate metabolism and have the ability to influence glycemic control. Carbohydrates are an essential dietary component for mammals as well as their gut microbiota. Mammals absorb simple sugars in the proximal jejunum and hydrolyze disaccharides (sucrose, lactose, and maltose) in their monosaccharide constituents or degrade starch into monosaccharides. However, they have limited ability to hydrolyze other polysaccharides (Hooper et al., 2002). As a result, undigested polysaccharides (cellulose, xylan, and pectin) and

partially digested starches reach the gut microbiota in the distal gut, where they are metabolized by bacterial enzymes (Musso et al., 2011).

Monosaccharides hydrolyzed from polysaccharides are converted by bacteria to pyruvate through glycolysis, resulting in the production of adenosine triphosphate (ATP). In a highly anaerobic environment of the intestinal lumen, distal carbons and greater energy are obtained from microbial fermentation of pyruvate. To recover the nutritional value of the degraded polysaccharides, mammals have developed mechanisms for the uptake and utilization of products of bacterial fermentation such as short-chain fatty acids (SCFA), the main end product of bacterial fermentation.

Acetate, propionate, and butyrate have different metabolic pathways. Butyrate is the preferred energy source for the epithelial cells of the colon, where it is converted to ketone bodies or oxidized to carbon dioxide (Louis et al., 2007). The colonic epithelium takes 60–70% of energy from butyrate (Louis and Flint, 2009). Propionate and acetate are absorbed into the hepatocytes, which use most of these compounds for gluconeogenesis and lipogenesis. The SCFA have important effects on other aspects of intestinal physiology: they decrease the pH of the proximal colon and significantly affect the composition of the colonic microbiota. At pH 5.5, a higher concentration of ethyl butyrate was observed in the colon, when compared with that at pH 6.5. Furthermore, at pH 6.5, higher propionate production was noted, which induced changes in the composition of the microbiota, reducing the number of Roseburia spp. and increasing the number of Bacteroides spp. (Walker et al., 2005).

It is also noteworthy that the SCFA can improve insulin sensitivity owing to its metabolic effects in reducing the levels of free fatty acids through inhibition of phosphorylation of insulin receptor substrate. In addition, butyrate supplementation has been reported to improve glycemic control and insulin resistance in C57BL/6J mice (Gao et al., 2009). Thus, the SCFA, so far considered only as an indirect nutrient supplying energy to the bacteria and intestinal enterocytes, are regarded as regulators of other metabolic processes.

Firmicutes are usually involved in the transport of nutrients, and facilitate the absorption and fermentation of SCFA in non-digestible carbohydrates (Turnbaugh et al., 2009). Among the main phenotypic characteristics of Bifidobacterium spp., production of lactic acid and acetic acid as the main products of carbohydrate utilization in bowel is noteworthy (Ishibashi et al., 1997).

The following paragraphs briefly present a review of the efficacy of probiotics in the management of T2DM in vivo conditions. Studies included in this systematic review were conducted with people already diagnosed with diabetes or animals models of diabetics. To broaden the search, additional articles were sought from the references cited in the selected articles. To confirm the number of experimental studies and clinical trials in each database (PubMed/Medline and ISI Web of Science), we selected the advanced search options "clinical trial" or "animal species" after typing the descriptors. The keywords searched were: Gut microbiota, Probiotics, Lactobacillus, and Bifidobacterium, used individually or in combination with Insulin sensitivity, Diabetes, Insulin resistance, Oxidative stress, and Inflammation.

Primary effects of the gut microbiota on host metabolism

Currently, studies have been focused on understanding how the gut microbiota can influence the host metabolism and thus contribute to the development of diabetes and its complications. The metabolites derived from bacterial metabolism and reactions of bacterial cells with the host immune system represent the triggers for the development of metabolic abnormalities (Gravitz, 2012). Furthermore, changes in the gut microbiota can influence the levels of gut hormones involved in the regulation of satiety and glycemic control, such as glucagon-like peptide-1 (GLP-1) (Baggio and Drucker, 2007; Tolhurst et al., 2012; Yadav et al., 2013). The GLP-1 is a hormone secreted by the L-cells of the small intestine and distal colon, which produces antidiabetic effect by stimulating insulin secretion from pancreas, which can be modulated by SCFA, particularly, butyrate, after the intake of prebiotics (Tolhurst et al., 2012; Yadav et al., 2013). In addition, in a previous study, the consumption of probiotics by diabetic rats was observed to increase the bioavailability of gliclazide, an oral sulfonylurea class antidiabetic drug (Al-Salami et al., 2008a).

Probiotic consumption has also been observed to decrease the oxidative stress and inflammatory markers. This is an interesting effect because individuals with diabetes often exhibit changes in the levels of these markers, which are known to be involved in some way in glycemic control, and thus, in the pathogenesis and progression of T2DM (Ceriello and Motz, 2004; Stephens et al., 2009). Inflammation and oxidative stress may be induced by bacterial components such as lipopolysaccharide (LPS), the major component of the extracellular membrane of Gram-negative endotoxin. The increase in the circulating levels of endotoxin is currently

known as "metabolic endotoxemia," and can occur owing to changes in intestinal permeability. The decrease in the number of Bifidobacterium spp. and Lactobacillus spp. in the intestine, together with the derangement of intestinal cell junctions, have been noted to increase the intestinal permeability (Cani et al., 2007b; Cani et al., 2008), suggesting the role of microbiota in modulating intestinal permeability. Figure 1 shows the major mechanisms involving the intestinal microbiota and the main metabolic changes observed in diabetic subjects.

The general protective mechanisms involving probiotics, which are the most commonly reported are as follows: competition among pathogenic microorganisms for intestinal epithelial receptors; release of antimicrobial compounds that fight pathogens, such as SCFA (lactic, acetic, and propionic acids), hydrogen peroxide, free fatty acids, and bacteriocins; stimulation of mucin secretion for binding of probiotics to the intestinal mucosa and hindering pathogens; and stimulation of host immunity by inducing the production of interleukins as well as stabilization and improvement of the intestinal barrier associated lymphoid tissue (O'Hara AM and Shanahan F, 2006; Williams NT, 2010). Thus, the biological activity of the probiotic bacteria is partly associated with its ability to adhere to enterocytes, which inhibits the binding of enteric pathogens through competitive exclusion. The attachment of probiotic bacteria to the cell surface receptors of enterocytes also initiates signaling events, resulting in the production of cytokines. Moreover, butyric acid production by some probiotic bacteria affects the turnover of enterocytes and neutralizes the activity of carcinogens such as nitrosamines (Kailasapathy and Chin, 2000).

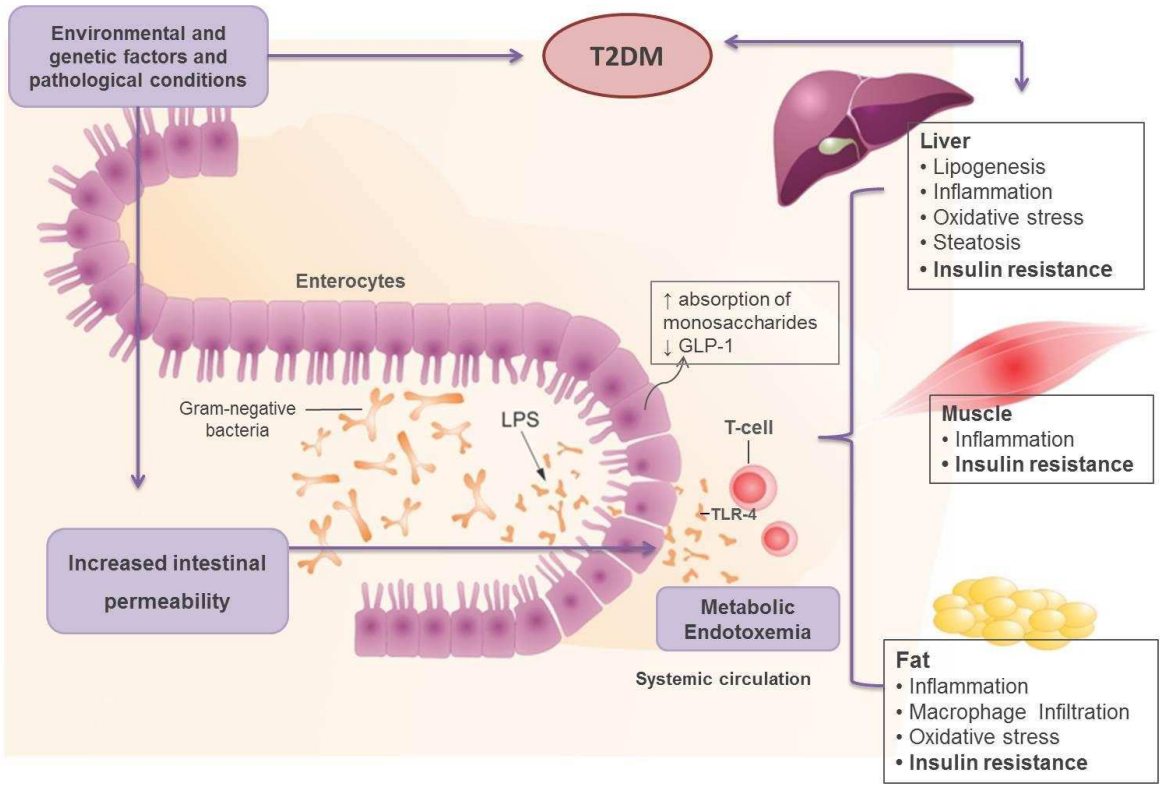


Fig 1. The low grade, systemic and chronic inflammation often associated with metabolic diseases, such as T2DM, developed due to influences of the gut microbiota. The origin of metabolic diseases is multifactorial and new evidences have demonstrated that gut microbiota could be an importante factor in the development of metabolic diseases. The diet is able to influence intestinal microbiota composition, increasing level of Gram-negative bacteria, and also the absorption of molecules from the intestinal lumen. These bacterias can influence the assembly of proteins from the tight junctions in intestinal epithelium, leading to an increased intestinal permeability (leaky gut). This in turn may favor the translocation of lipopolysaccharides (LPS), a component of gram-negative cell wall, from the intestinal lumen to the circulation. Once in the circulation, LPS can bind and activate toll-like receptor-4 expressed by different cell types (immune cells, adipocytes and Kupfer cells), triggering inflammatory responses such as the release of cytokines (TNF-α, IL-6) and oxidative stress. In consequence, insulin signaling is impaired, leading to the development of insulin resistance. An increased ability to ferment substrates from the diet may augment the availability and absorption of monosaccharides and short chain fatty acids. Microbiota can also influence the secretion of hormones such as decreasing the glucagon-like peptide (GLP-1), which is well known to increase insulin sensitivity.

Main effects of probiotics consumption in T2DM

Experimental studies

Studies using animal models of diabetes were the first to show that different species of bacteria such as Lactobacillus acidophilus and Lactobacillus casei reduce oxidative stress and exhibit antidiabetic effect (Harisa GI et al., 2009; Yadav et al., 2007; Yadav et al., 2008). In diabetic animal models, oxidative stress caused by the accumulation of free radicals leads to the damage of multiple tissues, including the β-cells of the pancreas, as well as to the distortion and dysfunction of several organs, including the liver and kidney (Hamden et al., 2008).

In non-diabetic C57BL/6J mice fed on fat diet was demonstrated that Lactobacillus rhamnosus GG (10^8 CFU/mL) administered for 13 weeks was able to achieve glycemic control by enhancing insulin sensitivity and increasing GLUT4 expression and adiponectin production (Kim et al., 2013). Similar results were also observed in diabetic mice using the same strain of bacteria administered for 6 weeks (Honda et al., 2012).

Male Sprague–Dawley rats were fed a high-fructose diet with or without Lactobacillus reuteri GMNL-263 administration for 14 weeks. The levels of serum glucose, insulin, leptin, C-peptide, glycated hemoglobin, GLP-1, liver injury markers, lipid profile in serum and liver were significantly increased in high-fructose-fed rats. However, after Lr263 administration, the elevation of these parameters was significantly suppressed. Furthermore, concentrations of IL-6 and TNF-α in adipose tissue which were elevated in high fructose treatment were markedly decreased after Lr263 feeding and decreased levels of PPAR-γ and GLUT4 mRNA after high fructose treatment were significantly enhanced by Lr263 administration. Interestingly, Lr263 consumption significantly increased the number of Bifidobacterium spp. and Lactobacillus spp., and on the contrary, decreased the number of Clostridium spp. in the feces of treated rats (Hsieh et al., 2013). These results implicated that L.reuteri administration might exert its therapeutic effect on diabetes via increasing the beneficial as well as decreasing the harmful gut microbiota species. However, the relevant mechanisms underlying this phenomenon needed further investigation.

In another study, the effect of dahi, a fermented milk product containing Lactobacillus acidophilus NCDC14 and L. casei NCDC19 (10^8 CFU/g – final product) on progression of streptozotocin (STZ)-induced diabetes in rats for 28 days was investigated. Feeding of probiotic dahi significantly suppressed STZ-induced oxidative

damage in pancreatic tissues by inhibiting the lipid peroxidation and formation of nitric oxide, and preserving antioxidant pool such as glutathione content and activities of superoxide dismutase, catalase and glutathione peroxidase (Yadav et al., 2008). The feeding of the same probiotic dahi to the fructose-induced diabetic rats significantly decreased the blood glucose and glycosylated haemoglobin, free fatty acids and trigycerides (Yadav et al., 2007).

Interestingly, L. acidophilus, Bifidobacterium lactis (strain not informed) and L. rhamnosus GG have been reported to reduce blood glucose levels and further improve the bioavailability of gliclazide, a sulphonylurea drug used to treat T2DM in alloxan-induced diabetes rats (Al-Salami et al., 2008a). A possible explanation for the increase in gliclazide systemic absorption is that probiotics activate the efflux drug transporter, Mrp2 (Al-Salami et al., 2008b).

Recently, the oral administration of Lactobacillus plantarum TN627 was noted to significantly improve the immunological parameters, reduce the pancreatic and plasmatic a-amylase activities and level of plasma glucose in Alloxan-induced diabetic rats. Furthermore, this probiotic treatment was observed to markedly reduce serum triglyceride and LDL-cholesterol rates and to increase the level of HDL-Cholesterol (Bejar et al., 2013). In a different previous study, a synbiotic product containing Enterococcus faecium CRL 183 or Lactobacillus helvetians 416 (10^8 - 10^9 CFU/mL each) and 1 mL/kg body weight/day of soybean and yacon extract (yacon of 60.00% to 25.86%) for 7 weeks, observed improvement in the lipid profile, but no effects on glycemic control in diabetic animals (Roselino et al., 2012). Improvement in the lipid profile, even if not accompanied by improved glycemic control, may be important effect in patients with T2DM.

A dose-dependent effect on control glycemic has also been reported after ingestion of probiotics. The blood glucose levels decreased from 4480 to 3620 mg/L (with 10^7 CFU/d) and 3040 mg/L (with 10^9 CFU/d) in STZ-induced DM animal models treated with L. reuteri GMN-32. Probiotic treatment also reduced the changes in the heart caused by the effects of DM (Lin et al., 2014).

The main features and results obtained in studies involving diabetic animals are shown in Table 1. The study period ranged from 2 to 14 weeks. Lactobacillus spp. was the most evaluated in the experimental studies. In some cases, the results regarding glycemic control were dependent on the strain used, although in most of the studies, an improvement in some parameters (insulin, glucose, or glycated hemoglobin) related to

glycemic control was observed. Honda et al., (2012) showed that only L. rhamnosus GG, and not Lactobacillus bulgaricus LB3, was able to improve glycemic control in fasting and postprandial diabetic mice.

Table 1 Experimental studies on the effects of probiotic consumption on glycemic control in diabetic animals

References	Animals models	Probiotic Strain / dose	Duration (wk)	Main outcomes after ingestion of probiotics
(Matsuzaki et al., 1997)	KK-A^y mice	L. casei (0.05%)	16	↓ blood glucose and insulin ↓ IL-2 and INF-y
(Tabuchi et al., 2003)	Streptozotocin	L. rhamnosus (CFU - not reported)	9	↓ HbA_{1c} and improvement in glucose tolerance ($P < 0.05$)
(Yadav et al., 2007)	Rats/ High-fructose diet	L. acidophilus and L. casei (CFU and strain - not reported)	8	Lower elevations in: HbA_{1c}, insulin, blood glucose, TC, TG, LDL-C and NEFA ($P < 0.05$) HDL-C decreased slightly Lower values of thiobarbituric acid-reactive substances and higher values of reduced glutathione in liver and pancreatic tissues ($P < 0.05$)
(Yadav et al., 2008)	Rats/ Streptozotocin	L. acidophillus NCDC14 L. casei NCDC19 (10^8 CFU/mL)	4	Suppressed the incremental peaks and area under the curve and delayed reduction of insulin secretion during OGTT Suppressed STZ-induced oxidative damage in pancreatic tissues by inhibiting the lipid peroxidation and formation of nitric oxide, and preserving antioxidant pool such as GPx, SOD and catalase ↓ TC, TG, LDL-C and VLDL-C; ↑ HDL-C levels ($P < 0.05$)

(Harisa GI et al., 2009)	Rats/Streptozotocin	L. acidophilus or L. acidophilus + acarbose (10^7 CFU/mL) (strain not reported)	2	↓ fasting blood glucose, HbA_{1c}, TG and MDA ($P < 0.001$) after administration of Lactobaillus alone or with acarbose.
(Yun et al., 2009)	db/db mice	L. gasseri BNR17 or rosiglitazone (10^{10} CFU/mL)	12	↓ fasting and postprandial (2h) glucose levels ($P < 0.05$) after intake of L. gasseri and rosiglitazone ($P < 0.001$) ↓ HbA_{1c} ($P > 0.05$)
(Roselino et al., 2012)	Rats/Streptozotocin	Enterococcus faecium CRL 183 + L. helveticus 416 + soybean and yacon extract (60%) (10^8- 10^9 CFU/mL of each microorganism)	7	No change was observed in blood glucose ↑ 23.7% in HDL-C and ↓ of 33.5% in TG levels in symbiotic groups
(Honda et al., 2012)	KK-A^y mice	L. rhamnosus GG ou L. bulgaricus LB3 (0.5%)	6	↓ fasting and postprandial blood glucose and HbA_{1c} ($P < 0.05$) of the L. rhamnosus group
(Bejar et al., 2013)	Rats/alloxan	L. plantarum TN627 (10^9 CFU/mL)	4	↓ plasma glucose ($P < 0.05$) ↓ serum TG and LDL-C ($P < 0.05$) ↑ HDL-C ($P < 0.05$)

(Kang et al., 2013)	Mice/ High-sucrose diet (50%)	L. gasseri BNR17 (10^{10} CFU/mL)	10	↑ GLUT4 mRNA expression ↓ leptina ($P < 0.01$) and insulin ($P < 0.05$) ↓ body weight and white adipose tissue weight ($P < 0.01$)
(Hsieh et al., 2013)	Rats/ High-fructose diet	L. reuteri GMNL-263 (10^{9} CFU/mL)	14	↓ fasting blood glucose, insulin, leptin, C-peptide, HbA_{1c}, GLP-1, liver injury markers, lipid profile in serum and liver ↓ IL-6 and TNF-α in adipose tissue ↑ PPAR-γ and GLUT4 mRNA expression ↑ number of Bifidobacterium and Lactobacillus and ↓ Clostridium in faeces
(Lin et al., 2014)	Rats/Streptozotocin	L. reuteri GMN-32 (10^{7} and 10^{9} CFU/day)	4	↓ blood glucose ($10^{9} > 10^{7}$ CFU) ↓ the changes in the heart caused by the effects of DM.

KK-Ay, a model of genetic type 2 diabetes. IL-2, interleucin 2. INF-y, interferon-y. HbA_{1c}, glycosylated hemoglobin. TC, plasma total cholesterol. TG, triacylglycerol. LDL-C, low-density lipoprotein cholesterol. NEFA, free fatty acids. HDL-C, high-density lipoprotein cholesterol. STZ, streptozotocin. OGTT, oral glucose tolerance test; GPx, glutathione. SOD, superoxide dismutase. MDA, malondialdehide. GLP-1, glucagon like peptide. IL-6, interleucin 6. TNF- α, tumor necrosis factor α. PPAR - γ, peroxisome proliferator-activated receptor γ; GLUT4, glucose transporter 4. ↑, increase; ↓, decrease.

Clinical trials

A few clinical trials assessing the effects of probiotics on diabetics have been reported (Table 2). Initially, studies were performed in healthy subjects (Naruszewicz et al., 2002; Songisepp et al., 2005). The effects of probiotics on glycemic control have also been reported in pregnant women, who are predisposed to changes in glucose metabolism. Luoto et al. (2010) reported a lower prevalence of gestational diabetes in the pregnant group (n = 256) who received L. rhamnosus GG and Bifidobacterium lactis BB-12 (10^{10} CFU/d each) taken from the first trimester of pregnancy to the end of exclusive breastfeeding, with a significant difference (P = 0.003), when compared with the control group (36%) who received normal diet according to the dietary guidelines. No adverse effects were observed in relation to probiotics consumption throughout the pregnancy. On the other hands, probiotic capsule treatment (Lactobacillus salivarius UCC118, 10^9 CFU/d) of 4 weeks during pregnancy did not influence maternal fasting glucose, the metabolic profile, or pregnancy outcomes in 175 obese women (Lindsay et al., 2014).

Recently, a randomized controlled trial with 156 overweight men and women does not support the hypothesis that L. acidophilus La-5 and B. animalis subsp lactis BB-12 for 6 weeks, either in isolated form or as part of a whole food (yoghurt), benefit short-term glycaemic control. Indeed, there is weak data for an adverse effect of these strains on glucose homoeostasis (Ivey et al., 2014).

Table 2 Clinical trials on the effects of probiotic consumption on the metabolism of diabetics subjects

References/ Design	Sample	Strain / daily dose	Duration (wk)	Main outcomes after ingestion of probiotics	Methodological limitations
(Andreasen et al., 2010) R, PC, DB	IG: 21 CG: 24	L. acidophilus NCFM (10^{10} CFU) in capsule form	4	L.acidophilus was detected in the faeces of 75% of the probiotic group. Insulin sensitivity (clamp) was preserved only in probiotic group ($P < 0.05$). No change was observed ($P > 0.05$) in IL-6, TNF-α, CRP, IL-1 and insulin plasma levels.	Inclusion of individuals with newly diagnosed T2DM and normal tolerance glucose. Not evaluated the characteristics of the diet during the study.
(Ejtahed et al., 2011) R, PC, DB	IG: 28 CG: 28 (DM2; LDL-C ↑)	L. acidophilus La-5 and B. lactis BB-12 in yogurt (10^6 CFU)	6	↓ levels of TC and LDL-C ($P < 0.05$) No change was observed in HDL-C plasma levels ($P > 0.05$).	-
(Ejtahed et al., 2012) R, PC, DB	IG: 30 CG: 30	L. acidophilus La-5 and B. lactis BB-12 (10^{10} CFU) in yogurt	6	↓ FPG ($P < 0.01$) and HbA_{1c} ($P < 0.05$). ↓ SOD, GPx and TAC ($P < 0.05$). ↓ MDA ($P < 0.05$) compared with the baseline value in both groups. No significant changes from baseline were shown in insulin and CAT.	-

(Moroti et al., 2012) R, PC, DB	IG: 10 CG: 10	L.acidophilus, B. bifidum (10^8 CFU) and 2g of oligofructose (strain not reported)	4	↓ TC and TG ($P < 0.05$) ↓ FPG ($P < 0.05$) and ↑ n-HDL-C ($P < 0,05$) after ten days of symbiotic ingestion	Small number of subjects per group. Inclusion of individuals with impaired glucose tolerance. Inclusion of smokers. Not evaluated the characteristics of the diet.
(Asemi et al., 2013) R, PC, DB	IG: 27 DM2 CG: 27 DM2	Multispecies probiotic supplement ($> 10^9$ CFU) and 100 mg fructo-oligosaccharide	8	Lower elevations in FPG ($P = 0.01$) ↑ serum insulin and LDL-C levels in both groups. ($P < 0.05$) ↑ HOMA-IR in both groups ($P = 0.001$). ↑ plasma GPx levels compared to placebo ($P = 0.03$).	-
(Mazloom et al., 2013) R, PC, SB	IG: 16 CG: 18	L. acidophilus, L. bulgaricus, L. bifidum, and L. casei. (CFU and strain not reported)	6	No change was observed in: FPG, insulin, lipids profile, IL-6, MDA e CRP.	CFU not reported Not evaluated the characteristics of the diet. Some subjects were using statins. Control group received 2.0g of magnesium as placebo. Waist circumference was greater in the intervention group.

(Asemi et al., 2014) R, PC, CO	IG: 62 DM2 CG: 62 DM2	Bacillus coagulans (10^7 CFU) + 1.08g of inulin (strain not reported)	6	↓ insulin (P = 0.03) and CRP (P = 0.01) ↑ GPx (P < 0.001) No change in the HOMA-IR, FPG, LDL-C e TAC levels (P > 0.05)	Used only the CRP as a marker of inflammation.

R, random study. PC, placebo-controled. DB, double-blind. IG, intervention group. CG, control group. IL-6, interleucin 6. TNF- α, tumor necrosis factor α. CRP, C-reactive protein. FPG, fasting plasma glucose. TC, plasma total cholesterol. SOD, erythrocyte superoxide dismutase. GPx, glutathione peroxidase activities. TAC, total antioxidant capacity. MDA, malondialdehyde. CAT, catalase activit. TG = triacylglycerol. n-HDL-C = non HDL-C. CO, crossover. SB, single-blinded. ↑, increase; ↓, decrease.

In subjects with normal or impaired insulin sensitivity, the effects of oral supplementation with the probiotic bacterium L. acidophilus NCFM (capsule – 10^{10} CFU/d) on insulin sensitivity and the inflammatory response were investigated. Forty-five males with type 2 diabetes, impaired or normal glucose tolerance were enrolled and allocated to a 4 weeks treatment course. The results showed that L. acidophilus NCFM was detected by denaturating gradient gel electrophoresis in 75% of the faecal samples after treatment with the probiotic bacterium. Insulin sensitivity was preserved among volunteers in the probiotic group, whereas it decreased in the placebo group. Both baseline inflammatory markers and the systemic inflammatory response (TNF-a, IL-6, IL-1 and C-reactive protein) were, however, unaffected by the intervention (Andreasen et al., 2010). The authors highlight that the improving effect observed on insulin sensitivity of L. acidophilus NCFM was evidently not related to its anti-inflammatory properties, at least not through any apparent effects on circulating cytokines, and our findings lend no support to the contention that insulin sensitivity is improved through probiotic-induced anti-inflammatory mechanisms. Alternatively, the variation in plasma cytokines due to the intervention may be so discreet that no significant changes during the treatment period were detected with the current number of volunteers included in the present study.

In a different study, the reduction of fasting blood glucose and hemoglobin A1c was associated with an improvement in antioxidant status after intake of probiotic yogurt containing L. acidophilus La-5 (10^6 CFU/mL) and B. lactis BB-12 (10^6 CFU/mL) for 6 weeks (Ejtahed et al., 2012).

In subsequent study carried out at the same time in 20 diabetics, but using a symbiotic drink, a combination of L. acidophilus, B. bifidum (10^8 CFU/100 mL each, strain not informed) and 2 g oligofructose showed a significant increase in HDL cholesterol, non-significant reduction in total cholesterol and triglycerides and a significant reduction in fasting glycemia after 30 days. No significant changes were recorded in the placebo group (Moroti et al., 2012).

Besides the effects on glucose metabolism, probiotics have also been investigated for possible impacts on lipid metabolism. Such assessments are important because diabetics have greater risks for cardiovascular diseases. Randomized trials conducted with hypercholesterolemic subjects also reported a significant reduction in total cholesterol after 6 weeks of yogurt intake with L. acidophilus (L1 and strains not informed) and/or B. lactis BB-12 (Anderson and Gilliland, 1999; Ataie-Jafari A et al.,

2009). However, these results are still controversial, especially with respect to evaluation in healthy subjects without hypercholesterolemia, and only a few studies have reported improvement in lipid profile in diabetic subjects (Ejtahed et al., 2011; Greany et al., 2008; Moroti et al., 2010; Moroti et al., 2012).

A meta-analysis involving clinical trials using milk products containing probiotics (E. faecium and Streptococcus spp.) reported that probiotic treatment for 4–8 weeks led to 4% decrease in total cholesterol and 5% decrease in LDL-C in healthy subjects (Agerholm- Larsen L et al., 2000). Recently, other meta-analysis examined the effects of probiotics on low-density lipoprotein cholesterol (LDL-C) and the potential of probiotic intake as a therapeutic lifestyle change dietary option. A significant LDL-C reduction was observed for four probiotic strains: *Lactobacillus reuteri* NCIMB 30242, Enterococcus faecium, and the combination of L. acidophilus La-5 and Bifidobacterium lactis BB-12. Two synbiotics, *L. acidophilus* CHO-220 plus inulin and L. acidophilus plus fructo-oligosaccharides, also decreased LDL-C. Of the probiotics examined, *L. reuteri* NCIMB 30242 was found to best meet therapeutic lifestyle change dietary requirements by 1) significantly reducing LDL-C and total cholesterol, with robustness similar to that of existing therapeutic lifestyle change dietary options, 2) improving other coronary heart disease risk factors, such as inflammatory biomarkers, and 3) having "generally recognized as safe" (GRAS) status (DiRienzo, 2014) .

The clinical trials that examined the effect of probiotics on the metabolism of diabetic subjects, which have been cited in this review, are shown in Table 2. The duration of intervention employed in studies that evaluated the relationship between consumption of probiotics and metabolism in diabetic patients was 4–6 weeks. The primary endpoints were fasting glucose, insulin, glycated hemoglobin, lipid profile, and markers of oxidative stress and/or inflammation. The probiotics used in the clinical trials were Lactobacillus acidophilus NCFM and La-5, L. bifidum (strain not reported), Bifidobacterium lactis BB-12, B. bifidum (strain not reported) and Bacillus coagulans (strain not reported) used alone or in combination. Among them, only one study quantified the bacteria in the feces of the study population (Andreasen et al., 2010). It is important to analyze of the faecal samples once the increase of bacteria tested can provide evidence of the presence of the strain in significant amounts in the gastrointestinal tract. Such results indicate a satisfactory compliance of participants, as well as an ability of the bacterium to survive gastrointestinal passage. Moreover, only a

few clinical trials had qualitatively and quantitatively evaluated the nutrient intake, which can influence the interpretation of the results.

Recently, the protocol of a double-blind, randomized, placebo-controlled clinical trial developed by researchers in Saudi Arabia was published. This study will be conducted with 120 individuals recently diagnosed with diabetes, who will be administered with probiotics in one formulation (B. bifidum, B. lactis, L. acidophilus, Lactobacillus brevis, L. casei, Lactobacillus salivarius, and Lactococcus lactis – 10^9 CFU/g) for 26 weeks. Subsequently, the effects of probiotics consumption on the levels of plasma endotoxin and inflammatory cytokines will be evaluated (Alokail et al., 2013).

Some exclusion criteria such as time of T2DM diagnosis, insulin and type of medication used, and treatment duration are important and should be standardized, especially for those trials that involve markers determination of oxidative stress (Choi et al., 2008). Pioglitazonas and rosiglitazones are the hypoglycemic agents that have been demonstrated to produce intracellular antioxidant effects (Dobrian et al., 2004) and affect inflammatory markers such as C-reactive protein and interleukin-6 (IL-6) (Agarwal, 2006). However, such effects have been observed 12 weeks after the initiation of treatment, and have not been evaluated after long periods of use.

T2DM is a condition that often requires treatment with various drugs such as statins and anti-hypertensives, in addition to hypoglyceminates (sulfonylureas, biguanides, thiazolidinediones, and/or insulin). This limits the clinical trials because large number diabetics cannot be included in such studies, and the sample size becomes increasingly smaller, not representing the present context, because most of the people with diabetes have other associated pathologies (Stratton et al., 2000; Scheffel et al., 2004).

Probiotics and oxidative stress

A major characteristic of T2DM is the systemic increase in oxidative stress (Rains and Jain, 2011). One way to counter balance the increase of reactive oxygen species and depletion of antioxidants is to supplement the latter through diet. In addition to the polyphenols, intestinal bacteria such as Bifidobacterium species and dietary interventions with probiotics containing Lactobacillus acidophilus La-5 and B. animalis subsp. lactis BB-12 have improved glucose tolerance and total antioxidant status in T2D patients (Ejtahed et al., 2012).

Therefore, previous studies have shown that different species of lactic acid bacteria exhibit antioxidant activity (Lin and Chang, 2000; Uskova MA and Kravchenko LV, 2009). The most frequently reported lactic acid bacteria exhibiting antioxidant activity are Lactobacillus spp., Bifidobacterium spp., Saccharomyces boulardii, Streptococcus thermophilus, Bacillus cereus, and E. faecium SF68. Only the genus Lactobacillus comprises more than 120 species, and L. acidophilus, L. rhamnosus, L. casei, L. bulgaricus, L. plantarum, and L. reuteri are the most commonly used in experimental and clinical trials.

Studies examining the relationship between oxidative stress and consumption of probiotics were first reported in healthy smokers (Naruszewicz et al., 2002; Songisepp et al., 2005). When compared with the control group, consumption of a functional drink containing L. plantarum 299v (10^7 CFU/mL) by smokers for 6 weeks resulted in a significant reduction in arterial blood pressure, levels of leptin and fibrinogen, as well as plasma levels of F2-isoprostane (37%) and IL-6 (42%) (Naruszewicz et al., 2002). Furthermore, Songisepp et al. (2005) evaluated the effect of the intake of goat's milk, goat's milk fermented with Lactobacillus fermentum ME-3 (10^9 CFU/mL, n = 19), or capsules containing L. fermentum ME-3 (10^8 CFU/g, n = 21) on healthy subjects. After 3 weeks of consumption, a significant increase in the number of Lactobacillus spp. in the feces, along with a significant improvement in the capacity and total antioxidant activity of plasma, were observed in both the groups that received the probiotic. On the other hand, a reduction in the ratio of oxidized glutathione/reduced glutathione was observed only in the group that received fermented milk containing L. fermentum ME-3. In another study with healthy subjects resistant to lipoprotein oxidation, reduced levels of peroxidized lipoproteins (oxidized LDL), 8-isoprostane, and oxidized glutathione/reduced glutathione ratio, and increased plasma total antioxidant capacity were noted after 3 weeks of consumption of fermented goat's milk containing L. fermentum ME-3 (10^9 CFU/mL) and L. plantarum LB-4 (10^8 CFU/mL). Furthermore, the use of probiotics was also observed to change the prevalence and proportion of lactic acid bacteria (Lactobacillus spp.) in the gut microbiota (Kullisaar et al., 2003).

In the context of diabetes mellitus, experimental and clinical studies have also demonstrated that different species of bacteria reduce oxidative stress, showing antidiabetic effect (Harisa GI et al., 2009; Yadav et al., 2007). A randomized, double-blind, placebo-controlled study, showed that consumption of yogurt containing B. lactis BB-12 and L. acidophilus La-5 for 6 weeks significantly reduced the levels of blood

glucose and glycated hemoglobin (HbA_{1c}), and increased the levels of erythrocyte superoxide dismutase (SOD), glutathione peroxidase (GPx) activity, and total antioxidant capacity, when compared with consumption of conventional yogurt. Furthermore, the concentration of malondialdehyde (MDA) was significantly reduced in both the groups, whereas the insulin concentration and activity of erythrocyte catalase remained unchanged (Ejtahed et al., 2012).

Besides the presence of probiotics, fermented milks have been described as dietary sources of natural antioxidants, and have been reported to contain bioactive peptides that are capable of promoting changes in the concentration of oxidative stress markers in the absence of probiotics (Fabian and Elmadfa, 2007). Most of the identified bioactive peptides derived from casein have been noted to be able to reduce free radicals and inhibit lipid peroxidation enzyme and non-enzymatic reactions (Muro Urista et al., 2011). Furthermore, the antioxidant peptides from whey protein rich in cysteine and glutathione precursor amino acid have been observed to act as a potent antioxidant (Hayes et al., 2007). Although fermented milk may exert antioxidant effects through its bioactive peptides, some studies have shown that milk beverages containing probiotics are most effective in improving antioxidant activity than conventional fermented dairy beverages (Sabeena Farvin et al., 2010).

The precise mechanisms involved in the effects of probiotic drinks on glycemic control can be partly explained by the effects on oxidative stress as a result of inhibition of ascorbate autoxidation, metal-ions chelation, and activity reduction and excretion of free radicals such as superoxide anion and hydrogen peroxide (Lin and Chang, 2000; Wang et al., 2013). Furthermore, in the context of improving oxidative stress, the importance of increasing activity of catalase and GPx, which should be higher than SOD activity, to remove reactive oxygen species has also been observed (Maritim et al., 2003).

Probiotics and low-grade systemic inflammation

The intestinal microbiota may be crucial to the development and homeostasis of the immune system since most immune cells in the body are conditioned in the gut and the gut microbiota interact closely with intestinal immune cells (Magrone and Jirillo, 2013).

According to the ‘‘metainflammation’’ hypothesis, T2DM is also considered as a state of chronic, systemic and low grade inflammation (Hotamisligil, 2006).

Circulating levels of several inflammatory mediators such as acute-phase protein, cytokines and markers of endothelial activation are elevated in T2DM patients (Kolb and Mandrup-Poulsen, 2005).

Low-grade systemic inflammation is characterized by constant high levels of proinflammatory cytokines in the circulatory system such as TNF-α, IL-6, β kinase inhibitor (IKKβ), and Jun N-terminal kinase (JNK). All these molecules can phosphorylate insulin receptor substrate (IRS) and turn them into serine, which exerts a negative effect on insulin signaling and can cause insulin resistance (Carvalho-Filho et al., 2005; Wang et al., 2013). The major cytokines that are related to glycemic control are tumor necrosis factor-α (TNF-α), IL-6, resistin, adiponectin, and IL-10 (Esposito et al., 2002; Pickup et al., 2000; Spranger et al., 2003; Wang et al., 2013). Thus, the intestinal mucosa can contribute to or facilitate the development of inflammatory disorders when the signaling cascade involves increased levels of pro-inflammatory signaling molecules (interleukins and neutrophils) and stress-mediators (norepinephrine and corticosterone) (Maslowski et al., 2009), leading to the endotoxemia concept (Cani and Delzenne, 2007). Accordingly, subjects with T2D present an altered microbiota reported to be enriched in gram-negative bacteria (Larsen et al., 2010; Wu et al., 2010) which express lipopolysaccharides (LPS).

Growing evidence suggests that cross-talk between gut bacteria and host is achieved through specific metabolites (such as short-chain fatty acids - SCFA) and molecular patterns of microbial membranes (lipopolysaccharides) that activate host cell receptors (such as toll-like receptors and G-protein-coupled receptors). Furtermore, the endocannabinoid (eCB) system is an important target in the context of T2DM, once insulin is a potent regulator of eCB metabolism. It has been demonstrated that eCB system activity is involved in the control of glucose and energy metabolism, and can be tuned up or down by specific gut microbe (Cani et al., 2014; D'Eon et al., 2008).

In this way, dietary intervention has represented an attractive way to restore immune function (Chandra, 1992; Santos et al., 1996). Different probiotic strains have been shown to enhance the intestinal barrier function, decreasing the translocation of microorganisms and their derivatives such as LPS (Cani et al., 2007a). When LPS enters the bloodstream, it activates Toll-like receptor 4 (TLR4), which is located at the surface of immune cells, leading to the release of proinflammatory cytokines and inflammation (Guha and Mackman, 2001).

Interestingly, evidence suggests that LPS is associated with marked changes in glucose metabolism. The activation of TLR4 signalling can induce both insulin resistance and pancreatic b-cell dysfunction (Tsukumo et al., 2007). In addition, LPS inhibited insulin secretion and insulin gene expression in isolated islets of Langerhans and in b-cell lines (Garay-Malpartida et al., 2011; Kiely et al., 2009). In LPS-treated mice, a decrease in both inflammation and mortality was reported when the plasma glucose level was strictly maintained at normal values, suggesting that appropriate/tight glucose control is a major determinant of outcome. Whether abnormalities in glucose control may vary according to the endotoxemic insult is unknown, and the molecular mechanisms involved are poorly understood (Nguyen et al., 2014).

Recently, the insulin response to experimental hyperglycemia was studied in mice with LPS mediated inflammation. The authors demonstrated that LPS increased glucose-stimulated insulin secretion (GSIS), which has been shown to be due, at least in part, to an increase in the level and activity of glucagon-like peptide 1 (GLP-1) (Nguyen et al., 2014).

Additionaly, microbiota components account for the production of SCFA, wich are endowed with anti-inflammatory (inhibition of NF-κB) and anti-neoplastic activities, also exerting a protective function in favor of intestinal epithelia (De Vuyst and Leroy, 2011). The lactic acid producing bacteria may provide clinical benefits for specific populations. However, reports on the ability of lactic acid bacteria to modulate immune and inflammatory condition of low grade in T2DM have been limited.

Reductions in Bifidobacterium spp. and Lactobacillus spp. during the onset of insulin resistance in hight fructose diet rats was related to increased plasma LPS (Cani et al., 2007b). Therefore, SCFA decrease may lead to an impaired secretion of mucins and easier entry of pathogens into the intestinal mucosa, especially Enterobacteriaceae. These Gram negative bacteria are able to release LPS or endotoxins, which, in turn, aggravate the inflammatory condition (Schiffrin et al., 2010).

In the context of cytokines, certain strains of lactobacillus and bifidobacteria have been shown to be able to modulate the production of cytokines by monocytes, and lymphocytes, presenting ability to regulate the immune system favorable for human health (Castellazzi AM et al., 2007; Kankaanpää et al., 2003). For example, L. casei shirota, L. acidophilus X37, and B. bifidum S131 were observed to increase the activity of NK cells in humans (Fink et al., 2007; Takeda and Okumura, 2007). In addition, B. lactis HN019 was proved to be effective as a probiotic dietary supplement, improving

some aspects of cellular immunity in the elderly after 3 weeks of consumption (Gill et al., 2001). The beneficial effects of different bacterial strains are primarily based on their ability to differentially regulate the production of anti-inflammatory cytokines (IL-10) and proinflammatory cytokines (IL-6, TNF-α, IL-12, and INF-γ) and balance the T helper cells (Th) 1/Th2 (Cross et al., 2004; Drago et al., 2010; Ghadimi et al., 2008). However, there is still little evidence on the role of probiotics in the regulation of immune response mediated by T cells and NK cells and also in immune response related cytokine production (Dong et al., 2012; Paineau et al., 2008).

Efficacy and safe use of probiotics

The term "probiotic" should be used only for products that meet the scientific criteria established for this classification; in other words, products that contain an adequate dose of live microorganisms and show beneficial effects on human health (Sanders et al., 2008). It is important to stress that the biological effects of probiotics are strain specific and that the success or failure of one strain cannot be extrapolated to another strain. Thus proper strain identification using novel molecular and based technologies is imperative (Azaıs-Braesco et al., 2010).

To explore the question “are probiotics safe?” using a drug based framework assumes that the literature will include drug like safety and toxicology data. The scientific community report the need to consider that traditional foods and food components are not studied in the same way as drugs. Additionally, many probiotics pose a low risk to the general population, including diabetics, as shown by their native colonization in the gastrointestinal tract of humans. Researchers should recognize that in the absence of drug-like safety data, the safety of traditional foods should be based on the totality of evidence in healthy populations (Wallace and MacKay, 2011).

Significant progress in legislation for the safety evaluation of probiotics has been made in USA, Canada, and Europe (FAO/WHO, 2002; EFSA, 2009); however, no standard protocol has been defined as consensus. The Food and Agriculture Organization (FAO/WHO) recommends that probiotic strains be characterized at a minimum with the following tests: determination of antibiotic resistance patterns, assessment of certain metabolic activities (e.g., D-lactate production, bile salt deconjugation), assessment of side-effects during human studies, epidemiological surveillance of adverse incidents in consumers (post-market), assessment of toxin production and determination of hemolytic activity if the strain under evaluation

belongs to a species with known hemolytic potential. The assessment of lack of infectivity by a probiotic strain in immunocompromized animals demonstrate a measure of confidence in the safety of the probiotic (FAO and WHO, 2002).

Many probiotic strains belong to species normally found in dairy/fermented foods and have been consumed for centuries as constituents of such foods without any apparent side effects (Wallace and MacKay, 2011). However the lack of a definitive identification of the probiotic strain and of the numbers actually necessary to infer functionality are drawbacks within the probiotic market (Huff, 2004; Mattarelli et al., 2002; Wannaprasat et al., 2009). Therefore, a consistent identification and concentration of the probiotic microorganism(s) must be clearly indicated on the label in order to avoid misunderstanding and misuse of the probiotic product. Another important aspect that might to be observed is related to the viability of the microorganism(s) at the time of consumption (Czinn and Blanchard, 2009); often the number of probiotic bacteria found in the products were below the one declared or they were absent (Wannaprasat et al., 2009).

Although the vast majority of probiotics are generally regarded as safe (GRAS and QPS) and beneficial for healthy individuals (FAO and WHO, 2002; EFSA, 2009), caution is needed when selecting, monitoring and administering probiotics to immune compromised, leaky gut and patients with critical illnesses. In these situations sepsis, fungemia and gastrointestinal ischemia could occur (Whelan and Myers, 2010). Therefore, while the overwhelming existing evidence suggests that probiotics are safe, complete consideration of risk-benefit before prescribing is recommended (Fijan et al., 2014).

Recently, some systematic reviews have been published on the safety of probiotics used in research to reduce the risk of, prevent, or treat disease (Hempel et al., 2011; Whelan and Myers, 2010). In the context of diabetes, a case report was reported. Zein et al., (2008) describe a case of a 54-year-old present history of type 2 diabetes poorly controlled (Hb A1c = 11%), hypertension, mixed dyslipidemia, smoking, and a thyroidectomy for 24 years who developed Lactobacillus rhamnosus septicemia while under consumption of an oral probiotic self prescribed. The preparation contained Ferment lactique actif 5 x 10^9, B. bifidus 3 x 10^9, L. acidophilus 77 x 10^7, L. bulgaricus 76 x 10^7, L. casei 54 x 10^8, B. longum 8 x 10^7, L. rhamnosus 8 x 10^7 and Streptococcus thermophilus 8 x 10^7. The infection was resolved after amoxicilline administration. This case highlights the complication of probiotic use, and perhaps the patient's

predispositions to develop such complications.

A meta-analysis involving 622 clinical trials (a total of 24,415 reviews) after consumption of different genera of microorganisms (Lactobacillus, Bifidobacterium, Saccharomyces, Streptococcus, Enterococcus, and Bacillus: 10^5–10^{11} CFU/mL), used alone or in combination. Only 5% of the studies had evaluated the safety of long-term use of probiotics (more than 1 year). Furthermore, from the 622 articles, 38% reported that no adverse effects (AEs) were observed, meaning that the intervention was only "well tolerated." Most of the studies with Lactobacillus spp. and/or Bifidobacterium spp. as the probiotic had gastrointestinal, followed by infection and "other" reported as the most common AEs (Hempel et al., 2011). Fungemia, bacteremia, sepsis, and other infections may be associated with the administration of probiotics, such as L. rhamnosus spp. and Bacillus subtilis, mostly in immunocompromised patients and/or patients with short gut syndrome (Kunz et al., 2004; De Groote et al., 2005; Zein et al., 2008). Among clinical trials, no infections were observed. The study concluded that despite the substantial number of publications, the current literature is not well equipped to answer questions on the safety of probiotic interventions with confidence. Thus, the scientific community must recognize that there is a need to include in the publications the strains evaluated as probiotic once the phenotypes of one strain could not be extrapolated to others even when isolated from the same species.

Conclusion and perspectives

The proposal that intestinal microbiota plays a role in the development of T2DM is important, but further consideration needs to be given to the potential roles of probiotics on glucose metabolism due to lack of sufficient scientific evidence in support of this health claim. Additionally, there is a dearth of studies on characterization of the gut microbiota in diabetic individuals. Nevertheless, data from animal studies encourage to believe that probiotics do have the potential to decrease the risk and to reduce the severity of T2DM and other metabolic syndromes possibly through modulating the gut microbiota, the immune response and other putative mechanisms. In clinical trials, the use of probiotics in glycemic control of diabetic individuals has conflicting results, and only a few studies have attempted to evaluate the markers of oxidative stress and inflammation which may explain possible links between glycemic control and intestinal microbiota. Furthermore, no clinical trials have been carried out to evaluate GLP-1, LPS and the intestinal microbiota. The evaluation of probiotic efficacy in human population

is, however, far more complex than under controlled experimental conditions due to the plethora of the confounding factors such as diet profile, use of drugs, body mass index and endotoxin content of ingested food that may also affect the gut microbiota, glucose metabolism, insulin secretion, energy balance and other gut hormones such as incretins. Thus, the design of future research should attempt to neutralize such factors in order to better understand the effects of probiotics on the metabolism of diabetic individuals as well as the main mechanisms involved in this complex relationship. Since the efficacy of probiotics is directly linked to the strain level, it is also possible to select probiotics with strains that fits better the necessities of the patients with T2DM. Such studies would consolidate future nutritional interventions focused on glycemic control and other metabolic disorders commonly present in chronic diseases, especially T2DM.

Conflict of interest

The authors declare no conflict of interest related to the contente of the present paper. All authors contributed equally to all aspects of the article.

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Article 2 – PROBIOTIC FLAVORED FERMENTED GOAT MILK: PRODUCT DEVELOPMENT, ANTIOXIDANT PROFILE AND CONSUMER ACCEPTANCE

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ABSTRACT

TONUCCI, Lívia Bordalo, D.Sc., Universidade Federal de Viçosa, December, 2014. **Probiotic flavored fermented goat milk: product development, antioxidant profile and consumer acceptance**. Advisor: Hércia Stampini Duarte. Co-Advisor: Karina Maria Olbrich do Santos, Sônia Machado Rocha Ribeiro and Leandro Licursi de Oliveira.

The present study evaluated physicochemical properties, microbial viability, tolerance to simulated digestive steps, antioxidante profile, as well as sensory preference of flavored fermented goat milks produced with Streptococcus thermophilus TA-40, Lactobacillus acidophilus La-5 and Bifidobacterium animallis sub. lactis BB-12. Samples of fermented milks were submitted to pH, titratable acidity, texture and bacterial viability determinations in different periods of storage at 4 ºC. Total phenolics content, antioxidant activity and sensorial analysis were also performed. The texture characteristics and pH values increased during the storage, however, did not affect the quality of the product. Total phenolic contents and antioxidant activity of probiotic flavored fermented milk were significantly higher than conventional fermented milk. A higher loss in cell viability was observed for L. acidophilus than for the B. animalis. However, the viability of all bacteria was adequate (> 10^6 cfu/mL) until day 28 of storage, remaining above the minimum therapeutic level. The fermented milk showed good sensory characteristics. This study developed a beverage of good quality, in terms of sensory characteristics, nutritional quality and survival of bacteria to be subsequently evaluated in a clinical trial.

Keywords: Goat milk, Lactobacillus acidophilus, Bifidobacterium animalis, microbial survival, sensory analysis.

1. Introduction

Throughout the history of microbiology, most human studies have focused on the disease-causing organisms found on or in people. However, in the last 20 years, many reports have centered on the benefits of the numerous microorganisms, which are currently used as human probiotics. Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a benefit on the host (FAO/WHO, 2002). Lactobacillus and Bifidobacterium constitute the most frequently used genera, and are known as bacteria with beneficial effects (G. Vinderola, Binetti, Burns, & Reinheimer, 2011).

The increasing interest in a healthy nutrition is stimulating innovative development of novel scientific products in the food industry. Among the foods whose alleged health claims, the ones with probiotic strains stand out (Lourens-Hattingh & Viljoen, 2001). The dairy sector, which is strongly linked to probiotics, is the largest functional food market accounting for nearly 33% of the broad market (Leatherhead Food International, 2006; Siró, Kápolna, Kápolna, & Lugasi, 2008).

Probiotics in fermented milk products have been associated with increased lactose tolerance, a well-balanced intestinal microbiota, stimulation of the immune system, anticholesterolaemic and antioxidative properties in human subjects (Andreasen, et al., 2010; Cross, Ganner, Teilab, & Fray, 2004; He, et al., 2008; Songisepp, et al., 2005; Zarrati, et al., 2014).

Additionally, the use of non-bovine milk as an alternative milk product has increased lately. In comparison to bovine milk, goat milk proteins and lipids are more easily digested because of the small size of fat globules (Ceballos, et al., 2009; Meena, Rajput, & Sharma, 2014). The unique composition of goat milk, combined with its nutritional value, is related to the release of protein fragments during digestion or technological processing, which are able to perform specific biological activities (Park, Juárez, Ramos, & Haenlein, 2007). However, it is not well accepted by many consumers, due to its typical flavor derived from caprylic, capric, and caproic acids present in this milk (Costa, et al., 2014).

Interestingly, the supplementation of probiotic fermented milks with functional ingredients, such as fruit pulp or juice, is increasing. Purple grape juice contains rich flavonoids, specifically anthocyanidins and resveratrol (Kanner, Frankel, Granit, German, & Kinsella, 1994; Mazza & Francis, 1995). Evidences indicate that these compounds have a wide range of biological functions and health benefits, such as anti-

oxidative and anti-inflammatory properties (Montagut, et al., 2010; Zafra-Stone, et al., 2007).

One of the main concerns in the probiotic fruit yogurt/fermented milk production is the acidic environment that most of the fruits may confer to the product. However, yogurts incorporated with fruit preparation (mango, mixed berry, passion fruit and strawberry), did not exhibit a greater loss in the viability of probiotic bacteria compared to plain yogurt during the storage period, although a correlation between the post-storage pH in yogurts and the survival of probiotic bacteria was observed (Kailasapathy, Harmstorf, & Phillips, 2008).

Only a few publications on these dairy products are available in literature, and data regarding the possible impact of these fruits on the viability of the probiotic microorganism and antioxidant profile in the food product are scarce (Kailasapathy, et al., 2008; C. G. Vinderola, Costa, Regenhardt, & Reinheimer, 2002).

The present study aimed to develop a functional flavored fermented goat milk, with the addition of probiotic Lactobacillus acidophilus La-5 and Bifidobacterium animalis subsp. lactis BB-12 cultures, and purple grape juice, evaluating physicochemical properties, probiotic viability, antioxidant profile, and sensory acceptability.

2. Materials and Methods

Formulation and Fermentation of dairy beverages

Goat milk provided by Embrapa Goats and Sheep (Sobral, Ceará, Brazil) was supplemented with 5% (w/v) sucrose and pasteurized at 90 ºC for 15 min. The pasteurized milk was cooled to 43 ± 2 ºC for the addition of the starter culture and the probiotic cultures. The probiotic fermented milk (PFM) and conventional fermented milk (CFM) were produced using the commercial starter culture Streptococcus thermophilus TA-40 (Danisco, Sassenage, France; 0.003 g/100 g). The PFM was enriched with B. animallis subsp. lactis BB-12 and L. acidophilus La-5 (Chr. Hansen, Hørsholm, Denmark; 0.024 g/100 g), as freeze-dried Direct Vat Set cultures. The fermentation process was conducted at 40 ± 1 ºC until reaching pH 5.0 ± 0.1. Next, the fermented milk temperature was decreased to 4º C up to the following day, and then the beverages were flavored with 20% (w/v) of purple grape juice obtained from Embrapa Grapes and Wine (Bento Gonçalves, Rio Grande do Sul, Brazil). All ingredients were mixed with a blender in order to form a homogeneous product. The final product were

packed in polypropylene bottles and stored at 4 ± 1 ºC for further analysis. The CFM was used for analysis of physicochemical properties and sensory quality in comparison with PFM.

Compositional analysis

Total solids, total dietary fiber, ash, fat and protein content were determined for PFM on the seventh day of storage and analyzed using standard AOAC procedures (AOAC, 2012). Protein was estimated by measuring the nitrogen content of samples through the micro Kjeldahl method and applying a nitrogen-to-protein conversion factor of 6.38, according to the AOAC official methods 690.52 and 991.20. All the analyses were performed in triplicate and were expressed as g/100g of whole matter.

Physicochemical properties and instrumental analysis

During fermentation and at 1, 7, 14, 21 e 28 days of storage at 4 ºC, PFM samples were taken to determine pH (pH meter Jenway 3510, Staffordshire) and titratable acidity. Titratable activity was determined according to the appropriate standard methods and expressed in terms of g/100 g lactic acid (IAL, 2008). Firmness, consistency, cohesiveness and viscosity index were evaluated in CFM and PFM samples after 1, 14 and 21 days of storage using a back extrusion cell (A/BE) on a Texture Analyzer TA-XTPlus (Stable Micro Systems, Surrey, UK). An acrylic compression disc (35 mm diameter) was thrust into a cylindrical container (50 mm diameter x 70 mm height) filled with samples at 4º C up to a height of 50 mm (ca.100 mL). The starting distance of the disc was set at 30 mm above the top of the sample surface. The disc penetrated into the sample to a depth of 30 mm at a 1 mm/s speed, and returned at 10 mm/s speed. The parameters measured consisted of firmness, consistency, cohesiveness and index of viscosity, obtained by using the Exponent Lite software e version 4.0.13 (Stable Micro Systems, Hamilton, US). The analyses were performed in quadruplicate.

Total phenolic content and antioxidant activity

The total phenolic (TP) contents of the CFM and PFM were estimated according to the Folin-Ciocalteu method (Singleton VL, Orthofer R, & Lamuela-Raventos RM, 1999) on the first day of storage. Briefly, an aliquot of each fermented milk extract (100 µL, in triplicate) was added to the Folin-Ciocalteu reagent (1 mL, 1:10 v/v in purified

water) and after 5 min of reaction at room temperature (25 °C), 1.0 mL of 7.5% NaCO3 was added. After 7 min, 5 mL of distilled water was added. After 30 minutes, the absorbance was measured at 726 nm using a microplate reader (Thermo Scientific®, Multiskan GO, Canadá, US). The TPC of each sample was assessed by plotting against a gallic acid calibration curve (0.0 to 250 μg/mL, R2 = 0.999) and the results were expressed as mg gallic acid equivalents (GAE)/mL.

The percentage of antioxidant activity of each beverage was assessed by DPPH free radical assay as previously described (Blois, 1958), with some modifications. Briefly, aliquots of samples (PFM and CFM) were diluted in 60% methanolic solution (1:10 v/v) and kept under agitation at 100 rpm during 20 min. Subsequently, 100μL of the extracts, in triplicate, was added to 1.5 mL of fresh 0.1 mM solution of DPPH in methanol and allowed to react at 37 °C in the dark. After thirty minutes, the absorbance was measured at 517 nm (Thermo Scientific Multiskan GO, Canada, US). The scavenging activity was estimated based on the percentage of DPPH radical scavenged (AA%) as the following equation: scavenging ability (%) = (control absorbance – sample absorbance) / (control absorbance) x 100. The results were expressed as % of radical scavenging activity.

Microbial viability

The flavored fermented milk was analyzed after 1, 7, 14, 21 and 28 days of storage at 4º C. The samples were serially diluted in sterile peptone water (0.1% v/v) and subsequently plated onto the appropiate media, in duplicate. S. thermophilus enumeration was performed on M17 agar, containing lactose (Vetec, Duque de Caxias, Brazil, 5 g/L) and incubated aerobically at 37º C for 48h (Codex Alimentarius, 2010). Bifidobacterium animalis were determined by pour plating 1mL of adequate dilution in modified DeManRogosaSharpe (MRS) agar (Oxoid, Basingstoke, UK), prepared as a basal medium, an added dicloxacillin (Sigma, St. Louis, US), cysteine hydrochloride (Cromoline, Diadema, Brazil) and lithium chloride (Cinética®, Jandira, Brazil) sterile solutions to reach a concentration of 0.5 mg/L, 0.5 g/L and 1 g/L, respectively, followed by anaerobic incubation (Anaerobic System Anaerogen, Oxoid) at 37 ºC for 72 h (Flávia C. A Buriti, Okazaki, Alegro, & Saad, 2007). Lactobacillus were pour plated into MRS agar (Oxoid) followed by incubation at 37 ºC for 72 h. The results were expressed as of log colony forming units per gram (cfu/mL).

Resistance to simulated gastrointestinal conditions

Fermented milk samples were collected at 7 days of storage for the evaluation of L. acidophilus and B. animalis survival to gastric and enteric simulated conditions according to the method previously described (Liserre, Ré, & Franco, 2007), with modifications. Samples were decimally diluted in a sterile 0.85% (w/v) NaCl solution and, for the gastric phase simulation the pH was set at 2.5 with 1 M HCl solution, and pepsin and lipase solutions were added to reach final concentrations of 3.0 g/L and 0.9 g/L, respectively. The flasks were incubated at 37 °C for 2 h under agitation (150 rpm). Subsequently, enteric conditions were simulated in two phases. In the enteric phase 1, the pH was increased to 5.0 with a sterile alkaline solution, and bovine bile and pancreatin were added to reach a concentration of 10 g/L and of 1 g/L, respectively. After 2 hours of incubation at the same conditions, the pH was adjusted to 6.5 - 7.0, and the respective bile and pancreatin concentrations were adjusted to 10 g/L and 1 g/L for the second enteric phase, followed by an additional incubation period of 2 hours. In order to enumerate the viable L. acidophilus and B. animalis cells, aliquots were taken at the assay baseline (0h) and after 2, 4 and 6 hours, serially diluted in peptone water solution, and 1 mL of adequate dilutions were pour plated in acidified MRS agar, followed by anaerobic incubation at 37 ^{o}C for 48 hours. A survival ratio (SR%) was calculated based on the initial and final populations to estimate the relative resistance of each strain to the simulated TGI conditions. All results are presented as log cfu/mL.

Sensory Analysis

The sensory evaluation of the probiotic flavored fermented goat milks was approved by the Federal University of Viçosa Human Ethics Research Committee, Brazil (Process No. 219.644; CAAE: 13380413.8.0000.5153 – Appendex II and III) and was carried out at the Laboratory of Sensory Analysis of INTA. Sensory evaluation was carried out with CFM and PFM samples after 7 days of cold storage (4 ± 1 ºC) through acceptability tests (Appendex IV), using the hybrid hedonic scale (1 = disliked extremely, 5 = neither liked nor disliked, 9 = liked extremely) focusing on attributes of color, taste, flavor, consistency and overall acceptability (Peryam & Pilgrim, 1957). Sixty-three consumers (untrained panelists) were recruited based, primarily, on interest and goat dairy products consuming habits. The consumers who did not regularly eat fermented milks (i.e., at least once per week), did not like fermented milks, or had allergies related to any of the ingredients used in the experiment were excluded from the

sensory test. The samples were maintained under refrigeration prior the tests and served, monadically, in individual disposable plastic cups (approximately 30 mL) codified with three random digits. The sensory analysis was performed in individual booths. Water and unsalted crackers were available during a 1 min rest period between sample sets to refresh the palate. The consumers were also instructed to report the sensory attributes related to flavor, texture, appearance and aroma that they liked and disliked most in the samples, and they were free to mention none or more than one attribute.

Statistical analysis

The experimental data were analyzed by SPSS software version 20 (IBM, Armonk, NY) and the results were expressed as mean ± standard deviation (SD). Values were the average of quadruplicate/triplicate/duplicate experiments. Before analysis, data were checked for the normality, homogeneity of variances and sphericity using the Shapiro-Wilk, Levene's and Mauchly's tests, respectively. Differences between trials (CFM and PFM) in a single moment were tested using unpaired t test or Mann-Whitney test. Differences between experimental storage periods were statistically analyzed using repeated measures analysis of variance (RM ANOVA), followed by the post hoc Bonferroni test, taking on $P < 0.05$. When normality was not found, the equivalent non-parametric tests were applied. Differences at $P < 0.05$ were considered to be significant.

3. Results and discussion

Composition and physicochemical analysis

Probiotic flavored fermented goat milk (PFM) had the following chemical composition: total solids 17.3 ± 0.04 g/100g, protein 2.47 ± 0.02 g/100g, fat 2.64 ± 0.02 g/100g, ash 0.74 ± 0.01 g/100g and total dietary fiber 0.14 ± 0.01 g/100g. Total solids, protein and fat contents of PFM were found to be lower than previous studies (Martín-Diana, Janer, Peláez, & Requena, 2003; Salva, et al., 2011), reflecting higher moisture content in flavored fermented milks due to addition of fruit juice. Changes in these parameters, especially total solids and fat content may affect certain other physico-chemical properties such as viscosity, syneresis and water holding capacity (Senaka Ranadheera, Evans, Adams, & Baines, 2012).

The texture parameters analyses of the dairy beverages are presented in Table 1. The firmness, consistency, cohesiveness and viscosity index of conventional fermented milk (CFM) increased during the 28 days of storage, but the increase observed between

14 and 28 days did not differed significantly ($P > 0.05$). Interestingly, these parameters values increased only until day 14 ($P < 0.05$) for PFM, however, did not affect the quality of the product. When the sampling periods were compared between trials, no significant difference was verified for firmness and consistency ($P > 0.05$). However, PFM presented a lower viscosity index on the first day of storage ($P = 0.04$) and cohesiveness at day 28 ($P = 0.03$) compared to CFM.

Usually, the viscosity of flavored fermented milks tends to be lower, in line with the lower level of total solids in this fermented milk (Martín-Diana, et al., 2003; Tamime A.Y & Robinson R.K, 1999). However, little information exists on the influence of probiotic strains on physicochemical properties of yogurts and fermented milks, especially, for goat milk products.

Table 1. Texture analyses of fermented goat milk beverages during 28 days of storage at 4 ± 1 ºC

Beverages	Time (days)	Firmness N x10^2	Consistency N x 10^2 s	Cohesiveness N x10^2	Viscosity index N x 10^2 s
CFM	1	12.19 ± 0.18^{A}	241.70 ± 5.96^{A}	8.06 ± 0.16^{A}	$14.94 \pm 0.87^{A,a}$
	14	15.99 ± 0.40^{B}	369.52 ± 5.35^{B}	10.75 ± 0.30^{B}	17.59 ± 1.31^{B}
	28	16.41 ± 0.77^{B}	369.60 ± 4.32^{B}	$11.71 \pm 0.59^{B,a}$	19.42 ± 1.06^{B}
	Overall mean	14.87 ± 2.03	326.60 ± 62.88	10.17 ± 1.64	17.31 ± 2.16
PFM	1	12.06 ± 0.23^{A}	234.86 ± 2.70^{A}	8.01 ± 0.13^{A}	$13.26 \pm 0.48^{A,b}$
	14	15.99 ± 0.20^{B}	369.61 ± 2.63^{B}	10.75 ± 0.15^{B}	17.63 ± 0.55^{B}
	28	16.15 ± 0.29^{B}	369.81 ± 3.60^{B}	$10.95 \pm 0.20^{B,b}$	18.50 ± 0.75^{B}
	Overall mean	14.73 ± 2.10	324.76 ± 66.61	9.90 ± 1.43	16.46 ± 2.43

Values are mean ± SD of four replicate determinations. $^{A-C}$ Different superscript capital letters in a column for a same beverage denote significant differences ($P < 0.05$) between sampling days. $^{a-b}$ Different superscript letters in a column denote significant differences ($P < 0.05$) between trials for a same moment. There was no significant difference between trials for overall mean. CFM = conventional fermented milk, PFM = probiotic fermented milk.

The use of a combination of starter cultures and probiotic adjunct cultures requires an adequate formulation that should guarantee rapid acidification during fermentation and reproducibility of the fermented milks. The time needed to reach pH 5.0 ± 0.1 during the fermentation was lower for PFM as compared to CFM (2h 30 min vs 5h00; $P < 0.001$). In this way we obtained a PFM with adequate technological quality, with time of fermentation lower than others study developed with non-flavored fermented goat milk containing S. thermophilus and L. rhamnosus CRL1505 (4 h to reach pH 4.8) at the incubation temperature of 37 or 42 ºC (Salva, et al., 2011) or S. thermophilus ST-20Y, L. acidophilus La-5 and B. animalis BB-12 (6h to reach pH 5.0) (Martín-Diana, et al., 2003).

During refrigerated storage, the pH of PFM ranged from 4.10 to 4.19 ($P < 0.001$), indicating no further acidification, probably due to the presence of three bacteria that show a lower proteolytic activity (Shihata & Shah, 2000). Values of pH below 4.0 are generally considered detrimental to the survival of probiotic organisms (Dave & Shah, 1997a, 1997b). Titratable acidity remained stable during the storage period, ranged from 0.82 to 0.85 mg lactic acid g^{-1}. Others studies have been reported small reductions in the pH and increase in the titratable acidity for goat's milk beverages (Buriti et al., 2014; Senaka Ranadheera et al., 2012; Guler-Akin & Akin, 2007).

Total phenolic contents and antioxidant activity of dairy beverages

The total phenolic (TP) contents of fermented milks varied from 0.179 ± 0.001 mg GAE/mL in CFM to 0.264 ± 0.01 mg GAE/mL in PFM. The antioxidant activity ranged from 65.17 ± 0.24 for CFM to 83.30 ± 0.68 % for PFM. TP contents and antioxidant activity of PFM were significantly higher ($P < 0.01$) than CFM (Figure 1).

There are a limited number of studies about the capacity of potential probiotics such as lactic acid bacteria and bifidobacteria to metabolize polyphenol compounds (Tabasco, et al., 2011). However, a high proportion of polyphenols from the diet are not directly absorbed and the transformation these compounds in the gut depends on microbial esterase and glucosidase, as well as on demethylation, dehydroxylation and decarboxylation activities (Aura, 2008). In this way, gut bacteria may play a major role in the production of new phenolic compounds "in situ", which could have better bioavailability and higher biological activity than their parent compounds (Requena, et al., 2010). This can explained the higher TP contents and antioxidant activity observed in PFM in this study.

Interestingly, antioxidant activity determined by DPPH assay showed higher activity in probiotic fermented milk obtained from goat milk (93 %) followed by product from camel milk (86 %) and then product from cow milk (79 %). Furthermore, the results suggested that probiotic bacteria are able to utilize the nutrients in goat and camel milk more efficiently compared to cow milk and increase in antioxidant activity and fatty acid profile of fermented milks enhances the therapeutic value of the products (Balakrishnan & Agrawal, 2014). Several studies have highlighted the association between the consumption of foods with high antioxidant activity and the development and progression of chronic low-grade inflammation and metabolic diseases (Chen, Song, & Zhang, 2013; Moazen, et al., 2013; Yamagata, Tagami, & Yamori, 2014).

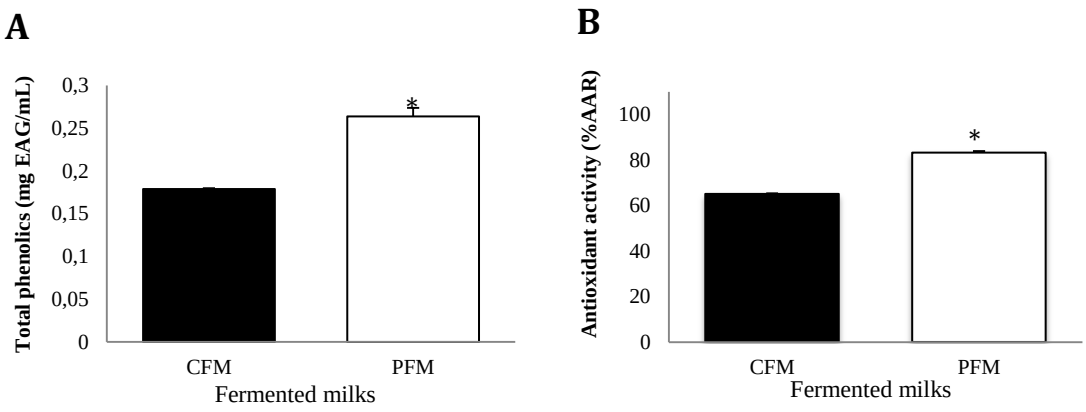

Figure 1. Total phenolic contents (A) and antioxidant activity (B) of Conventional (CFM) and Probiotic Fermented Milks (PFM). Values are means and errors bars indicate standard deviations (n = 3). * P < 0.01 from unpaired t test.

Microbiological analysis

The S. thermophilus population of the PFM remained stable during the entire period of cold storage, with a viability of 8.91 log cfu/mL at day 1 and 9.2 log cfu/mL at day 28. On the other hand, L. acidophilus and B. animalis subsp. lactis populations reduced significantly during the storage (P < 0.001), reaching concentrations of 6.10 and 7.98 log cfu/mL at day 28, respectively. L. acidophilus maintained viable cell counts $\geq 10^7$ cfu/mL for 2 weeks, whereas S. thermophilus and B. animalis subsp. lactis maintained this concentration throughout the 28 days. The viability values of bacteria in PFM during the cold storage period are shown in Table 2.

The health benefit of FMs containing probiotic depends on the viability of the

probiotic microorganisms in the refrigerated product (Patrignani, Lanciotti, Mathara, Guerzoni, & Holzapfel, 2006). In the present study, probiotic bacteria had variable viability in fermented milk during cold storage. In spite of having no agreement about the effective dose, many authors have been suggesting a minimum dose between 10^6 - 10^9 cfu/day to assure the therapeutic effect (Vasiljevic & Shah, 2008). The Codex Alimentarius states that the minimum viable quantity of probiotic culture should be 10^6 cfu/mL and 10^7 cfu/mL for starter culture (Codex Alimentarius, 2010). Probiotic bacteria and starter culture achieved this condition in the present study.

In agreement with other studies (Senaka Ranadheera, et al., 2012; C. G. Vinderola, et al., 2002) a higher loss in cell viability was observed for L. acidophilus La-5 than for the bifidobacteria strain. Also, the viability of S. thermophilus remained well above that of lactobacilli at the end of the storage period, similarly at previous studies (Dave and Shah 1997a, 1997b). However, L. acidophilus La-5 was able to maintain the minimum therapeutic level (> 10^6 cfu/mL) up to 4 weeks storage, in contrast to Ranadheera et al. (2012) in stirred fruit goat`s milk yogurts.

The viability of the probiotic strain depends on several factors (Shah, 2000), being the drop in pH the most important cause of the decrease in the viability of the probiotic culture (Ongol, et al., 2007). However, the pH obtained in the present study did not affect the viability of L. acidophilus La-5 and B. animalis BB-12, since there was no pH reduction during the storage period. In addition, it has been supposed that mixed cultures of probiotics in fermented milks may result in poor growth and subsequently poor viability in storage compared to pure cultures, most probably due to competition for nutrients (Timmerman, Koning, Mulder, Rombouts, & Beynen, 2004). Thus, it seems that B. animalis subsp. lactis has an advantage compared to the L. acidophilus (Bedani, Rossi, & Isay Saad, 2013; Jayamanne & Adams, 2006; Senaka Ranadheera, et al., 2012).

Additionally, other studies have reported that food polyphenols are able to selectively modify the growth of susceptible microorganisms (Tabasco, et al., 2011). Lactobacillus acidophilus and L. vaginalis strains showed a inhibition of growth by the phenolic extracts. However, within Bifidobacterium Genus, B. animals subsp. lactis BB-12 also showed an elevated sensitivity. On the other hand, L. plantarum, L. casei, and L. bulgaricus strains reached maximal growth in the presence of polyphenol extracts (343 mg/g of total phenolic content) (Tabasco, et al., 2011), being most appropriate for use in fermented beverages containing high proportion of polyphenols.

Table 2. Viability of S. thermophilus TA-40, L. acidophilus La-5 and B. animalis subsp. lactis BB-12 in the probiotic fermented milk (PFM) during 28 days of storage at 4 ± 1 ^{o}C

Microorganism (log cfu/mL)	1 day	7 days	14 days	21 days	28 days
S. thermophilus	8.92 ± 0.01	8.39 ± 0.42	8.90 ± 0.02	8.88 ± 0.02	9.20 ± 0.49
L. acidophilus	7.89 ± 0.02^{A}	7.76 ± 0.10^{A}	6.18 ± 0.05^{B}	6.16 ± 0.03^{B}	6.11 ± 0.02^{B}
B. animalis	8.65 ± 0.02^{A}	7.26 ± 0.01^{B}	7.19 ± 0.01^{C}	7.19 ± 0.01^{C}	7.98 ± 0.02^{D}

Values are mean ± SD of three batches, in duplicate, at each sampling day. $^{A-E}$ Different superscript capital letters in a same row denote significant differences ($P < 0.01$) between sampling days.

The survival of a probiotic bacteria during the gastrointestinal transit should be investigated in each food matrix, complementing the study of probiotic viability in the product, since most probiotic effects depends on the viable cells action at intestinal level and the food matrix which vehicles the probiotics might exert an important role in probiotic protection. Overall, reductions in L. acidophilus and B. animalis subsp. lactis was observed throughout exposition to simulated gastrointestinal conditions, however, in contrast to previous studies, lactobacilli and bifidobacteria counts were within the detection limits throughout the entire assay (Botes, van Reenen, & Dicks, 2008; Flávia C. A. Buriti, Castro, & Saad, 2010; Corsetti, et al., 2008; Liserre, et al., 2007). During the gastric phase, the populations of L. acidophilus and B. animalis subsp. lactis decreased significantly ($P < 0.001$ and $P < 0.01$, respectively). Populations of L. acidophilus reduced 1.2 log cfu/mL, in average, after 60 min of exposure to the simulated gastric conditions, and B. animalis subsp. lactis populations reduced 1.8 log cfu/mL in the same phase.

The probiotic L. acidophilus La-5 survived well in the enteric phase, maintaining almost the same population. On the other hand, populations of Bifidobacterium animalis BB-12 reduced significantly during the simulated enteric phase (Table 3). It is possible that bile affects the phospholipids and proteins of bacterial cell membranes, disrupting cellular homeostasis and Gram positive bacteria seem to be more susceptible to the deleterious effects of bile than Gram-negative.

However, the tolerance to the bile is a strain-dependent characteristic that should not be generalized in terms of species (Begley, Gahan, & Hill, 2005). In the present study, although both probiotic bacteria are gram-positive, B. animalis BB-12 had a higher sensitivity in these conditions.

Table 3. Survival of L. acidophilus La-5 and B. animalis BB-12 under simulated gastrointestinal conditions in the fermented milk at 7 days of storage at 4 ± 1 ºC

Microorganism (log cfu/mL)	0 hour	2 hours	4 hours	6 hours
L. acidophilus	$6.95 \pm 0.01^{A,a}$	$5.75 \pm 0.08^{B,a}$	$5.55 \pm 0.02^{B,a}$	$5.25 \pm 0.15^{B,a}$
B. animalis	$8.75 \pm 0.20^{A,b}$	$6.94 \pm 0.01^{B,b}$	$5.68 \pm 0.03^{C,b}$	$5.12 \pm 0.02^{D,a}$

Values are mean ± SD of three replicate determinations. $^{A-D}$ Different superscript capital letters in a same row denote significant differences ($P < 0.01$) between different sampling periods of the in vitro assay. $^{a-b}$ Different superscript letters in a column denote significant differences ($P < 0.05$) between probiotics for a same moment. pH at 0, 2, 4 and 6 hours = 4.39; 2.46; 5.0 and 6.3, respectively.

A high susceptibility of L. acidophilus La-5 and Bifidobacterium animalis subsp. lactis to simulated gastric juice containing HCl and pepsin has been observed in previous studies (Bedani, et al., 2013; Flávia C. A. Buriti, et al., 2010; Fávaro-Trindade & Grosso, 2002). This corroborates with the highest decrease in strains viability observed in the gastric phase. Fávaro-Trindade and Grosso (2002) reported that free cells of L. acidophilus La-5 reduced only 1 log cycle after 2 h of exposure to pH 2. Reductions varying from 0.1 to 2.5 log cycles of the 6 strains of L. acidophilus isolated from commercial probiotic yoghurts have been reported during 90 min in simulated gastric juice containing pepsin at pH 2.0. However, strains of the L. acidophilus group were more tolerant to the low pH 2.0 than strains of L. paracasei and L. rhamnosus which rapidly lost their viability (Schillinger, Guigas, & Heinrich Holzapfel, 2005).

The minimum populations of probiotic bacteria observed during the in vitro assay remained above 5 log cfu/mL, for both bacteria. However, the survival rate of L. acidophilus (75.71%) was higher than B. animalis (58.55%) considering the entire assay ($P < 0.01$). A different study reported that L. acidophilus La-5 exhibited greater survival rates than B. animalis supsp. lactis BB-12 in the more acidic stirred fruit yogurts,

confirming their capacity for acid tolerance (Senaka Ranadheera, et al., 2012). Recently, Casarotti and Penna (2014) showed that the addition of the fruit flours (apple, banana and grape flours - 1%) in fermented milks improved L. acidophilus tolerance to simulated gastrointestinal conditions, and only banana flour had a protective effect on B. animalis subsp. lactis. Thus, the microenvironments produced by the food matrix or ingredient in the intestine may protect the probiotic microorganism from the harsh conditions present in the gastrointestinal tract. Moreover, food components could bind to bile acids, reducing their toxic effect on probiotic cells (Begley, et al., 2005).

Interestingly, the observed patterns of survival after exposure to simulated gastrointestinal conditions were in contrast with the trends observed in the viable counts recorded during the refrigerated storage of PFM, since B. animalis subsp lactis showed higher viability than L. acidophilus. However, considering the usual portion of dairy beverages consumed, at least 100 mL, these results obtained in vitro suggests a relevant number of probiotic viable cells, ensures in the beverages a minimum dose of 10^6 cfu/mL suggested (Codex, 2010). Furthermore, the population of B. animalis was higher than L.acidophilus ($P < 0.01$) until 4h at pH 5.0 of assay.

Sensory evaluation of dairy beverages

A total of 63 panelists (41 females and 22 males; mean age = 24.2 ± 4.40 years old) participated in the study. There was no significant sensory preference between the PFM and the CFM, although the overall acceptability of the PFM was slightly higher than CFM (7.0 vs 6.6). The fermented goat milks showed scores greater than 6.5 in a 9-point hedonic scale in sensory evaluation for flavor, appearance, texture, color, and overall impression. Among the tested sensory characteristics, flavor received the lowest scores for all fermented milks. The color of the beverages was scored most highly for all two preparations. The results of the sensory evaluation of the dairy beverages are shown in Table 4.

It has been suggested that probiotic bacteria addition creates sensory advantages in dairy products (Castro, et al., 2013; Gomes, et al., 2011; Vinderola C. G & Reinheimer J. A, 2000). Furthermore, the L. acidophilus La-5 may produce flavor compounds, such as acetaldehyde, which are recognized as important flavor components (Ekinci & Gurel, 2008; Güler-Akın & Akın, 2007). However, in this study, despite the good score of flavor, no increase in acceptance by consumers was observed for PFM compared to CFM, possibly reflecting the contribution of the flavor

compounds in grape fruit juice. Martín-Diana et al., (2003) obtained lower scores for all sensory attributes for a non-flavored fermented goat's milk containing L. acidophilus La-5 and B. animalis BB-12. The incorporation of natural sugars into the dairy beverages base through addition of fruit juice has been proposed a key factor in the higher consumer acceptability of goat's milk beverages (Senaka Ranadheera, et al., 2012; Tranjan, et al., 2009), besides contributing with nutrients, which are not contained in milk, particularly, polyphenolics.

Additionally, the flavored fermented milks demonstrated good acidity levels, contributing to higher consumer acceptability in the present study.

General comments by the panellists regarding sensory attributes were also evaluated. The most common criticisms were related to the semi-liquid texture of the beverages and ‘‘goaty’’ taste. Senaka Ranadheera et al., (2012) related that complaints regarding the characteristic unpleasant ‘‘goaty’’ taste were not recorded for the 10% and 15% stirred fruit yogurts.

Table 4. Sensory evaluation scores of the fermented milks

Fermented milk	Flavor	Appearance	Texture	Color	Overall acceptability
Conventional	6.76 ± 1.99	6.87 ± 1.46	7.08 ±1.41	7.43 ± 1.34	6.65 ± 1.79
Probiotic	6.86 ± 1.89	7.14 ± 1.67	7.06 ± 1.50	7.27 ± 1.73	7.00 ± 1.53

Values are mean ± SD. Scores vary between 1 (dislike extremely) and 9 (like extremely). There were no significant difference between fermented milks.

4. Conclusion

The functional beverage tested in the present study showed to be adequate vehicle for the probiotics L. a cidophilus La-5 and B. animalis subsp. lactis BB-12 and exhibited a good sensory quality. Furthermore, the antioxidant profile of the probiotic fermented milk was higher than conventional fermented milk. Thus, this study presents relevant information on physicochemical, sensory, microbial and antioxidant properties of a probiotic fermented milk, which could guide the dairy industry in developing new probiotic products, primarily from caprine origin, besides characterize the product for use in future clinical trial.

Conflict of Interest

The authors declare no conflict of interest.

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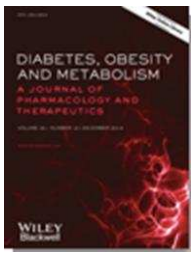

Article 3 – CLINICAL APPLICATION OF PROBIOTICS IN TYPE 2 DIABETES MELLITUS: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY

Article unpublished. Journal suggestion: Diabetes, Obesity and Metabolism. Impact Factor 5.456.

ABSTRACT

TONUCCI, Lívia Bordalo, D.Sc., Universidade Federal de Viçosa, December, 2014. **Clinical application of probiotics in type 2 diabetes mellitus: a randomized, double-blind, placebo-controlled study**. Advisor: Hércia Stampini Duarte. Co-Advisor: Karina Maria Olbrich do Santos, Sônia Machado Rocha Ribeiro and Leandro Licursi de Oliveira.

Aims: Type 2 diabetes has been associated with dysbiosis and one of the possible routes for restore a healthy gut microbiota is by the regular ingestion of probiotics. The aim of this clinical trial was to investigate the effects of probiotics on glycemic control, lipid profile, inflammation, oxidative stress and short chain fatty acids in T2D. **Methods:** In a double-blind, randomized controlled trial, 50 subjects were assigned to two groups: probiotic, consumed daily 120 g/d of fermented milk containing Lactobacillus acidophilus La-5 and Bifidobacterium lactis BB-12 or control, consumed daily 120 g/d of conventional fermented milk for 6 weeks. Anthropometric measurements, body composition, fasting blood and faecal samples were taken at baseline and after 6 weeks. **Results:** The study demonstrated a significant decrease in fructosamine levels ($p = 0.05$) and haemoglobin A_1c tended to be lower ($p = 0.07$) in probiotic group. TNF- α and resistin were significantly reduced ($p < 0.05$) and acetic acid was increased ($p < 0.01$) in both groups at the end of trial, while IL-10 was significantly reduced ($p < 0.001$) only in the control group. There was a significant difference between groups ($p < 0.05$) concerning mean changes of HbA_{1c}, total cholesterol and low-density lipoprotein. No significant changes from baseline were detected in plasma total antioxidant status and F2-isoprostane. **Conclusion:** Probiotic consumption improved the glycemic control in T2D subjects, however, the intake of fermented milks seems to be involved with others metabolic changes.

Keywords: Type 2 diabetes; gut microbiota; probiotics; inflammation; stress oxidative.

Introduction

Diabetes is undoubtedly one of the most challenging health problems of the 21st century affecting more than 382 million people [1]. Type 2 diabetes (T2D) accounts for 85% to 95% of all diabetes and is a complex chronic illness requiring multifactorial risk reduction strategies [2].

T2D is often associated with systemic inflammation [3] and increased oxidative stress [4], and preclinical evidence links both to β-cell dysfunction and/or insulin resistance [5-7]. The intake of probiotics have been demonstraded to reduce inflammation and oxidative stress markers, beyond the improvement of glycemic and insulin metabolims [8-11].

In addition, the intestinal microbiome also seems to be important to the pathophysiology of T2D [12-13]. Findings from two studies that used faecal samples suggested that functional changes in the gut microbiome might be directly linked to the development of T2D [13-14]. Various others mechanisms have been proposed to explain the influence of the microbiota on insulin resistance and T2D, such as metabolic endotoxemia [15], modifications in the secretion of the incretins [16] and short-chain fatty acids (SCFA) production [17-18].

Alterations in the gut microbiota as a result of probiotics and prebiotics intake have been reported [10, 19]. However, studies on the effects of probiotics on characteristics of T2D are mostly performed in animal models, reporting beneficial effects by some strains of Lactobacillus on glycemic control, stress oxidative or inflammation [10, 20-22].

The objective of this study was to investigate the efficacy of the intake of a flavored fermented milk containing Lactobacillus acidophilus La-5 and Bifidobacterium animalis BB-12 on glycemic control, lipid profile, inflammation, oxidative stress and faecal short chain fatty acids in T2D subjects.

Patients and Methods

Study design and subjects

The randomised, double-blind, parallel-group, placebo-controlled trial was carried out in Ceará, Brazil, during July 2013 to February 2014. The study was performed on 50 patients with T2D recruited from endocrinology clinics. For a value equal to 0.05 and a power of 80%, the sample size was computed as 22.5 per group, considerend serum interleukin (IL) - 6 levels obtained from the study by Mazloom et al.

[23]. This number was increased to 25 per group to accommodate the anticipated dropout rate. The randomization was stratified by gender and was done in 11 blocks of 4 and 1 block of 6 subjects, which were assigned equally between intervention groups. Subjects and investigators were blinded to the treatment. The clinical trial protocol was approved by the ethic committe (Federal University of Viçosa, MG, Brazil; protocol: 219.644/2013 – Appendex II), and the study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Harmonized Tripartite Guideline for Good Clinical Practice. All patients provided written informed consent (Appendex V).

Patients were eligible for inclusion if they were aged 35 – 60 years, body mass index (BMI) lower than 35 kg/m^2 and type 2 diabetes diagnosed for at least 1 year. Exclusion criteria included clinical evidence of chronic illness or gastrointestinal disorders; the presence of renal, hepatic, haematological or immunodeficiency diseases; acute coronary syndrome, stroke, or transient ischemic attack; history of cancer; smoking; any intake of probiotics, supplements, antiobesity and anti-inflammatory drugs and antibiotics in the three months preceding recruitment and pregnancy or breast-feeding. Recruitment was done by telephone and advertisements after indication of subjects by endocrinologist (Appendex VI).

Intervention

T2D subjects were randomly assigned to consume either 120 g/d of probiotic fermented milk containing Lactobacillus acidophilus La-5 and Bifidobacterium lactis BB-12 (probiotic group) or consumed 120 g/d of conventional fermented milk contained Streptococcus thermophilus TA-40 (control group) for 6 weeks. The fermented milks (FM) was delivered every 2 weeks and the patients were instructed to keep FM under refrigeration (4 ºC).

The subjects were instructed not to change their usual diets, level of physical activity, or other lifestyle factors, to avoid any changes in medication and unusual or excessive food and drink consumption throughout the intervention period. The study coordinator contacted the volunteers once a week to ask for adverse health events and to assess the adherence to treatment.

Fermented milks formulation and analyses

Probiotic and conventional FMs were developed in parthership with Embrapa

Goat and Sheep (Ceará, Brazil). Before fermentation, the goat milk was supplemented with sucrose and pasteurized at 90 ^{o}C for 15 min. Pasteurised milk was cooled to 43 ± 2 ^{o}C for the addition of the starter culture (S. thermophilus TA-40; Danisco, Sassenage, France), and the probiotic cultures (L. acidophilus La-5 and B. animallis subsp. lactis BB-12; Chr. Hansen, Hoersholm, Denmark). The fermentation process was conducted at 40^{o} ± 1 ^{o}C until reaching pH 5.0 ± 0.1. Next, the temperature of the FM was decreased to 4° C up to the following day. The beverages were flavored with of grape juice obtained from Embrapa Grape and Wine (Rio Grande do Sul, Brazil). Ingredients, chemical composition and antioxidants status of the probiotic fermented milk are shown in Table 1.

Fermented milks were sampled immediately after manufacture and delivered on the following day. Samples were refrigerated at 4°C and microbiological analysis was conducted every week during the trial. The continuous quality control of cell counts was done by plating serial dilution on Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) and incubating in an anaerobic jar with AnaeroGenTM (Oxoid Ltd, UK) at 37 °C for 48 h, or aerobically on M17 agar, containing lactose (Vetec, Duque de Caxias, Brazil, 5 g/L), for S. thermophiles TA-40 (Mortazavian et al., 2007). The analyses were performed in duplicate.

Microbiological analyses of the probiotic fermented milk showed that the average colony counts of probiotic bacteria on days 1, 7 and 14 were 7.72 x 10^{7}, 5.82 x 10^{7}, and 1.62 x 10^{6} cfu/g of L. acidophilus La-5 and 4.45 x 10^{8}, 1.84 x 10^{7}, and 1.56 x 10^{7} cfu/g of B. lactis BB-12, respectively. Both probiotic bacteria showed an appropiate survival rate during a 14 days storage time.

Sensory evaluation was carried out in fermented milks samples (probiotic and control groups) during the trial through acceptability tests, using the hybrid hedonic scale (1 = disliked extremely, 5 = neither liked nor disliked, 09 = liked extremely) focussing on attributes of colour, taste, flavour, consistency and overall acceptability (Peryam & Pilgrim, 1957). The overall acceptability of the probiotic and conventional FMs was 8.17 and 8.20, respectively.

Table 1. Ingredients, chemical composition and antioxidant capacity of the probiotic fermented milk

Item	Values
Ingredients (g/100g)	
Goat milk	75
Saccharose	5
Grape juice	20
Lactobacillus acidophilus	0.024
Bifidobacterium animalis subsp. lactis	0.024
Streptococcus thermophilus	0.003
Chemical composition (g/100g)[a]	
Ash	0.74 ± 0.01
Protein	2.18 ± 0.02
Lipids	2.64 ± 0.02
Total Dietary Fiber	0.14 ± 0.01
Carboydrate	11.5 ± 0.01
Energy (kcal)	78 ± 0.8
Phytochemicals antioxidants[b]	
Phenolics in CFM	0.179 ± 0.001
Phenolics in PFM	0.264 ± 0.01[†]
Antioxidant activity in CFM	65.35 ± 0.01
Antioxidant activity in PFM	83.30 ± 0.68[*]

Mean ± SD. The contents were determined on the seventh day of storage. [a] whole matter [b] Three replicate determinations. The total phenolic contents were estimated according to the Folin-Ciocalteu method [24] and expressed as mg gallic acid equivalents (GAE)/mL. The free radical scavenging capacity was determined by the DPPH assay as previously described [25] and expressed as % of radical scavenging activity. [†]$p < 0.001$ and [*]$p = 0.01$, from unpaired t test. CFM = conventional fermented milk, PFM = probiotic fermented milk.

Study measurements

All anthropometric and biochemical measures were conducted in a fasting state taken at baseline and after 6 weeks intervention. For the anthropometric measurements, participants removed their shoes and emptied their pockets. A single experienced

examiner performed all anthropometric measurements. Body weight and body composition were assessed by bioelectrical impedance (Tanita, model TBF-300, Tanita Corporation) in full compliance with the manufacturer guidelines. Waist circumference was measured using a nonstretchable measuring tape (Sanny, São Paulo, Brazil).

Dietary assessment was done using food records, which were conducted by the nutritionist in the first and last week of the intervention. A photographic record of the portions of food was used so as to improve the quality of the data collected. Dietary intake was analysed using the Avanutri Revolution software (Rio de Janeiro, Brazil) based on Brazilian food composition tables [26-27]. It was considerated regular physical activity when subjects did it for at least 30 minutes three times a week.

For the biochemical measurements, blood samples were obtained after a 12 hours overnight fast. Before the test day, the subjects were instructed not to consume alcohol and to refrain from heavy physical activity during 72 and 24 hours, respectively. Samples were centrifuged at 1,000 g for 10 min at 4 °C, aliquoted and analysed or immediately stored at – 80 °C for cytokines and oxidative stress analyses.

Clinical and laboratory assessments

Serum samples were analyzed for total cholesterol (TC) and lipoproteins by enzymatic colorimetric method (Bioclin kits); fasting plasma glucose (FPG) was quantified by enzymatic colorimetric method of glucose-oxidase (Beckman Synchron LX System; Beckman Coulter kit) and insulin through electrochemiluminescence method using the Modular Analytics DXI800 analyser (Beckman Coulter kit). Fructosamine was assayed by colorimetric method with reduction of nitroblue tetrazolium, NBT, using the AU 5800 analyser (Biosystems kit), and haemoglobin A1c (HbA_{1c}) by HPLC**,** using the Variant II analyser (Bio-Rad kit). The homeostasis model assessment index (HOMA-IR) was used as an indicator of insulin resistance and calculated as follows: HOMA-IR = fasting plasma insulin (μU/mL) x fasting plasma glucose (mmol/ L)/22.5 [28]. Insulin resistance was diagnosed using a cutoff value of 2.71 [29].

Determination of oxidative stress markers

Plasma samples were collected in vacutainers containing sodium citrate. Colorimetric assay (Sigma-Aldrich antioxidant assay Kit) was used to measure the plasma total antioxidant status (TAS). Plasma TAS is determined by the ability of

antioxidants in the sample to inhibit the peroxidase-mediated formation of the 2.2-azino-bis-3-ethylbensthiazoline-6-sulfonic acid ($ABTS^{+}$) radical by metmyoglobin. The amount of $ABTS^{+}$ produced was read at 405 nm. Plasma total F2 –isoprostane was measured by ELISA kit (Cayman`s 8-isoprostane EIA kit). Briefly, the plasma samples (400 μL) were hydrolysed with (10N) NaOH (100 μL) at 45 °C for 2 hours to measure both free and esterified isoprostane. After incubation, 100 μL of (10M) HCl was added and the samples were centrifuged at 1,500g for 10 minutes to remove precipitated proteins. This assay is based on the competition between 8-isoprostane and an 8-isoprostane- acetylcholinesterase (AChE) conjugate (8-Isoprostane Tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm.

Cytokine analyses

Cytokine concentrations were determined using a multiplexed bead immunoassay. This is a bead-based suspension array using the Luminex xMAP technology in which fluorescent-coded beads, known as microspheres, have cytokine capture antibodies on the bead surface to bind the proteins. The measures of 5 cytokines (IL-6, IL-10, TNF-α, adiponectin and resistin) were measured using the human Cytokine/Adipokine magnetic bead kits (Millipore Coproration). Assays were performed in 96-well filter plates, as previously described [30]. The cytokine concentrations were analyzed in the MagPix instrument (Luminex Corporation). The concentration of the samples was estimated from the standard curve using a fifth-order polynomial equation (Software xPonent/Analyst versão 4.2). Concentrations of cytokines were expressed in pg/mL for IL-6, IL-10 and TNF-α, ng/mL for adiponetcin and μg/mL for resistin.

Faecal SCFA analysis

Feces samples (5 - 10g) were collected at enrollment and on week 6 for SCFA analysis. Samples (blinded) were immediately placed at - 80°C and stored until analyzed. The extraction of SCFA (acetate, butyrate and propionate) was based on the method of Smiricky- Tjardes et al. [31]. Briefly, around 600 mg of frozen faeces were weighed and homogenised with the addition of 1ml of m-phosphoric acid solution (25 %). After incubation at room temperature for 30 min, the samples were centrifuged (Refrigerated microcentrifuge, Himac CT 15; Hitachi Koki Co) at 720 g for 30 min at 4

°C. Then, the supernatants were transferred to a new Ependorf tube. After a second centrifugation, the supernatants were collected and subsequently frozen at - 20°C. Before analysis, a third centrifugation and supernatant collection were performed. The final volume of supernatants from each duplicate was mixed together and homogenised. Butyric, propionic and acetic acids were measured by GC (model GC-17A; Shimadzuw). N2 was used as the carrier gas and the flux in the column was 1.0 ml/min. The temperatures of the injector and detector were set at 220 and 250 °C, respectively. Initial column temperature was 100°C sustained for 5 min, rising at 108 °C/min until it reached 185 °C. Next, the samples were injected (1 ml) through a Hamiltonw syringe (10 ml) in split system 5. The results were represented as per100 mg of faeces (% w/w).

Statistical Analysis

Statistical analysis was done using SPSS Statistics version 20 (IBM, Armonk, NY). All data were checked for normal distribution using Shapiro-Wilk test and Skewness/ Kurtosis. Data were reported as mean ± standard deviation (SD) and median and interquartile interval (P25 and P75 %), since some variables were not normally distributed (HbA_{1c}, HOMA-IR, IL-6 and acetic acid). Comparison between probiotic and control groups at baseline was tested using unpaired Student's t test or Mann-Whitney test. Paired Student's t test or Wilcoxon matched-pairs signed-rank test were used to analyze differences between baseline and endpoint values. Pearson or Spearman's correlation tests were performed to measure the degree of correlation between cytokine concentrations and glycemic control. A p value ≤ 0.05 was considered statistically significant.

Results

A total of 45 (90 %) subjects aged 35 to 60 (mean 51.40 ± 6.80) years concluded this study. Five patients were excluded from the statistical analysis because they needed to change their medication during the trial (n = 2, 1 from each group), use of antibiotic (n = 1, control group) or they did not consume the fermented milk according to the plan (n = 2, 1 from each group). Abdominal discomfort was the only reported adverse effect. This subject (n = 1) belonged to the palcebo group and was withdrawal from the study.

Baseline characteristics are shown in Table 2. The main drugs used were metformin (94% in the probiotic group and 95.5% in the control group) and

glibenclamide (44% in the probiotic group and 41% in the control group). At the baseline, no relevant differences could be detected in the general characteristics between control and probiotics group, except for HbA_{1c} ($p = 0.04$), which was higher in probiotic group. There were no statistically significant differences in anthropometric and body composition values between or within groups at the end of the study.

Table 2. Baseline characteristics of the type 2 diabetes participants by control and probiotic treatment

Characteristics	Control (n = 22)	Probiotic (n = 23)	p value
Age (y)	50.95 ± 7.20	51.83 ± 6.64	0.73
Sex (M/F)	14/8 (63/37)	12/11 (53/47)	0.45
Diabetes duration (y)	4.5 (2 – 15)	6.0 (2 -17)	0.51
Regular physical activity	12 (54.5)	09 (39.1)	0.31
Weight (kg)	77.15 ± 13.85	71.70 ± 12.43	0.16
BMI (kg/m^2)	27.94 ± 4.15	27.49 ± 3.97	0.65
Total body fat (%)	32.95 ± 8.96	33.92 ± 8.24	0.70
HbA_{1c} (%)	5.35 (4.86 – 6.15)	6.07 (5.39 – 7.0)	0.04*
FPG (mmol/L)	7.38 ± 2.39	7.97 ± 2.31	0.41
HOMA-IR	2.15 (1.71 – 3.26)	2.63 (1.57 – 3.51)	0.55
Metformin (mg)	997.74 ± 460.50	1123.91 ± 578.76	0.36
Glibenclamide (mg)	5.02 ± 12.60	9.78 ± 23.84	0.83

Data are presented as mean ± SD, n (%) or median (P25-P75). *Significant difference between groups at baseline ($p < 0.05$ from Student's t test or Mann–Whitney test). Y = years; M= masculine; F= female; BMI = body mass index; HbA_{1c} = haemoglobin A1c; FPG = fasting plasma glucose.

Energy and nutrient intakes

At the beginning of the study, no significant differences were found between the two groups in terms of dietary intakes. Comparing the dietary intakes throughout the study separately in each group, we observed no significant within group differences (Supplementary table – Appendex VII).

Impact of the intervention on glicemic control

Biochemical markers after probiotic treatment are show in Table 3. At 6-week follow-up, probiotic fermented milk consumption significantly decreased fructosamine levels ($p = 0.05$) and HbA_{1c} levels tended to be reduced ($p = 0.07$), while in the control group any significant effect was detected on glycemic control ($p > 0.05$). When the mean changes in HbA_{1c} were compared between groups, there was a significant difference (+ 0.11 for control group vs. - 0.40 for probiotic group, $p = 0.02$). The fasting plasma glucose (FPG), insulin concentrations, as well as insulin resistance, evaluated by the HOMA index, did not change significantly throughout the follow-up period in both groups ($p > 0.05$).

Effect of probiotics on lipid profile

Within group comparisons of lipid profile revealed that consumption of probiotic fermented milk prevented a rise in total cholesterol (TC) and LDL-C, while in the control group we observed a significant increase in the TC and LDL-c (11,35% and 16,10%; $p = 0.01$ and $p = 0.04$, respectively). Furtermore, when the mean changes were compared between the two groups, there was a statistically significant difference in the TC and LDL-C (+ 0.55 and + 0.36 for control vs. – 0.15 and – 0.20 mmol/L for the probiotic group, $p = 0.04$ and $p = 0.03$, respectively). At the end of trial, no significant effect in the HDL-C, VLDL and triglyceries were found in both groups ($p > 0.05$). The TC:HDL-C ratio was significantly increased by 8.94% in the control group during the study ($p = 0.03$), while no statistically significant changes was reported in probiotic group. Also, at the study baseline, no significant differences in lipid profile were found between probiotic and control groups (Table 3).

Table 3. Metabolic parameters of type 2 diabetes subjects at baseline and endpoint by fermented milk treatment

Measure	Control (n = 22)				Probiotic (n = 23)				
	Week 0	Week 6	Change[a]	P[b]	Week 0	Week 6	Change[a]	P[b]	P[c]
FPG, mmol/L	7.38 (2.39)	7.54 (2.52)	0.16	0.65	7.97 (2.31)	8.49 (2.43)	0.48	0.14	0.48
Fru, mmol/L	297.3 (65.52)	298.72 (65.17)	0.01	0.86	305.65 (55.99)	295.50 (56.06)	- 10.1	0.05*	0.33
HbA_{1c}, %	5.35 (4.8-6.1)	5.66 (4.9-6.7)	0.31	0.82	6.07 (5.3-7.0)	5.39 (5.1-7.2)	- 0.68	0.07	0.02*
Insulin, µU/mL	7.89 (2.80)	7.79 (3.29)	- 0,10	0.81	8.52 (4.60)	8.12 (3.87)	- 0,40	0.50	0.70
HOMA-IR	2.14 (1.7-3.2)	2.30 (1.5-3.2)	0.16	0.76	2.63 (1.5-3.5)	2.65 (1.7-4.2)	0.02	0.41	0.77
TC, mmol/L	4.85 (1.32)	5.30 (1.19)	0.55	0.01*	4.66 (1.38)	4.51 (1.11)	- 0.15	0.52	0.04*
LDL-C, mmol/L	2.24 (1.24)	2.60 (1.11)	0.36	0,04*	2.31 (1.17)	2.11 (0.82)	- 0.20	0.31	0.03*
HDL-C, mmol/L	1.51 (0.26)	1.53 (0.34)	0.02	0,59	1.56 (0.29)	1.53 (0.33)	- 0.03	0.50	0.38
TC:HDL-C	3.20 (1.06)	3.48 (0.97)	0.28	0.03*	2.98 (0.64)	2.94 (0.72)	- 0.04	0.75	0.35
LDL-C:HDL-C	1.48 (0.93)	1.69 (0.83)	0.21	0.08	1.48 (0.64)	1.37 (0.58)	- 0.11	0.30	0.10
TAG, mmol/L	1.83 (0.72)	1.99 (0.91)	0.16	0,73	1.60 (0.63)	1.68 (0.63)	0.08	0.28	0.62
TAS, (mM)	0.31 (0.12)	0.33 (0.08)	0.02	0.23	0.31 (0.09)	0.33 (0.11)	0.02	0.39	0.87
F2-iso, (pg/mL)	62.99 (33.67)	63.42 (32.35)	- 0.43	0.94	74.66 (30.20)	72.49 (34.70)	- 2.17	0.76	0.78

Data are means ± SD or median (P25-P75). [a] Change from baseline to follow-up. [b] Obtained from paired t test/Wilcoxon matched-pairs signed-rank test for the within-group comparisons (baseline vs. endpoint). [c] Obtained from unpaired Student's t test or Mann–Whitney test, as statistical significance between changes (control vs. probiótico). *$p \leq 0.05$. FPG = fasting blood glucose; Fru = fructosamine; TC = total cholesterol; TAG = triglyceride; TAS = total antixodant status; F2-iso = F2-isoprostane.

Effect of probiotics on markers of oxidative stress

At baseline, there was no difference between groups in TAS and F2-iso levels ($p = 0.86$ and $p = 0.25$, respectively). No significant difference was detected with regard to plasma TAS concentrations from baseline to postintervention in probiotic or control groups ($p = 0.40$ and $p = 0.23$, respectively); changes in TAS were also not significant over time between groups ($p = 0.78$). Plasma F2-iso concentrations did not change in the probiotic or control groups over time ($p = 0.76$ and $p = 0.94$, respectively). There was also no difference in plasma F2-iso change score between groups ($p = 0.78$).

Effect of probiotics on cytokines levels

At the end of trial, the two groups that consumed fermented milk presented reduction in TNF- α and resistin ($p < 0.05$ and $p \leq 0.01$, respectively). Anti-inflammatory markers, such as IL-10 and adiponectin, decreased after intervention in control group ($p < 0.001$ for IL-10 and $p = 0.07$ for adiponectin), while no significant change was observed in the probiotic group ($p = 0.38$ and $p = 0.14$, respectively). No significant differences in IL-6 levels were observed in any groups post intervention. Reductions in resistin were positively correlated with reductions in HbA_{1c} ($r = 0.320$; $p = 0.03$). No correlation was found between others cytokines and markes of glycemic control (data not shown). In Figure 1 it is possible to compare the cytokines concentration according the groups.

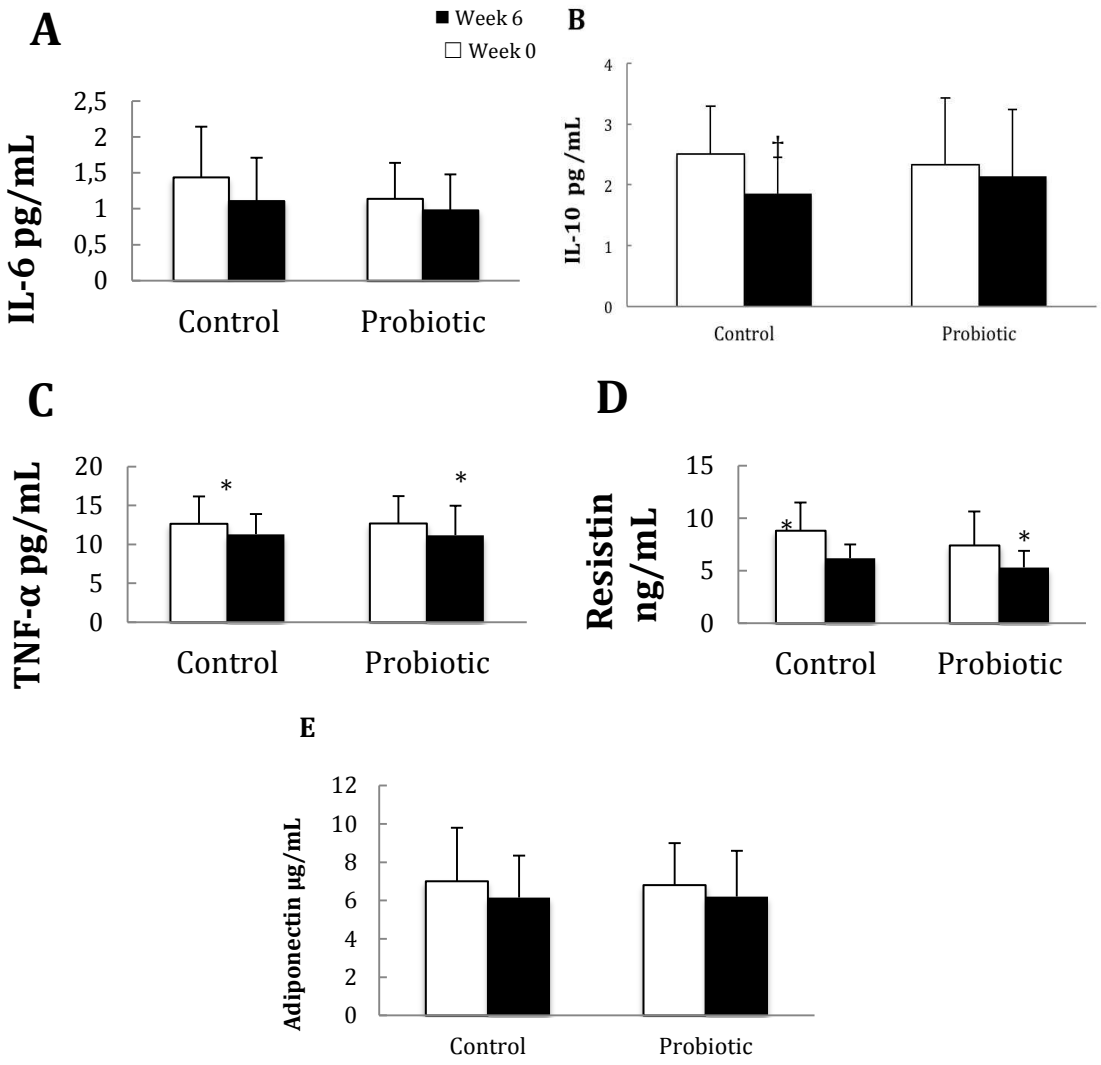

Figure 1. Effects of the fermented milks intake on cytokine levels. Data are shown as mean ± SD. $^{*}p \leq 0.05$, $^{\dagger}p < 0.001$ from paired t test or for Wilcoxon matched-pairs signed-rank test, as statistical within group differences (baseline vs. endpoint). IL = interleukin; TNF- α = tumor necrosis factor alpha.

Effect of probiotics on faecal SCFA analysis

Data from faecal analysis showed a significant increase in the acetic acid in both groups ($p < 0.05$) at the end of the 6 weeks treatment. However, there were no significant differences in the butyric and propionic acids after intervention in control and probiotic groups, as shown in Figure 2. Additionally, no significant difference was found between changes of the two groups in butyric, acetic and propionic acids ($p > 0.05$). Interestingly, a higher proportion of propionic acid and a lower proportion of butyric acid were recorded in both groups. At end of trial, the proportion of propionic:

acetic: butyric acids, taking into account the mean values, was also similar: 10: 8: 1 in the control group and 14: 10: 1 in the probiotic group.

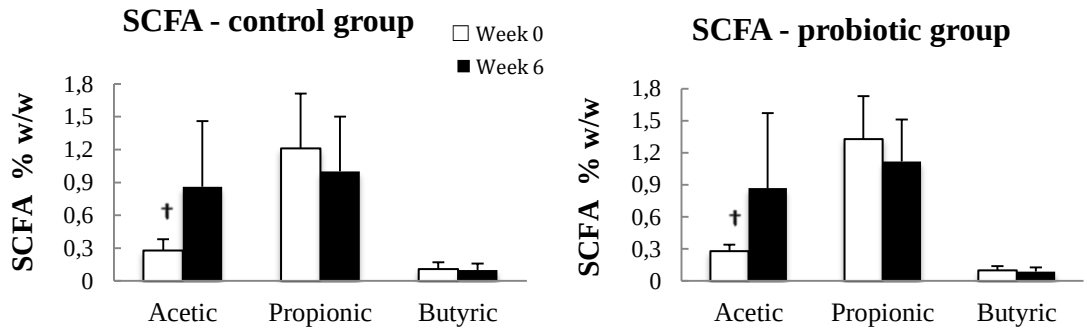

Figure 2. Effects of the fermented milks intake on faecal short-chain fatty acid concentrations. Data are shown as mean ± SD or median. $^{\dagger}p < 0.001$ from paired t test or for Wilcoxon matched-pairs signed-rank test, as statistical within group differences (baseline vs. endpoint).

Discussion

This is the first clinical trial to assess the impact of probiotic use on fructosamine, faecal SCFA, IL-10, resistin, adiponectin and F2-isoprostane in T2D patients. It is speculated that improvement in the inflammatory markers, stress oxidative status and SCFA contributes to diabetes control [19, 32-33].

The present study showed that the fermented milk consumption significantly decreased inflammatory cytokines (TNF-α and resistin) in both treatments (control vs probiotic), as well as caused a significant increase in the acetic acid, but only probiotic group showed improvement in glycemic control, as asseded by fructosamine and HbA_{1c}. The main differences between the results of the two treatments (control vs probiotic) were: significant decrease in IL-10, adiponectin levels tended to be lower and significant increase in TC and LDL-C, which occurred only in the control group. These results indicate that these last factors hinder the improvement in glycemic control of subjects in the control group. Furthermore, total phenolic content and antioxidant activity were significantly higher in the probiotic fermented milk (see Table 1).

Most of the previous animal studies that evaluated the effect of probiotics on glycemic control in T2D, related that probiotics, especially Lactobacillus, can reduced FPG, HbA_{1c} and insulin, beyond inflammatory markers, such as IL-2, INF-γ, IL-6 and TNF-α [10, 34-38]. The intervention time ranged from 4 to 16 weeks. Increase in

GLUT4 mRNA expression [10, 22] has also been reported.

Concerning clinical trials, previous studies including diabetic subjects have shown controversial results, especially in regard to glycemic control and oxidative stress. Similarly to present study, Ejtahed et al.,[39] reported reductions in HbA_{1c} ($p < 0.05$) after 6 weeks of intake of yogurt containing 10^{10} CFU of L. acidophilus La-5 and B. lactis BB-12. On the other hand, L. acidophilus NCFM capsule (10^{10} CFU) intake during 4 weeks, did not change the glycemic control, insulin, IL-1, IL-6, TNF- α and protein C reactive (CRP) in T2D subjects [40]. Also, multispecies probiotic supplementation (L. acidophilus, L. casei, L. rhamnosus, L. bulgaricus, B. breve, B. longum and Streptococcus thermophillus - 10^9 CFU each) associated with fructo-oligosaccharide (100 mg) during 8 weeks did not provide significant reductions in FPG and a significant increase in the levels of insulin, HOMA-IR and LDL-C was found in both groups [41]. Subsequently, this same research group reported that the daily consumption of Bacillus coagulans (10^7 CFU) and 1.08g of inulin during 6 weeks, not resulted in changes in FPG and HOMA-IR ($p > 0.05$), although a significant decrease in hs-CRP and a significant increase in glutathione peroxidase (GPx) activity was found [42].

The measurement of plasma F2-isoprostane is considered the best measure of plasma oxidative stress and TAS gives the sum total of both exogenous as well as endogenous antioxidants [43-44] showing the complete antioxidant status picture. It has been suggested that the effects of probiotic drinks on glycemic control can be partly explained by the effects on oxidative stress [45-46]. However, the present study did not show a significant impact of probiotics on F2-isoprostane or TAS plasma levels in T2D subjects, as well as previously clinical trial that evaluated TAS after 6 weeks intake of B. coagulans (10^7 CFU) and 1g of inulin in 124 diabetic patients [42]. This relationship was not also observed after consumption of probiotic yogurt containing L. acidophilus La-5 and B. lactis BB-12 for 6 weeks, which increased the erythrocyte superoxide dismutase (SOD) and GPx activities, and TAS ($p < 0.05$) but no significant changes were observed in HbA_{1c}, insulin and erythrocyte catalase activity [39].

We hypothesized that the improvement in glycemic control after probiotic treatment could be mediated, in part, through immune-modulatory effects. To capture these interrelations, were selected a set of key inflammatory and antiinflammatory citokines, including adipokines produced primarily by adipocytes (adiponectin), macrophages (resistin), or both (TNF-α), all related to glycemic control and insulin

resistance [3, 47-48].

Although previous studies mention the relationship between inflammation and gut microbiota [15, 49], few experimental and clinical studies investigated this association in the diabetes mellitus context [10, 34, 40]. Some experimental studies reported reductions in inflammatory cytokines, and/or improvements in glucose and insulin metabolims after ingestion of Lactobacillus spp during 14 weeks [10, 34]. Two randomized, control controlled trial reported no observed effects on inflammatory cytokines after 4 or 6 weeks of L. acidophilus NCFM (10^{10} CFU) or L. acidophilus, L. bulgaricus, L. bifidum and L. casei (CFU and strain not reported), respectively [23, 40]. However, in the present study, TNF- α and resistin levels decreased in both treatments while IL-6 not changed. As far as cytokine release is concerned, goat's milk seems to be a poor trigger of these mediators [50-51]. Thus, it is not appropriate to justify the results in this way. A parallel question was whether fermented milks can influence these biomarkers differently, once bioactive peptides seems to activate innate immunity by stimulating macrophages and cytokine production [52-55].

In regard to anti-inflammatory cytokines (IL-10 and adiponectin), no changes were observed in the probiotic group, unlike the control group, which showed a significant reduction at the end of trial. Thus, our results suggest that the immune-modulatory effect reflected on glycemic control of probiotic group without interfering with insulin and HOMA-IR. Recently, Mohamadshahi et al. [56], also reported that the consumption of probiotic yogurt enriched with L. acidophilus La-5 and B. lactis BB-12 (10^6 CFU) for 8 weeks caused significant decrease in HbA_{1c} and TNF-α levels in the intervention group, but no change was related in IL-6 and hs-CRP levels. In mice, L. rhamnosus GG orally administrated for 13 weeks improves insulin sensitivity by stimulating adiponectin secretion and consequent activation of AMPK [57]. These controversial results may be explained by some of the studies limitations, like the number of subjects and short period of intervention or be related to the type of microorganism used.

Additionally, microbiota components account for the production of SCFA, which are linked to anti-inflammatory mechanisms (inhibition of NF-κB) and also exerting a protective function in favor of intestinal epithelium [58]. However, reports on the ability of lactic acid bacteria to modulate SCFA at the intestinal level are limited. In our literature review [66], were not found experimental or clinical trials that evaluated the effect of the intake of probiotics on the SCFA in T2D context. Interestingly,

growing evidence suggests a cross talk between SCFA and improves in glycemic control and insulin sensitivity, especially butyric and propionic acids via the G protein coupled receptors FFA1, FFA2 and FFA3 [18, 59]. However, we found a significant increase in acetic acid after intervention in both groups, which seems not to induce insulin secretion from pancreatic b cells through fatty acid receptor 1 (FFA1 or GPR40) [60] or improve control glycemic through gut hormones peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) [18]. This increase in the acetic acid in both treatments may be attributed to phenolic compounds present in FMs [61]. Another important finding was the greater amount of SCFA in T2D subjects, especially propionic acid. Previous studies suggest that the higher faecal concentration of SCFA is associated with metabolic risk factors and thus may influence metabolic homeostasis [62-63].

With regard to lipid profile, controversial results have also been shown in the literature. L. acidophilus La-5 and B. lactis BB-12 (10^6 CFU) use contributed to decrease the TC (4.5%) and LDL-C (7.5%) levels ($p < 0.05$) after 6 weeks in T2D subjects (Ejtahed et al., 2011). In a different study, this same probiotics taken for 8 weeks, caused reductions in LDL-C/ HDL-C ($p = 0.01$) and increased HDL-C [64]. However, other studies using different species of Lactobacillus or B. coagulans and inulin for 6 weeks also observed no effects on the lipid profile in T2D subjects [23, 42]. In this present study, we demonstrated that consumption of probiotic FM prevented a rise in TC and LDL-C compared to the control group, which can be attributed, in part, by the higher total phenolic concentrations and antioxidant capacity in probiotic FM.

Finally, probiotics as functional foods offer great potential to improve health and/or help prevent certain diseases when taken as part of a balanced diet and healthy lifestyle. Our results showed low intake of micronutrients and dietary fiber and high intake of SFA.

Limitations of this trial include the HbA_{1c} dosage that was done imediatelly at end of the study, because subjects did not come back after one month, as would be the most appropriate. The same seems to have occurred or is not mentioned in the methodology of previous clinical trials [39, 56]. Furthermore, none of the above-mentioned studies directly assessed the relationship between the observed metabolic changes and the gut microbiota composition. In our study, analysis of the microbiota is being conducted and will be published elsewhere.

Conclusion

The results of this trial suggest that probiotic consumption improved the glycemic control in T2D subjects, however, the intake of fermented milks seems to be involved with others metabolic changes. The major findings of this study are the following: (i) L. acidophilus and B. lactis intake seem to be associated with a modulation of the fructosamine and HbA_{1c} levels; (ii) fermented milks consumption appears to be related to acetic acid content in faecal samples; (iii) a potential role of the probiotic product in counteracting the increase in CT and LDL-C and the reduction in anti-inflamatory cytokines. Because our study was short-term, our findings need to be confirmed in larger trials of longer duration to test the hypothesis that probiotic supplementation is effective to improve control glycemic, regulate levels of SCFA, low-grade inflammatory and stress oxidative in T2D subjects.

Conflict of Interest

The authors declare no conflicts of interest.

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GENERAL CONCLUSIONS

The probiotics, particularly, Lactobacillus sp. and Bifidobacterium sp. have potential therapeutic for metabolic control in diabetics. However, the presence of controversial results in this study and in other clinical trials, hinders the understanding of the real efficacy of probiotics in improving glycemic and metabolic control of diabetic subjects and to determine the main mechanisms involved in this process. The efficacy of the daily intake of probiotics in clinical trials is more complex than in experimental studies, since it becomes difficult to control all the variables which are currently proposed for interfering in the microbiota, such as, stress, diet characteristics contamination environmental, dose and time of use of drugs and hormonal factors. In addition, type 2 diabetes mellitus has a multifactorial etiology and a wide variety of drugs are used to improve metabolic control in these individuals and little is known about the concentrations of SCFA fecal and microbiota profile. Regarding to probiotic bacteria, a better understanding of their action is important to explore its potential biotherapeutic in diabetes control. In this sense, the therapeutic effects of probiotics in different clinical situations are strain-specific and closely related to the host. Thus, it is imperative to carry out well-designed clinical trials involving a wide range of diabetic subjects with clearly defined proven probiotic strains and their formulations, besides of adequate nutritional characterization of products to reach some meaningful conclusion as far as their efficacy against T2D is concerned.

APPENDEX

APPENDEX I

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Fergus M. Clydesdale,
Distinguished Professor and
Director of the Food Science Policy Alliance
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June 20, 2014

To whom it may concern:

This is to certify that the paper referenced , **BFSN-2014-1282.R1, Clinical Application of Probiotics in Diabetes Mellitus: Therapeutics and New Perspectives** , coauthored by Dr. Livia Bordalo Tonnuci (first author) was accepted June 10, 2014, for publication in the Journal Critical Reviews in Food Science and Nutrition of which I am Editor in Chief.

Sincerely

Fergus M. Clydesdale Distinguished Professor, Director of the Food Science Policy Alliance and Editor in Chief of Critical Reviews in Food Science and Nutrition

APPENDEX II

UNIVERSIDADE FEDERAL DE VIÇOSA - UFV

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: EFEITO DO CONSUMO DE BEBIDA LÁCTEA FERMENTADA CONTENDO PROBIÓTICOS SOBRE O CONTROLE GLICÊMICO, ESTRESSE OXIDATIVO E CITOCINAS INFLAMATÓRIAS EM INDIVÍDUOS COM DIABETES MELLITUS TIPO 2

Pesquisador: Hercia Stampini Duarte Martino

Área Temática:

Versão: 3

CAAE: 13380413.8.0000.5153

Instituição Proponente: Departamento de Nutrição e Saúde

Patrocinador Principal: FUNDACAO DE DESENVOLVIMENTO DA PESQUISA

DADOS DO PARECER

Número do Parecer: 219.644

Data da Relatoria: 05/04/2013

Apresentação do Projeto:

Projeto apresentado em parecer anterior, submetido ao Colegiado do CEP/UFV.

Objetivo da Pesquisa:

Os objetivos já foram apresentados em parecer anterior, submetido ao Colegiado do CEP/UFV.

Avaliação dos Riscos e Benefícios:

Apresentados em parecer anterior, submetido ao Colegiado do CEP/UFV.

Comentários e Considerações sobre a Pesquisa:

Não são necessários. Trata-se, no momento, de ajustes de documentos já avaliados anteriormente.

Considerações sobre os Termos de apresentação obrigatória:

Todos foram devidamente apresentados, com as determinações estabelecidas pelo CEP/UFV em reunião plenária.

Recomendações:

Nenhuma

Conclusões ou Pendências e Lista de Inadequações:

Parecer aprovado, tendo em vista trata-se de nova apresentação de documentos, não se faz necessário submeter, novamente ao Colegiado do CEP.

APPENDEX III

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO I

Convidamos o(a) Sr(a) a participar da avaliação sensorial do bebida láctea caprina adicionada de Lactobacillus acidophillus e Bifidobacterium lactis, sob a responsabilidade da Pesquisadora Karina Maria Olbrich dos Santos, da Embrapa Caprinos e Ovinos, em Sobral-CE e Livia Bordalo Tonucci, da Universidade Federal de Viçosa, Minas Gerias. O objetivo da pesquisa é o desenvolvimento de produtos lácteos caprinos com potencial probiótico utilizando bactérias láticas.

Sua participação é voluntária e se dará por meio da degustação de amostras da bebida láctea para avaliarmos a aceitação do produto por consumidores potenciais. Sua participação não implicará em riscos a sua saúde e, caso aceite participar, estará contribuindo para o desenvolvimento científico nacional.

Se depois de consentir a sua participação o Sr(a) desistir de continuar participando, tem o direito e a liberdade de retirar seu consentimento em qualquer momento da pesquisa, seja antes ou depois da coleta dos dados, independente do motivo e sem nenhum prejuízo a sua pessoa.

Os resultados da pesquisa serão analisados e publicados, mas sua identidade não será divulgada, sendo mantida em sigilo.

Para qualquer outra informação, o(a) Sr(a) poderá entrar em contato com o pesquisador no endereço Estrada Sobral/Groaíras Km 04, Sobral – CE, telefone (88) 3112-7562, ou com o Comitê de Ética em Pesquisa – CEP da Universidade Estadual Vale do Acaraú - UVA, na Av. Comandante Mauricélio Rocha Ponte, nº 150, Derby, Sobral - CE, Fone/Fax: 3677-4255.

Consentimento Pós–Informação

Declaro que fui informado sobre o projeto de pesquisa e sobre minha colaboração, e compreendi os objetivos. Por isso, concordo em participar da pesquisa, sabendo que a qualquer momento posso retirar meu consentimento em participar. Este documento é emitido em duas vias que serão ambas assinadas por mim e pelo pesquisador, ficando uma via com cada um de nós.

Data: / / .

Ciente: _____

Assinatura do participante

Knol

_____ _____

Assinatura do Pesquisador Responsável Assinatura do Pesquisador Responsável

APPENDEX IV

Teste de Aceitabilidade

Nome: _____ Data: ___/____/_____

Sexo: Masc. ( ) Fem ( ) Idade: _____

Produto: Leite fermentado caprino sabor uva

Prove a amostra e expresse o quanto você gostou ou desgostou de suas características utilizando a escala abaixo:

9- gostei muitíssimo
8- gostei muito
7- gostei moderadamente
6- gostei ligeiramente
5- nem gostei, nem desgostei
4- desgostei ligeiramente
3- desgostei moderadamente
2- desgostei muito
1- desgostei extremamente

Amostra nº: _____

Aparência _____
Sabor _____
Cor _____
Consistência _____
Aceitação global _____

 Cite a característica que você mais gostou na amostra e comente:

_____
_____

 Cite a característica que você menos gostou na amostra e comente:

_____
_____
_____

APPENDEX V

Termo de Consentimento Livre e Esclarecido II

Você está sendo convidado a participar, como voluntário, em uma pesquisa para realização de um trabalho de doutorado em Nutrição pela Universidade Federal de Viçosa/MG em parceria com a Embrapa Caprinos e Ovinos (CE). Após ser esclarecido sobre as informações da pesquisa, assine ao final deste documento caso aceite fazer parte deste estudo. Em caso de recusa você não será penalizada de forma alguma.

Informações sobre a Pesquisa:

Título do Projeto: "Efeitos do consumo de bebida láctea fermentada contendo probióticos sobre o controle glicêmico, estresse oxidativo e citocinas inflamatórias em indivíduos portadores de diabetes mellitus tipo 2"

Pesquisador responsável: Lívia Bordalo Tonucci (Telefone: 88 8809-9885)

- A pesquisa tem por objetivo avaliar o efeito de uma bebida láctea contendo probióticos sobre alguns parâmetros metabólicos, a fim de contribuir com o melhor controle glicêmico de pacientes diabéticos.

- Para participar do estudo todos os participantes deverão ingerir 120 mL de bebida láctea sabor uva, no período noturno, durante 45 dias. Distribuiremos, semanalmente, as bebidas para você.

- A pesquisa consistirá em avaliação nutricional e na realização de exames laboratoriais. Para isso precisaremos medir o peso, a altura, a circunferência da cintura, a composição corporal e coleta de sangue e fezes, todos em dois momentos: antes e após a realização do estudo. Também será realizado o preenchimento de um registro sobre a sua alimentação.

- A ingestão diária das bebidas lácteas não proporcionará efeitos adversos aos participantes do estudo.

- As informações coletadas ficarão sob responsabilidade do pesquisador que se comprometerá com o sigilo das mesmas.

- O voluntário poderá se retirar da pesquisa, a qualquer momento, antes do seu término.

- Sua participação neste estudo não implica em contrato de trabalho, e não haverá nenhuma compensação financeira.

_____

Profª. Dra Hercia Martino (responsável pelo projeto)

_____

Pesquisador responsável

_____

Voluntário

APPENDEX VI

Questionário de triagem e controle

CRITÉRIOS DE INCLUSÃO/ EXCLUSÃO	SIM	NÃO
Idade entre 30 e 59 anos?	☐	☐
IMC menor que 30 kg/m^2?	☐	☐
DM tipo 2 há mais de um ano?	☐	☐
Utiliza insulina atualmente?	☐	☐
É portador de alguma outra patologia?	☐	☐
Fuma ou usa bebida alcoólica frequente?	☐	☐
Faz uso de algum suplemento (vitamina)?	☐	☐

GRUPO: ( )A ( )B

CODIGO DO PACIENTE:____

Data: ____/_____/_____

I. Informações gerais:

Nome: _____Data de nascimento: ____/_____/____

E-mail:_____ _____Telefones:_____

Endereço: _____

Pratica de atividades física regular: Não □ Sim □

Descrição:_____

Diagnóstico de DM2 há (anos ou meses):_____

Medicamentos

utilizados:_____

Dosagens:_____

Dados antropométricos:

Dados	**Periodo antes da intervenção**	**Período pós-intervenção**
Peso (kg)		
Altura (m)		

CC (cm)		
IMC (kg/m^2)		
% de gordura		
% de massa magra		

Dados bioquímicos:

Dados	**Antes da intervenção**	**Pós- intervenção**
Glicemia de jejum (mg/dL)		
Frutosamina		
Hemoglobina Glicada (%)		
LDL-c (mg/dL)		
Colesterol Total (mg/dL)		
Triglicerídeos (mg/dL)		
HDL-c (mg/dL)		
Insulina (mmol/L)		

TCLE: ( ) Ok ( ) Não ok **Fezes:** ( ) Ok ( ) Não ok
Recordatorio habitual: ( ) Ok ( ) Não ok

CONTROLE DE ENTREGA DAS BEBIDAS (relate observações):

Semana 1:_____

Semana 2:_____

Semana 3: _____

Semana 4: _____

Semana 5: _____

Semana 6: _____

Algum efeito adverso observado?_____ _____

OBSERVAÇÕES:_____

APPENDEX VII

Table suplementary. Dietary intakes of type 2 diabetes participants at baseline and endpoint by fermented milk treatments

	Control (n = 22)		Probiotic (n =23)	
	Baseline	Endpoint	Baseline	Endpoint
Energy (kcal)	1997.62 ± 413.52	2064.84 ± 340.69	2004.10 ± 529.20	1950.60 ± 493.84
Carbohydrate (g)	256.92 ± 55.55	265.48 ± 44.78	261.20 ± 75.84	260.56 ± 68.95
Protein (g)	92.55 ± 23.27	91.26 ± 20.62	105.95 ± 49,13	100.85 ± 36.22
Total fat (g)	67.08 ± 24.42	69.61 ± 25.43	60.65 ± 21,90	61.65 ± 20.68
SFA (g)	23.24 ± 9.97	23.59 ± 10.66	21.82 ± 11.54	20.05 ± 8.46
MUFA (g)	16.33 ± 5.96	18.81 ± 6.38	14.43 ± 6,95	14.89 ± 8,03
PUFA (g)	10.00 ± 4.35	10.64 ± 4.53	7.43 ± 3.87	7.96 ± 3.71
Dietary fiber (g)	17.49 ± 10.80	15.12 ± 8.00	16.14 ± 7.30	14.48 ± 6.34
Vitamin A (μg)	353.17 ± 168.04	384.28 ± 162.77	387.02 ± 238.60	403.14 ± 276.40
Vitamin E (mg)	7.70 ± 5.16	8.68 ± 7.07	5.86 ± 5.00	7.49 ± 5.49
Vitamnin C (mg)	78.73 ± 65.70	64.54 ± 55.00	83.01 ± 61.21	74.80 ± 48.14
Copper (mg)	0.83 ± 0.35	0.85 ± 0.29	0.81 ± 0.29	0.75 ± 0.20
Zinc (mg)	8.43 ± 4.24	9.42 ± 4.16	8.13 ± 4.27	7.76 ±4.70
Calcium (mg)	834.64 ± 320.66	896.90 ± 290.60	626.80 ± 375.00	694.93 ± 351.40

Data are presented as mean ± SD. SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.