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Leite fermentado e tecido adiposo visceral – possível efeito emagrecedor em obesos e portadores de síndrome metabólica

Natália Pratis Perina

Tese para obtenção do grau de

DOUTOR

Orientadora:

Profª Drª Maricê Nogueira de Oliveira

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Natália Pratis Perina

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Tese apresentada à Faculdade de Ciências Farmacêuticas da Universidade de São Paulo para obtenção do título de Doutor em Ciências

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Banca Examinadora

Profª. Drª. Maricê Nogueira de Oliveira

Orientadora / Presidente

Professor_____Assinatura_____

1º examinador

Professor_____Assinatura_____

2º examinador

Professor_____Assinatura_____

3º examinador

Professor_____Assinatura_____

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Professor_____Assinatura_____

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À minha família,

Por todo o suporte, compreensão e amor incondicional.

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“A grande conquista é o resultado de pequenas vitórias que passam despercebidas.”

Paulo Coelho

RESUMO

Perina, N.P. **Leite fermentado e tecido adiposo visceral – possível efeito emagrecedor em obesos e portadores de síndrome metabólica.** 2015. 167 f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2015.

O trato gastrointestinal de humanos é rico em microrganismos que, podem tanto ser benéficos para a saúde do hospedeiro, prevenindo e/ ou tratando a intolerância à lactose, constipação intestinal, síndrome do intestino irritável, entre outras, quanto podem prejudicá-lo, afetando a aquisição de nutrientes e produção de mediadores inflamatórios. Estes distintos papéis da microbiota intestinal são tão marcantes que podem, inclusive, influenciar no desenvolvimento da obesidade em algumas pessoas, podendo levar até mesmo à Síndrome Metabólica. Probióticos e prebióticos podem conferir alterações nas propriedades da microbiota, afetando o crescimento bacteriano e seu metabolismo e, até mesmo, o uso de nutrientes. Assim, o objetivo geral deste trabalho foi avaliar o efeito do uso de leite fermentado simbiótico na obesidade e nos indicadores de Síndrome Metabólica, como circunferência de cintura, TGL, HDL-c, glicemia e pressão arterial, em pacientes com predisposição a esta doença, selecionados de acordo com os critérios de diagnóstico para síndrome metabólica. Para esta pesquisa foram desenhados quatro produtos, três deles inoculados com uma cultura comercial de probiótico - *Bifidobacterium lactis* (BL420), adicionados ou não de casca de maracujá em pó (prebiótico), sendo um deles preparado com uma emulsão de óleos vegetais, que apresenta efeito em prolongar a saciedade; e o quarto produto, fermentado apenas com a cultura clássica *Streptococcus thermophilus*. Avaliaram-se as propriedades tecnológicas dos produtos – físico-química, sensorial, microestrutura, microbiológica, e estudaram-se seus aspectos funcionais. Finalmente, os produtos foram testados em consumidores ao longo de um ensaio clínico durante 12 semanas, período no qual os voluntários tiveram que consumir 100 mL do produto, duas vezes ao dia, todos os dias. A avaliação dos voluntários foi feita antes do início do ensaio e também aos ao final dos 90 dias.

Palavras-chave: Probiótico, Fibra, Leite fermentado, Síndrome metabólica, Obesidade

ABSTRACT

Perina, N.P. **Fermented milk and adipose visceral tissue - possible slimming effects in obese and patients with metabolic syndrome.** 2015. 167 f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2015.

The gastrointestinal tract of humans is rich in microorganisms which can both be beneficial to host health, preventing and / or treating lactose intolerance, constipation, irritable bowel syndrome, among others, as they can harm, affecting nutrient acquisition and the production of inflammatory mediators. These distinctive roles of intestinal microbiota are so striking that can even influence the development of obesity in some people and may even lead to metabolic syndrome. Probiotics and prebiotics can confer alterations in the microbiota properties, affecting the bacteria growth and their metabolism, and the use of nutrients. Thus, the aim of this study was to evaluate the use of innovative symbiotic fermented milk in obesity and metabolic syndrome indicators such as waist circumference, TGL, HDL-C, blood glucose and blood pressure in patients with a predisposition to this disease, selected according to the diagnostic criteria for metabolic syndrome. For this, four fermented mik were designed, three of them inoculated with a commercial probiotic culture - *Bifidobacterium lactis* (BL420), added or not by passion fruit peel powder (prebiotic), one of them being prepared with an emulsion of vegetable oil, which has effect on prolonguing satiety; and the fourth product, fermented only with the classical culture *Streptococcus thermophilus*. Products´ technological properties were evaluated - physico chemical, sensory, microstructure, microbiology, and functional aspects were studied. Finally products were tested in consumers throughout a clinical trial during 12 weeks, period in which the volunteers had to consume 100 mL of the product twice a day, every day. The evaluation of the volunteers was done before the start of the test and also at the end of the 90 days.

Keywords: Probiotic, Fiber, Fermented Milk, Metabolic Syndrome, Obesity.

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LIST OF SYMBOLS

CFU	Colony-forming unit	**ml**	Millilitre
cm	Centimiter	**mm**	Millimiter
dL	Deciliter	**mmHg**	Millimeter of mercury
eV	electron volt	**MPa**	Megapascal
g	Gram	**MS**	Metabolic Syndrome
h	Hour	**ng**	Nanogram
hz	Hertz	**ºC**	Degree Celsius
kcal	kilocalories	**pH**	Potencial of Hydrogen
Kg	Kilogram	**psi**	Pounds per square inch
kV	Kilovolt	**s**	seconds
kw	Kilowatt	**s.d.**	Standard Deviation
L	Litre	**v**	Volt
m/z	Mass-to-charge ratio	**yr**	Year
mg	Milligram	**µL**	Microliter
min	Minutes	**µm**	Micron

CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTORY REMARKS

The gastrointestinal tract (GUT) of mammals contains a society of non-pathogenic bacteria, which is complex, dynamic and diverse (TEITELBAUM and Walker, 2002).

Evidence suggests that these trillions of bacteria that typically reside in the humans GUT, collectively called intestinal microbiota, affect the acquisition of nutrients and energy regulation. It is also suggested that lean and obese people have a distinct intestinal microbiota. Together these findings raise the possibility that intestinal microbiota plays an important role in the control of body weight and may be partially responsible for the development of obesity in some people (DIBAISE et al., 2008), which is currently one of the major public health problems that affects both developed and developing countries (SBEM, 2007).

The introduction presented in the following section comprises the main topics approached in this thesis. A brief overview of fermented milk as well as of probiotic bacteria and their importance in human health, especially in obesity, is presented.

1.2. GENERAL INTRODUCTION

1.2.1. Fermented Milk

Fermentating dairy foods are one of the oldest methods of long-term preservation of milk sources. Fermentating milk is quite old and can be placed in the Middle East and traced back long before the Phoenician era (VASILJEVIC; SHAH, 2008). According to the Codex standard for fermented milks (CODEX STAN 243-2003) (WHO / FAO, 2011), “Fermented Milk is a milk product obtained by fermentation of milk, which milk may have been manufactured from products obtained

from milk with or without compositional modification (…), by the action of suitable microorganisms and resulting in reduction of pH with or without coagulation (iso-electric precipitation). These starter microorganisms shall be viable, active and abundant in the product to the date of minimum durability. If the product is heattreated after fermentation the requirement for viable microorganisms does not apply.”

Although the preservation role of fermented dairy products was widely recognized and appreciated early, scientists realized in the late 19th century that a wide range of traditional milk products had further advantages in prolonging shelf-life and pleasant sensory properties. Actually, health benefits associated to these fermented dairy products consumption are of growing interests in reseach worldwide.

1.2.2. Probiotics and its beneficial health effects

The term probiotics is derived from Greek and means "for life" (SCHREZENMEIR; VRESE, 2001; NICHOLS, 2007). This name was first used in 1965, in contrast to the word antibiotic to describe substances secreted by a microorganism which stimulates the growth of another (SCHREZENMEIR; VRESE, 2001). Fuller definitions used to describe the word probiotic live bacteria normally associated with substances that promote bacterial growth, constituting components of food or added to foods to provide a pharmacological effect (UTERMOHLEN, 2003).

The original concept of using bacteria to prevent and treat disease appeared about 100 years ago, with the Nobel earner Eli Metchnikoff, who noted that the lactic fermentation prevented putrefaction of milk, and had the idea of using the same principle in the digestive tract, looking for the same purpose, which was proven to be true by the Bulgarian peasants, who had a regular consumption of fermented dairy products and had longer and healthier life expectancy (TANNOCK, 2004).

Currently, the most internationally accepted definition is that probiotics are live microorganisms that when administered in adequate amounts confers a health benefit on the host (FAO / WHO, 2002). According to Teitelbaum and Walker (2002) some of the current criteria used to define probiotics says that they must be of human origin; be non-pathogenic in nature; be resistant to destruction by technical processing; be resistant to destruction by gastric acid and the bile; adhere to the intestinal epithelial tissue; be able

to colonize the GUT, even for a short period of time; produce antimicrobial substances; modulating the immune response and influence human metabolic activities (e.g., cholesterol assimilation, the production of vitamins, etc.).

As the fermented milk used by Metchnikoff, other products containing probiotics can promote positive effects on health, such as yogurt, Yakult®, kefir, among others, that have the ability to positively change the intestinal flora (JONES, 2002).

Various bacteria have been identified according to the definition of criteria for probiotics (TEITELBAUM; WALKER, 2002). Bacteria belonging to the genera Lactobacillus and Bifidobacterium, and, to a lesser extent, Enterococcus faecium, are the most often used probiotics in food supplements (SAAD, 2006). Other types of bacteria studied, used or considered suitable for commercial use, which have probiotic properties such as: *L. casei* spp, *L.* delbrueckii bulgaricus, *Bifidobacterium bifidum*, *B. longum*, *Enterococcus faecium*, *Saccharomyces boulardii*, among others (NICHOLS, 2007).

Probiotic bacteria are associated with many beneficial health effects, which can contribute to the prevention and treatment of various medical conditions including, lactose intolerance, colon cancer, hypercholesterolemia, hypertension, immune system, mineral absorption in the GUT, diarrhea, constipation, irritable bowel syndrome, inflammatory bowel disease, allergies, among others (HASLER, 2002; NICHOLS, 2007). Other possible effects of probiotics are their role in prevention and treatment of hypertension, osteoporosis, atherosclerotic vascular disease associated with dyslipidemia, obesity, allergies and inflammation (TEITELBAUM; WALKER, 2002; Nichols, 2007).

Is important to emphasize that many probiotic bacteria express their most significant effect in improving the overall health of the individual through the gastrointestinal tract, which depends on their ability to adhere to and colonize the intestinal mucosa (NICHOLS, 2007).

1.2.3. Obesity and Metabolic Syndrome

Obesity is a chronic disease characterized by excessive fat accumulation (CARVALHO et al., 2009). Its etiology is complex and multifactorial, resulting of interactions of the genes, environment, lifestyle and emotional factors (ABESO, 2009).

Besides representing a risk factor for many other chronic diseases, obesity is associated with dyslipidemia, diabetes, hypertension and left vascular hypertrophy, which are coronary risk factors. Obesity also favors increased frequency of colon cancer, rectum and prostate in men and bladder cancer, endometrial and breast cancer in women (CARVALHO et al., 2009).

Following the global trend, the prevalence of overweight and obesity is increasing in Brazil. The number of overweight women increased by 67 % since the first evaluation in the period from 1974 to 1985, and among men the increase was even greater, 170 %, giving a jump of 18.5 % of overweight men in 1974- 1985 to 50.1 % in 2009. Obesity also gave big jumps, increasing 25 % among women and 37 % among men in the period 2002-2003 to 2008-2009 (IBGE, 2010).

The Ministry of Health also noted the high prevalence of obesity in Brazil, by the Risk and Protective Factors for Chronic Diseases Surveillance Study through telephone inquiry (VIGITEL). In this research, it was observed that 13.9% of adults are obese, with the highest rate in the population between 55 and 64 years, reaching 19.9 % for men and 21.3 % for women (BRASIL, 2010).

Metabolic Syndrome (MS), in turn, refers to the group of cardiovascular risk factors including diabetes, obesity, dyslipidemia and hypertension (DUVNJAK; DUVNJAK, 2009), increasing the overall mortality by about one and a half times, Cardiovascular in about two and a half times (DBSM, 2005).

According to the First Brazilian Guidelines for Diagnosis and Treatment of Metabolic Syndrome, MS represents the combination of at least three of the following risk factors: abdominal obesity, measured by waist circumference (> 102.0 cm in men and > 88.0 cm in women); high triglycerides (≥ 150 mg /dl); HDL cholesterol (< 40 mg /dl for men and <50 mg / dl for women); high blood pressure (≥ 130 mmHg or ≥ 85 mmHg); impaired fasting glucose (≥ 110 mg /dl, whereas the use of

antihypertensive medication or lipid-lowering, as well as the previous diagnosis of diabetes, in itself, meet the specific criteria (DBSM, 2005).

However, these values were reviewed by the IDF - International Diabetes Federation, which defines some lower parameters for the diagnosis of MS, such as waist circumference (WC), which must have less tolerance for individuals from South and Central America, being > 90.0 cm for men and > 80.0 cm for women, and also fasting glucose ≥ 100 mg /dl (IDF, 2006).

In line with the increasing prevalence of obesity, MS is also growing in developing countries. High prevalence of MS was observed in sub-Saharan Africa and the Middle East. South Africa, Morocco, Oman, Turkey, and Iran showed prevalence of 33.5, 16.3, 21.0, 33.4 and 33.7 %, respectively. Prevalence rates are also high in Venezuela (31.2 %) and in the urban area of Brazil (25.4 %). The situation appears to be similar in South Asian countries (MISRA; KHURANA, 2008).

1.3. THESIS OVERVIEW

Given the high prevalence of obesity and metabolic syndrome, which is strongly associated with increased cardiovascular risk, is evident the importance of seeking healthy food alternatives that can benefit both the control and treatment of individuals already affected by the disease, as well as to prevent this pathology. As seen, probiotics play an important role in modulating the intestinal microbiota, with possible beneficial effects in cases of obesity and metabolic syndrome, controlling hypertension, dyslipidemia, obesity and other risk factors. However, as these effects are not well established in the literature, more research seems necessary to increase knowledge of the role of probiotics in the prevention and treatment of chronic diseases, in particular, of the Metabolic Syndrome.

So, the ultimate goal of this thesis is to evaluate the metabolic effect of new fermented milk consumption in overweight patients and people in risk of Metabolic Syndrome. For this, innovative fermented milks were designed; their technological properties were evaluated - physico chemical, sensory, microstructure, microbiology, and functional aspects were studied. Finally products were tested in consumers throughout a clinical trial. In chapter 2 the effect of the addition of fiber and substituting

milk-fat by a vegetal-oil emulsion and homogenization on viable cell-counts and physicochemical properties of yoghurt were studied. Sensorial analyses and acceptation test of the first tested formulations were investigated in chapter 3, with the application of hedonic scale and projective mapping. In chapter 4 the survival of the probiotic specie and the starter culture was verified. Chapter 5 and 6 presents and discusses the results of the clinical trial realized at University of São Paulo Clinical Hospital HCFM/USP.

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CHAPTER 2

2. FUNCTIONALITY OF PROBIOTIC YOGHURT IS ENHANCED THROUGH ENRICHMENT OF MILK WITH PASSION FRUIT PEEL POWDER AND VEGETAL-OIL EMULSION

The main target of this work was to innovatively design a functional probiotic yoghurt with the enrichment of the milk matrix with passion fruit peel-powder and the vegetal-oil emulsion Fabuless™ in six different formulations, fermented with the starter cultures *Streptococcus thermophilus* (TA040) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB340) and the probiotic bacteria *Bifidobacterium lactis* (BL420) ir order to select the best formulations for future use. The effect of the addition of fiber and substituting milk-fat by a vegetal-oil emulsion and homogenization on viable cell-counts and physicochemical properties of yoghurt, such as composition, fatty acids' profile and firmness were studied. In addition, scanning electron microscopy (SEM) was used to evaluate the gel microstructure, and used to explain changes of the physical properties of yoghurts, after the addition of fiber and substituting milk-fat.

2.1. INTRODUCTION

The method of producing yoghurt has slightly changed since its discovery. Although there had been some refinements, especially in relation to the lactic acid bacteria that start fermentation, the essential steps remain the same (TAMIME; ROBINSON, 2007).

Factors as fortification level of solids-not-fat, fat content and other added materials as well as processing conditions of the milk-base (i.e. homogenization pressure or heat-treatment level) can affect the physical properties of the acidified gel of fermented milk such as: stability, viscosity, texture and microstructure (TAMIME; ROBINSON, 2007; SODINI et al., 2004; 2005). The textural properties of the yoghurt, in special, depends on the microstructure and physicochemical interactions between its different structural elements, such as fat globules, water, colloidal protein, aggregates and additives (TAMIME et al., 2007).

Milk fat contains saturated fat which is related to chronic degenerative diseases, mainly: coronary heart disease and hypertension, type 2 diabetes, hyperlipidaemia, cardio-respiratory failure, other chronic degenerative diseases, and also, obesity and metabolic syndrome. In addition, weight loss has been recognized as a key factor in the control and prevention of obesity and metabolic syndrome. Consumers in many countries are aware of these facts and prefer the use of unsaturated fats or oils in the place of milk-fat. The use of vegetable-oils to replace milk-fat in designed milk products, manufactured from reconstituted skimmed milk powder has been used in developing countries for a long time and could be an alternative for the consumers. Although few data are available on fermented milks, especially on yoghurts, made with vegetable-oils, Barrantes et al. (1996), in an experiment where they compared the rheological properties and microstructure of yoghurts rich in poly- and mono-unsaturated fatty acids (manufactured from reconstituted skimmed milk powder using vegetable-oils such as olive, groundnut, sunflower or maize) with yoghurt containing anhydrous milk-fat observed that separation was higher and firmness was lower for all vegetable-oil-containing yoghurts.

While there is a small number of studies' describing the effect of substituting milk-fat by vegetable oil in fermented milk, there are no studies regarding the use of a vegetable-oil emulsion, such as Fabuless™, which is formulated from palm-oil and oat-oil fractions. Recent studies have shown that Fabuless™ can increase and extend post-prandial satiety (HAENNI et al., 2009), reduce food intake (APPLETON et al., 2011), reduce the desire to eat and the preoccupation with thoughts of food (BURNS et al., 2001). Thus, the addition of Fabuless™ to yoghurt would help to reduce food intake and appetite, and therefore, control weight management.

Functional properties such as anti-hypertensive, hypocholesterolemic and the reduction of blood-glucose level have been attributed to the passion fruit peel (CHAU; HUANG, 2005; ZIBADI et al., 2007; JANEBRO et al., 2008; SALGADO et al., 2010). Although several researchers have studied the effect of dietary fiber on yoghurt (HASHIM et al., 2009), little is known about the effects of passion fruit-fiber on the physicochemical properties and microstructure of yoghurt.

Over the last two decades, probiotics have been added to different food matrices; especially to fermented milk of which the health benefits are mostly well-known (DAVE; SHAH, 1997; VASILJEVIC; SHAH, 2008). The effects of probiotic intakes on obesity and metabolic syndrome turn out to be a proposal (OUWEHAND; VESTERLUND, 2003; OUWEHAND et al., 2003; AMROUCHE, 2005; COMMANE et al., 2005; BOGSAN et al., 2011). Lahtinen et al. (2010) has shown a reduction in insulin resistance, tissue inflammation and observed less-total fat mass in mice, treated with *Bifidobacterium lactis* 420 for four weeks.

Based on its nutritional and technological properties and as fat and fiber content can interfere with the microstructure we aimed to design and investigate in the present study functional probiotic yoghurts enriched with passion fruit peel-powder and the

vegetable-oil emulsion Fabuless™ and its influence on the physicochemical properties of yoghurt.

2.2. EXPERIMENTAL SECTION

2.2.1. Material

Skimmed milk powder (SMP) and passion fruit-fiber were purchased from Nestlé (Araçatuba, Brasil) and Apis-Flora (Ribeirão Preto, Brazil), respectively. Fat from milk cream (Carambeí, Brazil) and fat from vegetal-oil emulsion - Fabuless™ (DSM Nutritional Products, Switzerland) were used to adjust the fat content of yoghurts at 3%. Mix of proteins according to Marafon et al. (2011) was used to enrich the protein content of yoghurts.

Three strains of pure commercial-starter cultures, both from Danisco (Sassenage, France), for direct inoculation of the processed milk, were used for the preparation of products:

(i) *S. thermophilus* strain TA040, (ii) *Lactobacillus delbrueckii* subsp. *bulgaricus* strain LB340 and (iii) *Bifidobacterium lactis* (species 420). The first two organisms are the 'classical' yoghurt starter-culture while the bifidobacteria is the probiotic.

2.2.2. Yogurt Production

Six different probiotic yoghurts denoted Y1 to Y6 were prepared considering fat's addition - two with milk cream, two with vegetable-oil-emulsion and two controls with skimmed milk powder (Table 2.1). As homogenization could affect oil emulsion's efficacy in appetite control (SMIT et al., 20011), two milk base process i.e. with or

without homogenization were applied to each yoghurt type. Control probiotic yoghurts with skimmed milk were studied without passion-fruit-peel-powder, but with and without homogenization.

Two milk-base processes were performed for each yoghurt type: (i) P1 - ingredients were reconstituted to their final concentration in potable water, pre-heated at 50°C, and shaken for 15min. Subsequently, milk base was heated at 90°C for 5min. Both heat treatments were performed in a Thermomix TM31 (Vorwek & Co. KG, Wuppertal, Germany), and then, cooled to 10°C in an ice water bath, and; (ii) P2 - ingredients were reconstituted to their final concentration in potable water, pre-heated to 55°C in a plate-heat exchanger (PHE) (Alfa Laval, type A3-HRB - Lund, Sweden), homogenized at 15 MPa, in double-stage equipment (i.e. 1st stage at 10 MPa and 2nd stage at 5 MPa) (Treu, Rio de Janeiro, Brazil), heated to 95°C for 5 min in closed circuit, and then, cooled to 10°C in the same PHE. Both milk bases prepared by P1 or P2 treatments were refrigerated overnight.

Table 2.1. Experimental design employed to evaluate the functionality of probiotic yoghurt enricmed with passion fruit peel powder and vegetal-oil emulsion

Probiotic Yoghurt	Passion fruit peel powder	Fat addition	Milk Base Process
Y1- control	-	-	P1
Y2- control	-	-	P2
Y3	+	Cream milk	P1
Y4	+	Cream milk	P2
Y5	+	Vegetal oil emulsion	P1
Y6	+	Vegetal oil emulsion	P2

P1: pre-heating to 50°C - 15 min / heating to 90°C – 5 min/ cooled to 10°C;

P2: pre-heating to 55°C / homogenization at 15 MPa/ heating to 95°C - 5 min/ cooled to 10°C.

On the following day, each milk base was tempered to 42°C, divided into two batches and inoculated with 'classical' yoghurt starter-culture plus the bifidobacteria. Each batch of milk was incubated at 42°C in a water bath until the pH was 4.7. The acidification profile of microbial blends was monitored using the Cinac (*Cinètique d´aciditication*) system (Ysebaert Frépillon, France), and $t_{pH\ 5.}0$ and $tpH_{\ 4.7}$ or fermentation time (time in hours to reach pH 5.0 and 4.7, respectively) was calculated. After reaching pH 4.7, yoghurts were immediately cooled in an ice bath until 10°C. After that, each probiotic yoghurt was manually agitated using a stainless steel plunger (i.e. consisting of a rod with a perforated disc) moved upwards and downwards for 60s, dispensed into 50mL polypropylene cups (heat sealed using Selopar equipment - BrasHolanda, Pinhais, Brazil) and stored at 4°C until further analysis. The trials were replicated twice on different days.

2.2.3. Analyses of yogurts

Total solid, fat and protein contents of the milk bases were determined according to recommendations of Venturoso et al. (2007) in triplicate for each sample.

Bacterial enumerations were carried out before fermentation (d0), and after 1 (d1) and 21 (d21) days of cold storage in two replicates of each batch according to Saccaro et al. (2011). Cell-counts were expressed as log CFU mL^{-1} of yoghurt and average values were calculated.

Lipids were extracted from yoghurt-bases and probiotic yoghurts according to the method described by the ISO method 14156 (ISO-IDF, 2001). The fatty acid methyl esters (FAMEs) were prepared by esterification, according to the ISO method 15884 (ISO-IDF, 2002).

Analyses of FAMEs were carried out in a Varian GC gas chromatograph (model 430 GC, Varian Chromatograph Systems, Walnut Creek, California, USA), equipped with a CP 8412 auto injector. The Galaxie software was used for the quantification and identification of peaks. Injections were performed in a 100-m fused silica capillary column (ID = 0.25 mm), coated with 0.2 µm of polyethylene glycol (SP-2560, Supelco, USA), using helium as the carrier gas at isobaric pressure of 37 psi; linear velocity of 20 cm/s; make-up gas: helium at 29 mL/min at a split ratio of 1:50; volume injected: 1.0 µL. The injector-temperature was set at 250°C and the detector-temperature was set at 280°C. The oven temperature was initially held at 140°C for 5 min, then programmed to 240°C at the rate of 4°C/min, and held isothermally for 30 min. Quantitative composition of fatty acids was calculated according to the AOCS Official Method Ce 1-62 (AOCS, 1997). All samples were analyzed in triplicate for each sample, before fermentation, and after one (d1) day of storage, at 4°C, the reported values are the average of the three runs.

Probiotic yoghurt's firmness was measured using a TA-XT2 Texture Analyser (Stable Micro Systems Ltd., Godalming, UK) equipped with 25P acrylic type probe (diameter of 2.5cm). The probe penetrated vertically for 10 mm into the products with a speed of 10 mm/s, and the maximum force (in Newton), at 10 mm depth was recorded to express the firmness. Firmness measurements were carried out in triplicate for each sample after one (d1) and 21 (d21) days of storage at 4°C, and average values were calculated.

After 24 h of storage at 4°C all yoghurt samples were freeze-dried using an Edwards' model L4KR 118 (BOC Edwards, Brazil). The freeze-dried samples were fixed with 25% glutaraldehyde, according to the method adapted from Oliveira et al.(2002). Samples underwent further dehydration in a graded alcohol series (50, 60, 70,

80, 90 and 95%), followed by three-time dehydration in an anhydrous alcohol and subsequently dried by the critical point method under CO_2 medium with a BAL-TEC CPD 030 apparatus (Liechtenstein, Germany). Samples were then placed on stubs, covered with copper double-face tapes and received a final gold-coating performed by the cathodic spreading of the samples in a Balzers Union SCD 040 (Liechtenstein, Germany). Samples observation and photomicrographs were done in a field emission scanning electron microscope (SEM) (JEOL model JSM-7401-F, JEOL Ltd, Japan), operating at a voltage of 1.0-10.0 kV. The images were registered under magnifications from 5.000 to 10.000 x, and almost six fields were observed.

2.2.4. Statistical analyses

One-way analyses of variance (ANOVA) tests were carried out using Statistica 9.0® software (Statsoft, Tulsa, OK, USA) to find differences ($P \leq 0.05$) among samples. Paired comparisons between means were carried out using the Tukey's test, when a significant difference was observed.

2.3. RESULTS AND DISCUSSION

2.3.1. Yogurt composition

The composition of the six experimental yoghurts presents no significant difference(s) in fat and protein contents ($P \leq 0.05$). Yoghurts had 52.6 ± 3.5, 27.1 ± 0.3 and 10.0 ± 0.1 g Kg^{-1} of protein, fat and fiber, respectively. Although a slight difference in total solid contents was found, the relation proteins/total solids (P/TS) were similar for the tested yoghurts (data not shown).

Beyond the content of 10-20% of pectin, a soluble fiber which is known for its prebiotic action, the passion fruit peel-powder is composed of an approximately 15 g of protein, lipids ≤ 8 g, 87 g of ash, 560 g of carbohydrates per Kg of dry matter, and is also a source of: iron, calcium, phosphorus and niacin (CORDOVA et al., 2005; YAPO; KOFFI, 2008). According to the producer, the passion fruit peel-powder under study has, per kilogram, 76 g of total protein, 37 g of lipids, 83 g of ash, 133 g of carbohydrates and 586 g of total fiber, being 132 g of soluble fiber and 454 g of insoluble fiber.

2.3.2. Changes in pH during fermentation and counts of viable bacteria

Initial pH values, which were not adjusted, were 6.44 ± 0.10. The enrichment of milk with vegetal oil emulsion and passion-fruit-peel-powder significantly affected the initial pH of the milk ($P \leq 0.05$).

As shown in Figure 1, the addition of fat and homogenization did not affect the time to reach pH 5.0 ($T_{pH5.0}$), which was faster for Y4 and Y6 than for Y1, Y2 and Y3. In contrast, $T_{pH5.0}$ of milk containing vegetal-oil emulsion not homogenized (Y5) was the highest.

The fermentation time lasted for 4.7 h (Y4) to 5.3 h (Y3 and Y5) in tested yoghurts (Figure 1). The supplementation of passion fruit peel powder could be considered satisfactory, in terms of their acidification and fermentation time. Other authors recently reported that fibers like inulin, maltodextrin, polydextrose, lactulose, and oligofructose supplemented to milk, in order to improve the probiotic effect, influenced fermentation either by bifidobacteria (AKALIN et al., 2007; DONKOR et al., 2007; DAMIN et al., 2008; OLIVEIRA et al., 2009), or lactobacilli (OLIVEIRA et al., 2009). Meanwhile,

Espírito Santo et al. (2012) reported that the addition of passion fruit peel-powder significantly reduced the fermentation time of the skimmed yoghurts, co-fermented by the strains of *Lactobacillus acidophillus* (L10 and NCFM), and *Bifidobacterium animalis* subsp. *lactis* (Bl04 and HN019).

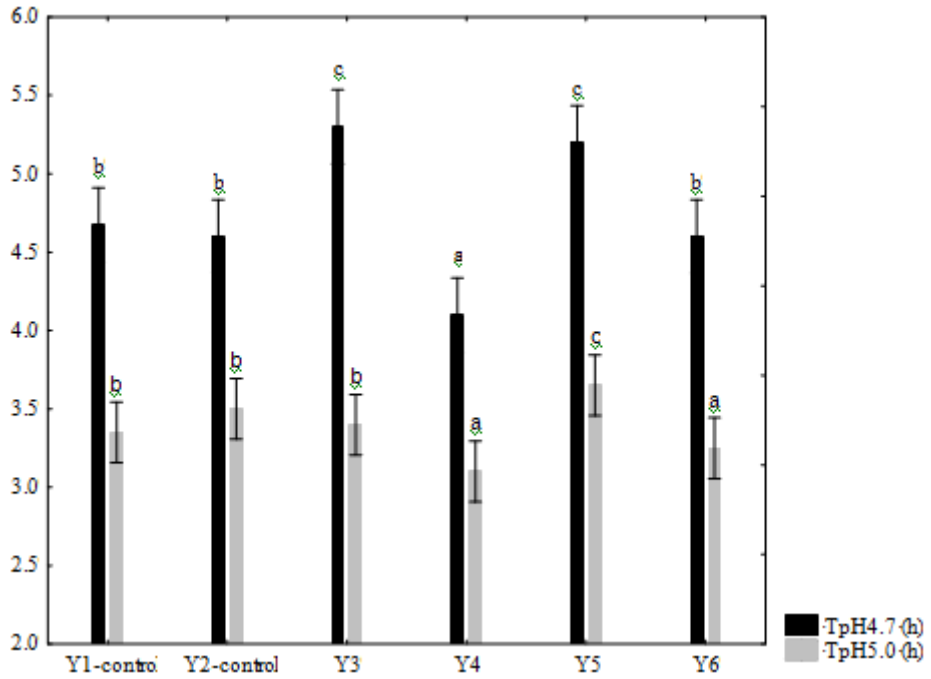


Figure 2.1. Fermentation time (time to reach pH4.7) and time to reach pH5.0, of milk containing passion fruit peel powder fermented by yoghurt bacteria - *Streptococcus thermophilus* strain TA040 and *Lactobacillus delbrueckii* subsp. *bulgaricus* strain LB340 and *Bifidobacterium lactis* - Bifidobacterium species 420*B* at 42°C. Means (n = 4) with different letters are significantly different; $P \leq 0.05$.

Y1-control: skimmed milk, P1; Y2-control: skimmed milk, P2;

Y3: fat from milk cream, P1; Y4: fat from milk cream, P2;

Y5: fat from vegetal emulsion, P1; Y6: fat from vegetal emulsion, P2.

P1: pre-heating to 50°C - 15 min / heating to 90°C – 5 min/ cooled to 10°C;

P2: pre-heating to 55°C / homogenization at 15 MPa/ heating to 95°C - 5 min/ cooled to 10°C.

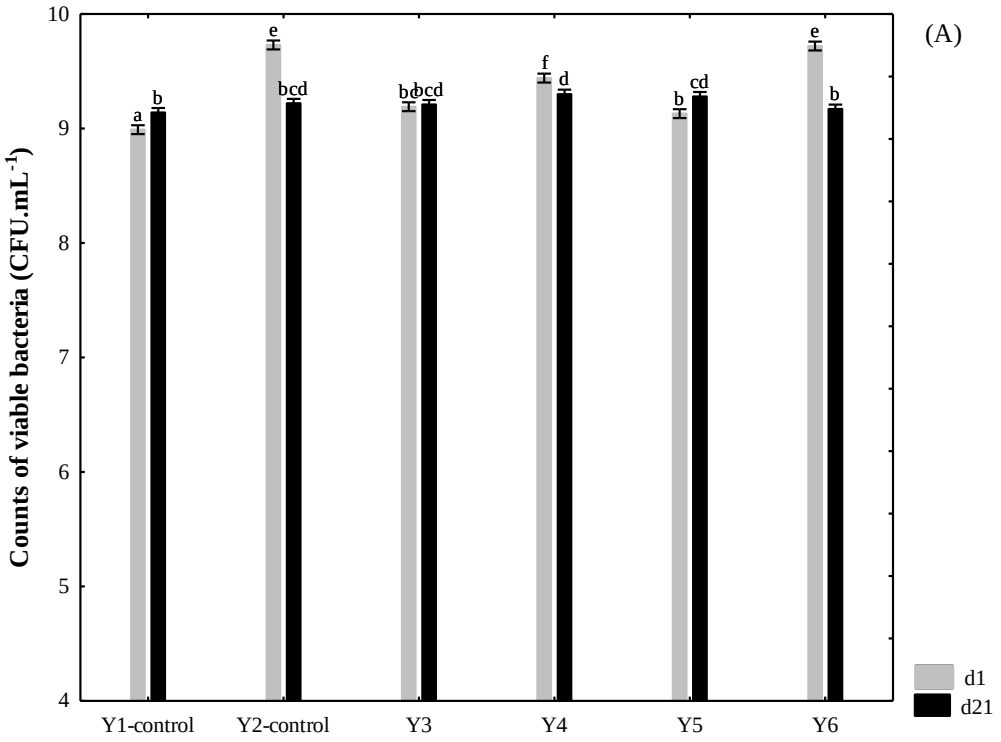
Considering this process in general, the fermentation was quicker in homogenized supplemented milk than in not-homogenized base ($P \leq 0.05$), while the addition of vegetal-oil emulsion significantly increased the fermentation in Y6, compared to Y4

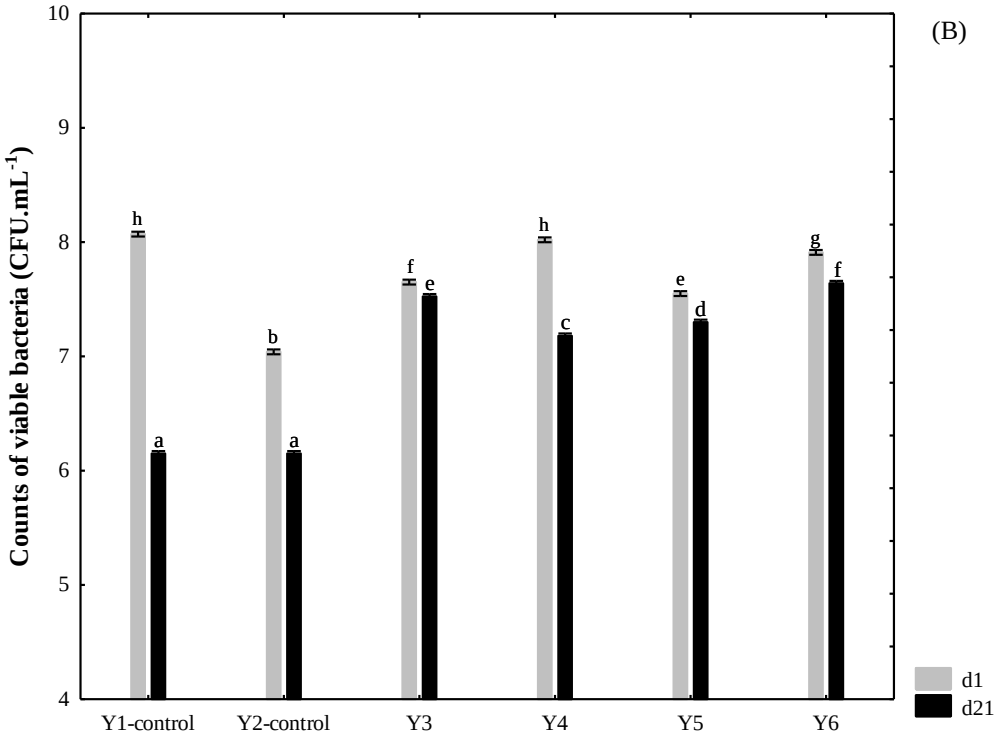
(Figure 2.1). The increase of T_{pH} 4.7 (~36 min), due to the vegetal-oil emulsion, could be considered not significant in industrial conditions and could improve bioactive products' formation. The reason for the slowest drop in pH due to the presence of vegetal-oil emulsion could be the protection that the emulsion confers on casein micelles. Further research is necessary to confirm these findings.

Although many other researchers have reported that probiotic bacteria have a poor acidification performance in milk when compared to a yoghurt starter-culture (KLAVER, 1993; MARSHALL; TAMIME, 1997; SAXELIN et al., 1999; OLIVEIRA et al., 2001; SODINI et al., 2002; ALMEIDA et al., 2008; DAMIN et al., 2009), the acidification profile of milk enriched with passion fruit peel-powder fermented by yoghurt bacteria - *S. thermophilus* strain TA040 and *Lactobacillus delbrueckii* subsp. *bulgaricus* strain LB340 plus *Bifidobacterium lactis* - Bifidobacterium species 420*B* at 42°C is suitable for probiotic yoghurt manufacture of milk containing vegetal-oil emulsion.

Counts of viable bacteria before fermentation (d0) were 6.48 ± 0.27 log CFU mL-1, 5.06 ± 0.35 log CFU mL^{-1} and 7.12 ± 0.13 log CFU mL^{-1} for *S. thermophilus*, and *L. bulgaricus* and *B. lactis*, respectively. After fermentation, counts increased in average 2.90 log, 2.65 log and 1.84 log, correspondingly.

Figure 2.2 shows the counts of viable bacteria in yoghurt containing passion fruit peel powder and after 1 (d1) and 21 (d21) days of cold storage. It could be seen that during the 21 days of storage, *S. thermophilus* counts were stable and ranged, as an average, from 9.22 to 9.37 log CFU mL^{-1} (Figure 2.2A). A similar behaviour was noted for *L. delbrueckii* subsp. *bulgaricus* that suffered a small decrease in its counts, which ranged in average from 7.71 log CFU mL^{-1} at d1 to 6.99 at d21 log CFU mL^{-1} (Figure 2.2B).


(A)
Counts of viable bacteria (CFU.mL-1)
10
9
8
7
6
5
4
a
b
e
bcd
bc
bcd
f
d
b
cd
e
b
Y1-control
Y2-control
Y3
Y4
Y5
Y6
d1
d21


(B)
Counts of viable bacteria (CFU.mL-1)
10
9
8
7
6
5
4
h
a
b
a
f
e
h
c
e
d
g
f
Y1-control
Y2-control
Y3
Y4
Y5
Y6
d1
d21

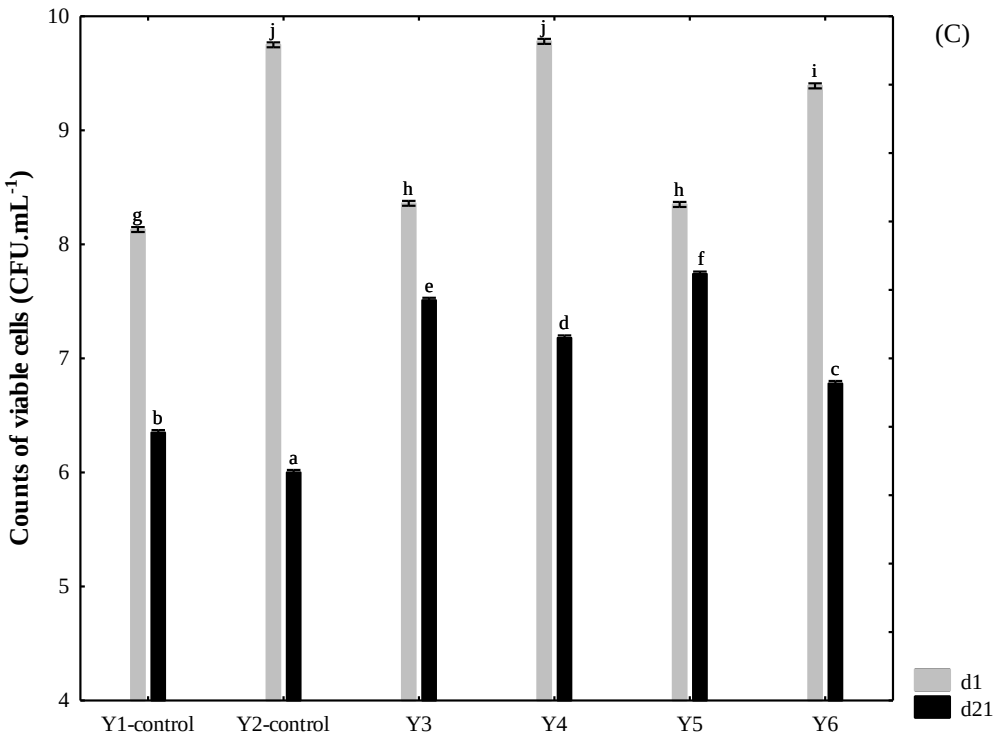


Figure 2.2. Counts of viable bacteria in milk base in yoghurt containing passion fruit peel powder after 1 (d1) and 21 (d21) days of cold storage. (A) *Streptococcus thermophilus* strain TA040, (B) *Lactobacillus delbrueckii* subsp. *bulgaricus* strain LB340 and (C) *Bifidobacterium lactis* - Bifidobacterium species 420*B*. Means (n = 4) with different letters are significantly different; $P \leq 0.05$.

Y1-control: skimmed milk, P1; Y2-control: skimmed milk, P2;

Y3: fat from milk cream, P1; Y4: fat from milk cream, P2;

Y5: fat from vegetal emulsion, P1; Y6: fat from vegetal emulsion, P2.

P1: pre-heating to 50°C - 15 min / heating to 90°C – 5 min/ cooled to 10°C;

P2: pre-heating to 55°C / homogenization at 15 MPa/ heating to 95°C - 5 min/ cooled to 10°C.

On the 1st day of cold storage, the probiotic counts varied from 8.13 to 9.78log CFU mL^{-1}. Regarding the control, a positive effect of passion fruit peel-powder was observed in counts of probiotic bacteria on day-1 except for Y6, which contained vegetal-oil emulsion ($P \leq 0.05$). The B. lactis counts rose 0.8 log when non-homogenized milk was used and 2.7 log to the homogenized milk. In the milk supplemented with cream or

vegetal-oil emulsion, *B. lactis* grew from 1.2 log (not-homogenized) to 2.5 log (homogenized), being almost twice in homogenized milk. In contrast, after 21 days of storage, the passion fruit peel-powder had a beneficial effect on the counts of *B. lactis* strains in tested yoghurts not-homogenized in which counts decreased by 3-4 times (Figure 2.2C). These findings agree with Kailasapathy et al. (2008) that had reported no effect in counts of the *B. lactis* B94, due to passion fruit-juice's addition to milk used to prepare yoghurt as well as with Espírito Santo et al. (2012b), concerning *B. lactis* HN019 in yoghurts supplemented with passion fruit peel-powder. At the end of shelf life counts of probiotic strains ranged, as a whole, from 6.0 to 7.7log CFU mL^{-1}, being higher in yoghurts supplemented with vegetal-oil emulsion, and homogenized. The initial pH of the milk containing passion fruit peel-powder - that was near the neutrality (pH 6.44) - may have attenuated the possible negative effect of the acidity from the fruit, on the viability of *B. lactis*. Besides, the concentration of passion fruit peel-powder may not have been enough to exert an inhibitory effect on the probiotics.

2.3.3. Fatty acids profile

Fatty acid compositions of the 6 experimental probiotic yoghurts at d0 and d1 are presented in Table 2.2; data from d21 were not presented as there were no statistical differences observed when compared with those from d1 ($P \geq 0.05$). The average of saturated fatty acids (SFA) (Figure 2.3) of the samples Y1, Y2, Y3 and Y4 prepared with skimmed milk or enriched with milk cream was found as 657.6 ± 30.9 gkg^{-1} and 658.9 ± 25.3 gkg^{-1} at d0 and d1, respectively. Fermentation did not affect the composition in SFA ($P \leq 0.05$) for these yoghurts.

Table 2.2. Fatty acids profile of probiotic yoghurts enriched with passion fruit powder (g kg^{-1})

Fatty Acid	d0						d1					
	Y1	Y2	Y3	Y4	Y5	Y6	Y1	Y2	Y3	Y4	Y5	Y6
C4:0, Butyric acid	Nd	31.8	27.4	13.0	0.1	4.5	Nd	25.7	27.4	9.5	0.1	3.2
C6:0, Caproic acid	12.1	2.3	18.0	2.1	0.2	1.8	12.1	2.8	18.0	5.4	0.2	1.1
C8:0, Caprylic acid	9.4	6.0	12.4	9.9	0.3	1.4	9.4	5.6	12.4	8.7	0.3	1.4
C10:0, Capric acid	22.4	18.9	27.3	22.8	0.5	2.9	22.4	18.4	27.1	20.2	0.5	2.9
C12:0, Lauric acid	28.1	25.1	34.9	28.8	1.6	2.2	28.1	25.5	33.4	26.1	1.6	2.2
C14:0, Myristic acid	113.1	108.4	119.8	112.0	16.0	4.0	113.1	109.1	119.8	102.3	11.6	13.7
C16:0, Palmitic acid	349.6	355.2	305.6	321.3	424.9	371.2	349.5	356.3	305.6	331.5	424.9	372.5
C18:0 , Stearic acid	142.0	138.1	107.8	109.6	42.0	42.3	142.0	137.4	107.8	122.4	42.0	41.1
C16:1, Palmitoleic acid	7.6	20.8	8.0	10.3	0.2	3.9	7.6	21.9	8.0	10.1	0.2	4.2
C18:1n9c, Oleic acid	278.1	272.6	305.7	328.9	401.3	441.0	278.1	273.6	305.7	321.4	401.3	441.0
C18:2n6c ,Linoleic acid	25.3	15.9	24.9	29.0	106.8	110.3	25.4	16.5	23.5	27.1	106.8	110.4
C18:3n3 , α-Linoleic acid	4.2	1.4	3.2	14.2	9.5	3.7	4.2	1.2	3.2	12.0	9.5	3.6
C18.2 10 trans, 12 cis, Conjugated linoleic acid	8.0	4.3	8.0	1.2	1.0	1.5	8.0	5.9	8.0	3.2	1.0	2.6

Y1: skimmed milk, P1; Y2: skimmed milk, P2; Y3: fat from milk cream, P1; Y4: fat from milk cream, P2; Y5: fat from vegetal emulsion, P1; Y6: fat from vegetal emulsion, P2. P1: pre-heating to 50°C - 15 min / heating to 90°C – 5 min/ cooled to 10°C; P2: pre-heating to 55°C / homogenization at 15 MPa/ heating to 95°C - 5 min/ cooled to 10°C. Nd: not determined

The range of MUFA (Figure 2.3) was 307.9 ± 24.0 gkg^{-1} and 306.6 ± 20.3 gkg^{-1} at d0 and d1, respectively for Y1 to Y4. Finally, PUFA was in an average of 34.5 ± 09.6 gkg^{-1}. The findings of this study are similar to the findings of Espírito-Santo et al.(2012) for yoghurts prepared with milk-fat and passion fruit peel powder.

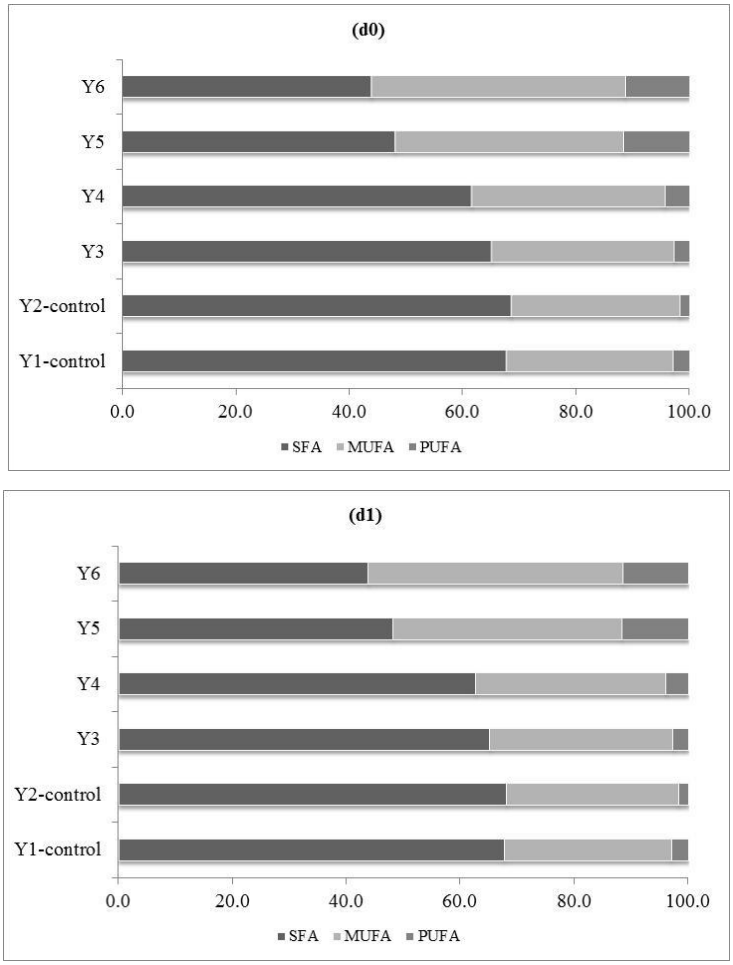


Figure 2.3. Fatty acids composition – SFA (Saturated fatty acids), MUFA (mono unsaturated) and PUFA (Poli unsaturated) of yoghurts containing passion fruit fiber fermented by yoghurt bacteria - *Streptococcus thermophilus* strain TA040 and *Lactobacillus delbrueckii* subsp. *bulgaricus* strain LB340 and *Bifidobacterium lactis* - Bifidobacterium species 420*B* at 42°C.

Y1-control: skimmed milk, P1; Y2-control: skimmed milk, P2;

Y3: fat from milk cream, P1; Y4: fat from milk cream, P2;

Y5: fat from vegetal emulsion, P1; Y6: fat from vegetal emulsion, P2.

P1: pre-heating to 50°C - 15 min / heating to 90°C – 5 min/ cooled to 10°C;
P2: pre-heating to 55°C / homogenization at 15 MPa/ heating to 95°C - 5 min/ cooled to 10°C

However, yoghurts prepared with vegetal-oil emulsion presented a reduction of almost 30% in SFA, as expected, compared to yoghurts containing milk-fat. In contrast

to the results of yoghurts prepared with skimmed milk or enriched with milk cream, MUFA for Y5 and Y6 varied from 402.5 to 447.1 gkg^{-1}. Finally, PUFA ranged from 114.0 to 116.3 gkg^{-1}. MUFA and PUFA in yoghurts containing passion fruit peel-powder and vegetal-oil emulsion, respectively improved by 38% and 238%. These results have never been reported before, but they are very important since these fatty acids (we are the first to show these new and important results). As shown, MUFAs and PUFAs can promote an efficient reduction in the total cholesterol and LDL-c, contributing more to the plasma lipids in comparison with a reduction in the ingestion of total fat in dyslipidemic individuals (LOTTENBERG, 2009). Thus, yoghurt industry should start thinking about substituting milk fat by vegetable-oil-emulsion in the manufacture of fermented milk products with enhanced functional properties, especially in the case of using this specific combination of vegetal-oil-emulsion that has been tested regarding sensorial aspects such as taste and global impression by the same group of researchers with good results (PERINA et al., 2015).

Conjugated linoleic acid (CLA) contents varied from 12 g to 80 g kg^{-1} for the yoghurts prepared from skimmed milk, or enriched with milk cream whilst 10 g to 26 g kg^{-1} for the yoghurts enriched with vegetal-oil emulsion (Table 2.2). As expected, these results are very low compared to Florence et al. (2009) which studied the increase in CLA content in organic milk, fermented by bifidobacteria or yoghurt cultures. Alternatively, the CLA content in yoghurts prepared with milk cream is very close to the results shown by Florence et al. (2009) to the control yoghurt (0.47 a 0.8%), and also to the yoghurts enriched with passion fruit-fiber and fermented by *Bifidobacterium animalis* subsp *lactis* (Bl 04, B94 e HN019) (ESPÍRITO-SANTO et al., 2012b).

2.3.4. Firmness and microstructure

After one day of fermentation, it was observed that yoghurts prepared with skimmed milk and enriched with cream milk not homogenized gave a lower firmness compared to yoghurts containing cream milk, homogenized (Figure 2.4). As expected, in general, firmness significantly increases during cold storage as reported before (MARAFON et al., 2011; DAMIN et al., 2008). It could be noted in Figure 4 that all yoghurts had higher firmness at d21 compared to d1. Additionally, the behaviour of yoghurts' firmness at d1 was similar to d21. The addition of cream milk followed by homogenization (Y4) resulted in higher firmness ($P < 0.05$).

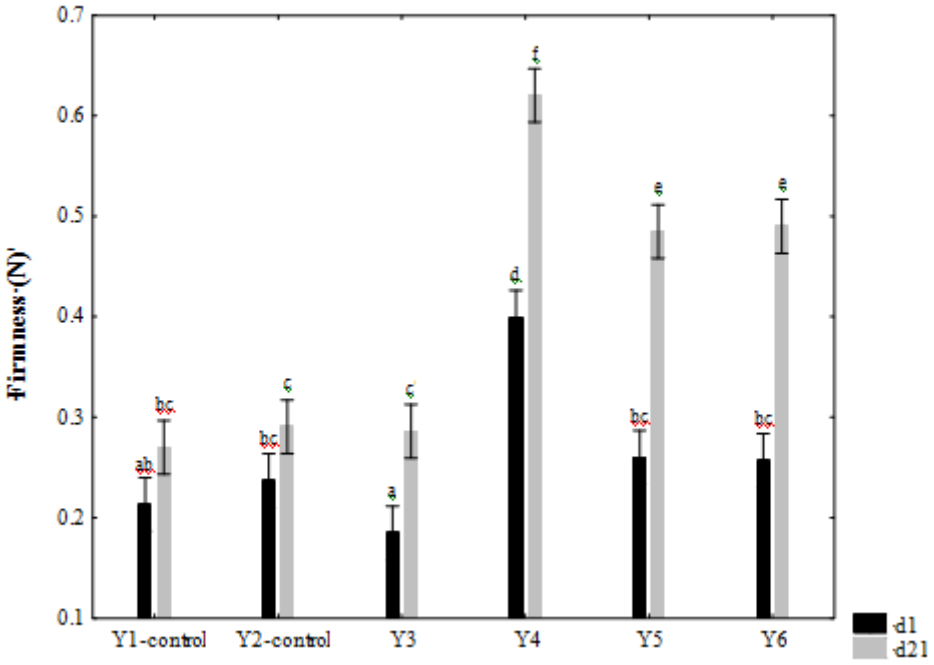


Figure 2.4. Firmness of probiotic yoghurt containing passion fruit fiber after 1 (d1) and 21 (d21) days of cold storage. Means (n = 6) with different letters are significantly different; $P \leq 0.05$.

Y1-control: skimmed milk, P1; Y2-control: skimmed milk, P2;

Y3: fat from milk cream, P1; Y4: fat from milk cream, P2;

Y5: fat from vegetal emulsion, P1; Y6: fat from vegetal emulsion, P2.

P1: pre-heating to 50°C - 15 min / heating to 90°C – 5 min/ cooled to 10°C;

P2: pre-*heating* to 55°C / homogenization at 15 MPa/ heating to 95°C - 5 min/ cooled to 10°C.

According to Damin et al. (2008), the firmness is higher in yoghurts' fermentation time that lasts longer. However, in our study, yoghurts supplemented with passion fruit peel-powder and vegetal-oil emulsion, in spite of the longer fermentation time, did not show higher firmness compared to the other treatments. In contrast, yoghurts with faster acidification profile (see Figure 2.1) presented higher firmness.

Dello Staffolo et al. (2004) observed that some rheological characteristics of yoghurt are modified by the addition of commercial fibers from: apple, wheat, bamboo, or inulin, but no syneresis was shown whilst acceptance of these products by the consumers was similar to the control product. Fiber improved the body and the texture of unsweetened yoghurt (FERNÁNDEZ-GARCÍA et al., 1998); while on the contrary, the pH of the yoghurt increased with increasing fiber (1.5, 3.0 and 4.5% by weight), and also syneresis (APORTELA-PALACIOS et al., 2005).

Figure 5 shows the microstructure of probiotic yoghurts 24 h after fermentation. For Y1 and Y2, a normal structure of non-fat yoghurt with a casein network, surrounded by a high number of pore-serum was seen compared to the other yoghurts. According to Lee and Lucey (2003), the presence of high numbers of pores corresponded to a weaker gel, suggesting that there were weak interactions between the casein particles.

The structures of yoghurt samples, enriched with passion fruit peel powder (Y3-Y6) were not much different from each other, except for the appearance of fat in Y6 image. In fact, the process parameters used to homogenize cream milk and vegetal-oil emulsion have not broken or spread fat into particles/fragments. As a result, fat recovered from the casein network, as can be seen in Figure 6(d) and (f), mainly when using vegetal-oil emulsion (Y6).

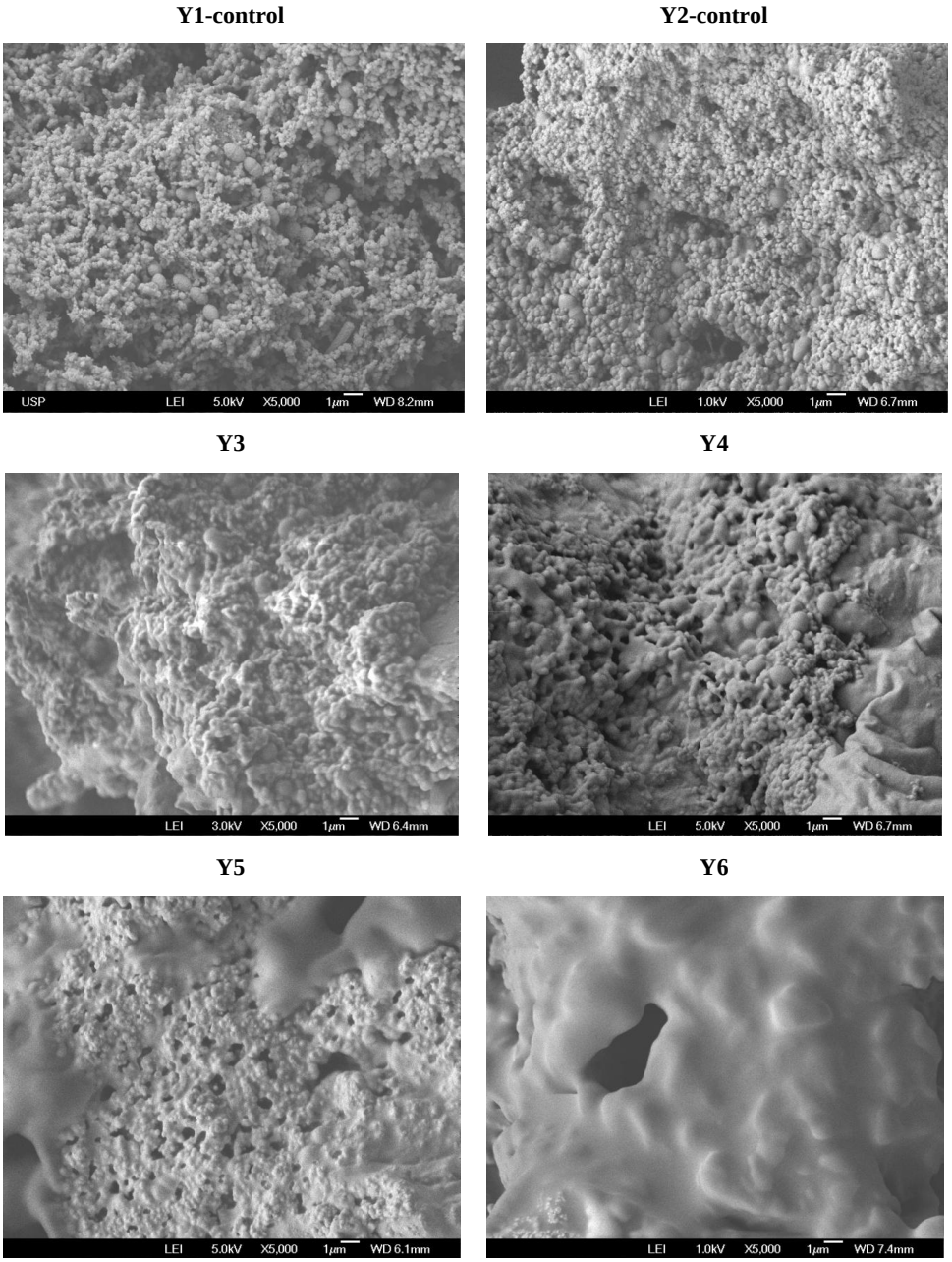


Figure 2.5. SEM of probiotic yoghurt containing passion fruit fiber after 1 (d1) day of cold storage.

Y1-control: skimmed milk, P1; Y2-control: skimmed milk, P2;

Y3: fat from milk cream, P1; Y4: fat from milk cream, P2;

Y5: fat from vegetal emulsion, P1; Y6: fat from vegetal emulsion, P2.

P1: pre-heating to 50°C - 15 min / heating to 90°C – 5 min/ cooled to 10°C;

P2: pre-heating to 55°C / homogenization at 15 MPa/ heating to 95°C - 5 min/ cooled to 10°C.

It is well established that the fortification of the protein content in the milk-base improves texture (VASILJEVIC; SHAH, 2008; SÉVERIN; WENSHUI, 2005) and is related to stronger protein matrices that lead to stronger firmness and more compact microstructures (MARAFON et al., 2011). The findings of the present study agree with Martín-Diana et al. (2004) who reported that the negative influence on the texture of fermented milks by milk fat replacement by oils rich in ω-3 polyunsaturated fatty acids was overcome by the supplementation of whey protein concentrate in products formulation.

It seemed that vegetal oil emulsion was less incorporated into the gel compared with the other samples. This fact is essential to warranty functional properties of yoghurts, as the whole of the vegetal-oil emulsion was indispensable to decreasing appetite (SMIT et al., 2011). In addition, the recovered casein network comprising the probiotic should increase its viability in the product, and protect it during digestion. It should be remembered that probiotics should survive in the gastrointestinal tract reaching the colon in high numbers (GUPTA; GARG, 2009), in order to promote health benefits to the host.

Our results confirm those of Tamime et al. (2007), who stated that the structure of the fermented milk is complex, mainly due to the aggregation of casein micelles in the milk, and the formation of a three-dimension network, with the fat embedded in the matrix.

Finally, in Figure 2.6, it can be seen how the structures of passion fruit peel-powder interact with casein networks. In a previous work, Espírito-Santo et al. (2013) showed throughout SEM micrographs that in yoghurts enriched with passion fruit-fiber, the casein gel overlay the fiber and sometimes was nestled in it. However, passion fruit-fibers entrap casein matrices network, as presented in Figure 2.6. To our knowledge,

this is the first time that such structure is shown in the literature. The integrity of passion fruits' fibers will certainly improve the functional properties of yoghurts when ingested.

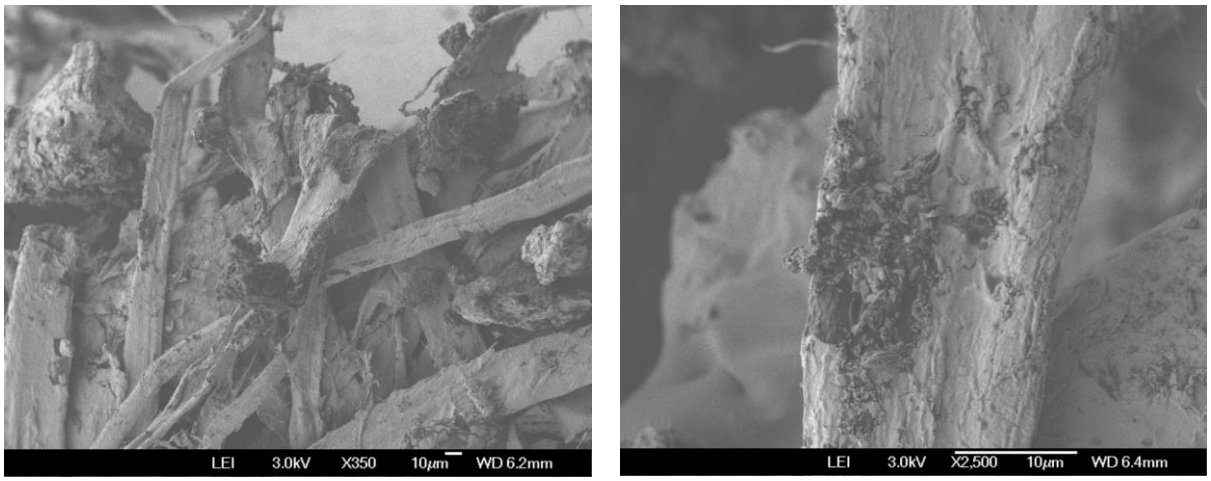


Figure 2.6. SEM of probiotic yoghurt containing passion fruit peel powder fiber showing interaction with casein network.

2.4. CONCLUSIONS

Probiotic yoghurt enriched with passion-fruit-peel-powder and vegetal-oil-emulsion could be a good alternative due to its technological behaviour on fermentation, counts of viable bacteria and texture. Besides, the improved fatty acids profile with reduced SFAs and increased MUFAs and PUFAs is an additional beneficial nutritional factor. Finally, microstructure should affect positively probiotic viability and its survival in the intestinal tract. More research should be conducted to confirm this proposal.

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CHAPTER 3

3. EFFECT OF VEGETAL-OIL EMULSION AND PASSION FRUIT PEEL-POWDER ON SENSORY ACCEPTANCE OF FUNCTIONAL YOGHURT

This work was developed with the objective of evaluating flavor, texture-in-spoon, creaminess-in-mouth, and global impression of functional yoghurt based on the hedonic scale and projective map approaches. As will be discussed in this chapter, these methods bring new opportunities for the sensory evaluation and acceptance of the incorporation of functional ingredients in yogurts.

3.1. INTRODUCTION

The prevalence of overweight and obesity is virally growing worldwide (IBGE, 2010), causing a high-rate of weight-related medical issues. Therefore, the implications associated with obesity, such as cardiovascular disease, no-insulin-dependent diabetes mellitus, cancer, and other non-transmissible diseases have increased considerably (SHETTY; SCHMIDHUBER, 2006).

The intensifying interest concerning healthy food is conveying food-markets towards a new category of products. The functional foods are defined as food, or food ingredients, which promote other health benefits other than just nutrition (DWYER, 2007). Besides the importance of developing strategies and new foods to improve health, especially in preventing weight-gain (APPLETON et al., 2011), the designed product must also be sensorally accepted by consumers (BAYARRI et al., 2011).

The most accepted international definition states that "probiotic are live microorganisms, administrated in adequate amounts, which confer benefits on the host" (FAO/WHO, 2002). Bacteria from *Lactobacillus* and *Bifidobacterium*, and in minor scale, *Enterococcus faecium* are the most often used probiotic supplements in a wide range of food products, as they were isolated from all portions of human gastro-intestinal tract (VASILJEVIC; SHAH, 2008). The significance of bifidobacteria's

addition to products for human consumption is associated, according to the strain to their survival through the gut-intestinal-tract, and their role for stimulating the immune system, and for decreasing the risk of microbial gastroenteritis (FOLIGNE et al., 2007). Moreover, vitamin production, anti-carcinogenic activity, hypocholesterolemic power, pathogen inhibition, and the improvement of immunity after physical exercise are related (EJTAHED et al., 2011; WANG et al., 2012).

The production of fermented milk using bifidobacteria is a big challenge in the dairy industry, because milk, on the whole, is not a suitable matrix for the growth of lactic and probiotic bacteria, since they lack essential proteolytic activity (OLIVEIRA et al., 2001; MARAFON et al., 2011). In furtherance, they could enhance levels of lactic and acetic acids together with volatile compounds in fermented products (CRUZ et al., 2012; OLIVEIRA et al., 2012) that could have a significant impact on flavour development and sensory acceptance of the food.

In addition, yoghurt is the most popular fermented milk, due to its association with good health (WANG et al., 2014). It is being preferred when supplemented with probiotic bacteria (ANNUNZIATA; VECCHIO, 2013). Moreover, its flavour has been considered very important to stimulate consumption (ROUTRAY; MISHRA, 2011). According to Reineccius (2006), although the perception of flavour is a complex phenomenon, and traditionally being limited to taste, olfaction and the somatic senses (irritation, tactile and thermal), and other signals are related. In this context, diverse modifications in the dairy matrix to be fermented for the production of yoghurt has been proposed, aiming at reducing calories or substituting fat (DOMAGALA et al., 2005), improving fermentation process and/or the stability of the product (OLIVEIRA et al., 2001), and enhancing texture and micro-structure (BHULLAR et al., 2002; SODINI et al., 2005).

Many reports focusing on sensory evaluation of dairy products are available (ANTUNES et al., 2009; CASTRO et al., 2013), but just a few concerning sensory properties of functional probiotic yoghurts (ALLGEYER et al., 2010; MAJCHRZAK et al., 2010).

The vegetal-oil emulsion - Fabulles™ previously called Olibra® is formulated from palm-oil and oat-oil fractions. Its ingestion is related with prolonging the time of food passage in the small intestine (HAENNI et al., 2009), reducing the caloric and

macronutrient ingestion, four hours after the ingestion of yoghurt added with vegetal-oil emulsion, and also, decreasing appetite, and promoting less desire to eat and less preoccupation with thoughts of food probably related to leptin mechanism (BURNS et al., 2001).

Passion fruit is the popular name for many different species from genus *Passiflora*, a very common fruit in the tropical and subtropical regions of the globe, like Brazil (ZERAIK et al., 2010). Its peel is rich in minerals and fibres, especially pectin, a soluble fraction of fibre that forms a gel in gastro-intestinal tract, preventing the absorption of some nutrients (SALGADO et al., 2010). The major effect of the soluble fibres as gums and pectin substances present in the passion fruit-peel (ZERAIK et al., 2010) occurs by the retarding gastric absorption of sugars and amino acids in the small intestine, reducing the postprandial blood glucose response's, contribution to the control of diabetes' response (REYES; AREAS, 2001), and as a possible source of natural flavonoids (ZERAIK et al., 2011). Therefore, a high consumption of dietary fibre promotes higher satiety, less energy ingestion, and also a contribution to obesity control (ARAYA; PAK, 2001).

Even though there are some studies showing the individual effect of Fabuless™ and passion fruit peel-fibres, respectively, in suppressing appetite as well as in anti-diabetic and in the prevention of dyslipidaemia (BURNS et al., 2001; BARBALHO et al., 2011; GUPTA et al., 2011; SILVA et al., 2011), scarce literature is available concerning its simultaneous influence on firmness and sensory properties when added to dairy products.

In this context, in the present study, vegetal-oil emulsion and passion fruit peel-powder were concomitantly incorporated to milk aiming to produce functional yoghurt, nevertheless chiefly with acceptable sensory characteristics. The influence of milk supplementation was investigated in instrumental firmness. In parallel, sensory attributes as flavour, texture (on spoon), creaminess (in the mouth) and overall liking were conducted by the application of two methods: hedonic scale and projective map.

3.2. EXPERIMENTAL SECTION

3.2.1. Yoghurt production and experimental design

Test and control yoghurts were formulated with the following ingredients: skimmed milk powder (SMP) Molico® - Nestlé (Araçatuba, Brazil), mix of proteins according to Marafon et al. (2011), sucrose, fat from milk cream (Carambeí, Brazil) and fat from vegetal emulsion - Fabuless™ (Lipid Technologies Provider AB, LTP, Karlshamn, Sweden), strawberry pulp (natural), unflavoured passion fruit peel-powder (Tango Alimentos, Curitiba, Brazil), corn starch, cochineal carmine colorant identical to natural strawberry flavour (Sunrise, São Paulo, Brazil).

Lactic Starter Cultures

Three strains of pure commercial starter cultures, all obtained from Danisco (Sassenage, France), for direct inoculation, were used for the preparation of yoghurt products: (i) *Streptococcus thermophilus* strain TA040, (ii) *Lactobacillus delbrueckii* subsp. *bulgaricus* strain LB340, and (iii) *Bifidobacterium lactis* (Bifidobacterium species 420). The former two organisms are the ‘classical’ yoghurt starter culture, whilst the bifidobacteria is the probiotic's, chosen by its health benefits in preventing gastrointestinal microbiota disbiose and weight gain (KALLIOMÄKI et *al.*, 2008; CANI; DELZENNE, 2009).

Formulation

The designed formulation, containing skimmed milk powder, protein mixture, fat (from vegetal oil emulsion or milk cream), passion fruit peel-powder and sucralose which amounts were defined in preliminary tests, aimed at assuring that test and control yoghurts had the same energetic content, macronutrient composition, as well as the aspect, taste and consistence.

Fruit and milk bases preparation

Fruit bases were separately prepared by mixing the strawberry pulp, strawberry flavoured colorant and starch, adding, when necessary, the passion fruit peel-powder (1%). The preparation was pasteurized in water bath at 65°C for 15 min, and cooled in ice bath.

For milk bases preparation skimmed milk powder was reconstituted to $9.5g.100g^{-1}$ in demineralised water and added with the mixture of proteins (1%) according to Marafon et al. (2011), and sucrose (7%). Supplemented milk was then divided into two different bases: (i) added with vegetal-oil emulsion as recommended by Burns et al. (2001), and, (ii) added with fat from cream milk. Both fat additions were made until reach 3%. Subsequently, both milk bases were heated in double-stage equipment at 65°C for 5min, and homogenized at 200bar in APV Rannie Copenhagen's homogenizer (Pittsburgh, USA). After that, bases were submitted to heat treatment at 90°C for 5min and cooled at 43°C.

Fermentation

Heat-treated milk (50 L) was transferred to aluminium cans (60 L), placed in a water bath Plurinox 380v/60hz/13, 5kw (Batatais, Brazil) until manufacture temperature stabilization. Two fermentation temperatures were tested – 42/ 37°C, which are the manufacture temperatures of conventional and containing *Bifidobacterium* spp. yoghurt, respectively, according to Tamime and Robinson (2007). When the milk in the cans reached the established temperature, they were inoculated with the bacterial cultures. Pilot essays were conducted to define the amount of bacteria to be used to ensure initial probiotic counts superior than 10^7 $cfu.mL^{-1}$. Acidification kinetics was followed by measuring the pH value during fermentation. When the milk reached pH 4.7; cooling the product in an ice bath stopped fermentation; clot was broken, subsequently, by stirring the product for 60 s with the aid of an electric stirrer with stainless steel rod.

Preparation of yoghurts

Milk and fruit bases were then mixed at a ratio of 80:20 pp. Summing five different products: (i) Y1: vegetable-oil emulsion, passion fruit peel-powder fermented

by StLbBl at 42°C, (ii) Y2: fat from cream milk, passion fruit peel-powder fermented with StLbBl at 42°C, (iii) Y3: vegetable-oil emulsion, passion fruit peel-powder fermented with StLbBl at 37°C, (iv) Y4: fat from cream milk, passion fruit peel-powder fermented with StLbBl at 37°C, and (v) YC or control yoghurt: fat from cream milk fermented with StLb at 42°C. The final products had an average of 4.7g.100g^{-1} of protein, 3.0g.100g^{-1} of fat, and 14.2g.100g^{-1} of carbohydrates, totalling 102.6 calories g.100g^{-1} of yoghurt. The complete experimental design is shown in Table 1.

The final products were packed into polypropylene recipients of 50mL and kept under refrigeration at 4°C until analysis. All analyses were performed in duplicate.

Table 3.1. Experimental Design used to study the incorporation of vegetal oil emulsion and passion fruit peel powder in probiotic yogurt

Yoghurt	Fat	Passion fruit peel powder	Culture	Fermentation temperature (°C)
Y1	Vegetable oil emulsion	+	*StLbBl*	42
Y2	Milk fat	+	*StLbBl*	42
Y3	Vegetable oil emulsion	+	*StLbBl*	37
Y4	Milk fat	+	*StLbBl*	37
YC – Control	Milk fat	-	*StLb*	42

StLb: *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*;

StLbBl: *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Bifidobacterium lactis* (Bifidobacterium species 420)

3.2.2. Enumeration of viable cells

Bacterial enumerations were carried out after 14 days of cold storage in two replicates of each batch; sampling time for enumeration was chosen according to Oliveira et al. (2001) as well as considering logistics conditions i.e. distance from pilot plant and laboratory where sensory analysis were conducted. Average of cell counts

were expressed as log cfu.mL^{-1} of yoghurt according to the methodology of Saccaro et al. (2011).

3.2.3. Firmness

The texture-profile-analysis of yoghurt was performed on samples stored under refrigeration (4°C) by a simple penetration testing using acrylic cylinder of 2.5 cm diameter in a texture analyzer TA-XT2 (Stable Micro Systems, Godalming, England), controlled by a micro-computer. The distance covered by the sample cylinder was 10mm at a speed of 10 mm.s^{-1}. Firmness was the force in Newton (N) corresponding to the height of the first peak of the curve of simple compression (DAMIN et al., 2008).

3.2.4. Sensory evaluation

The sensory evaluation of the probiotic yoghurts was performed using two different approaches: an affective test (consumer-test using a 9-point hedonic scale; Appendix 3.6.1) and a descriptive test (projective map or mapping; Appendix 3.6.2). Participation was free for anyone who wished to take part and had mainly students and staff of the University of São Paulo. As selection criteria people who were sick, flu or those with some kind of allergy or intolerance to milk were excluded, besides those consuming dairy foods at least weekly were included. A term of consent was given to all participants informing them of the objectives of the project, as well as the description of the products and the procedures to be performed. A sensory evaluation was conducted according to the guidelines outlined by the Standard 8586 ISO (2008), at the sensory laboratory of the Department of Pharmaceutical Sciences, São Paulo University, Brazil.

Acceptance test

After eating about 40g of one of the samples, the participant evaluated the product according to its degree of liking with the following attributes: (i) flavour, (ii) texture (on

spoon), (iii) creaminess (in the mouth), and, (iv) overall liking. For this evaluation, the participant indicated on the form how much he/she liked or disliked the product using the hedonic scale which includes grades 1-9, where 1 represents the highest degree of dissatisfaction (dislike, extreme hatred), and 9 represents the highest degree of satisfaction (extreme like, love it) (MORAIS et al., 2014). Initially, 250 people participated in this analysis. Some evaluations were excluded when the questionnaire was not completed or contained inaccuracies; therefore, the final number of panellists was 227 subjects, aged 17-60years being 106 men and 121 women.

Projective mapping

Projective mapping or mapping (PM) is a qualitative data collection technique that is used to create a "projection" of a product as a way to better understand a product in relationship to another product, or to better understand consumers and their reactions to products (JERVIS; DRAKE, 2014). PM is a sensory technique that allows consumers to express similarities and differences, as well as to group samples by positioning them on a two-dimension surface, using a piece of paper. The objective of this sensory technique is to provide a way in which consumers can assess samples in a global and simple manner (VARELA; ARES, 2012). Projective mapping has been used in perfumes (VERAMENDI et al., 2013), high alcoholic products (LOUW et al., 2013), granola bars (KENNEDY, 2010), wines (ROSS et al., 2012) and even reduced-calorie biscuit packaging (CARRILO et al., 2012).

For the projective map, a not trained panel received the five test samples monadically, about 40 g of each product, in plastic cups labelled with three random numbers. The consumers proved each sample and wrote five words to describe them in the evaluation form. Then, panellists were asked to draw the probiotic yoghurt samples on a piece of A4-paper (dimensions 40 x 60 cm) in a way that the distance between each pair would reflect their similarity: if close together, they were identical, if far apart, samples were regarded as different. Twenty-five subjects, aged 17-40 yr (12 women and 13 men) participated in this analysis. Between tasting each sample, the participants were oriented to eat a piece of salty biscuit, each and take a sip of water to cleanse the palate. Similar number of consumers had been used previously using projective mapping with probiotic yogurts according to Cruz et al. (2013a) which was considered an adequate panel number.

3.2.5. Statistical analysis

Initially, all the variables had their normality and their homogeneity of variances assessed by dispersion graphics and Hartley's test, respectively. As a second step, a one-way analyses of variance (ANOVA) was performed for the sensorial data (samples as variance source), followed by Tukey's test assuming $P \leq 0.05$. Kruskall-Wallis was applied to variables that did not present equality of variances. Linear correlation analyses, displayed by the Pearson's correlation coefficient (r) were calculated for the response variables. It was applied to variables which present data adjusted to the normality whilst for the opposite behaviour Spearman correlation was performed.

The data on the position of each product made by the panellists-X and Y axes - on the A4 sheet, on the projective map, as well as descriptors used to describe the samples were submitted to the multiple factor analysis (MFA) as recommended by Moussaoui and Varela (2010). MFA is a multivariate analysis for multiple block data, allowing use data groups of different nature simultaneously (quantitative, qualitative or frequency) which contribute in a similar potential form to the construction of the first dimension of the resultant map. In other words, the first dimension of the MFA is providing the maximum common information shared across the groups (WORCH, 2013). In this study, MFA data involved the establishment of a matrix where the rows are the yoghurts (6 lines), and the columns are the attributes used by the panellists to describe the samples (10 columns). A qualitative analysis of the terms used was done previously to avoid super-imposition, and similar terms were joined. The analysis consisted the terms cited by 20% of the consumers. Next, the terms used for describing each sample were counted. The acceptance data were considered additional variables, i.e., they were calculated, and the correlation coefficients with the factors of perceptual map generated by the MFA, but they did not participate in its construction (ARES et al., 2010).

Hierarchical clustering analysis (HCA) was also performed to find groups of samples with similar sensory characteristics, with samples coordinates in the first and second dimension of the sensory maps produced by PM though MFA (SANTOS et al, 2013).

3.3. RESULTS AND DISCUSSION

3.3.1. Acidification profile and counts of viable cells

The initial pH values of the milk bases were not adjusted, but were on an average of 6.63±0.07 varying from 6.51 to 6.71. The Incorporation of vegetal oil emulsion and passion fruit peel-powder significantly affected the initial pH values ($P \leq 0.05$). The distribution of the fermentation curves into two groups was distinctly visible according to the processing temperature: (i) Y1 and Y2 fermented at 42°C, and (ii) Y3 and Y4 prepared at 37°C. Temperature influenced the time of fermentation as the products prepared at 42°C had the fermentation time reduced in approximately 50min. The control milk without the passion fruit peel-powder's incorporation fermented at 42°C by starter cultures - *St* and *Lb*, reached pH 4.7 in about 4.5 h.

Samples Y1 and Y2 had similar fermentation time, 3.7 h. Compared to the control yoghurt (YC), it is possible to notice that the fermentation time of Y3 and Y4 were similar, lasting about 4.5 h. Considering the same process, the temperature incorporation of vegetal oil emulsion and the presence of passion fruit peel-powder did not affect the fermentation time of the different milk bases. This result corroborates with those obtained by Espírito-Santo et al. (2012) that did not observe significant statistic effect on time to reach pH 4.5 in whole yoghurts containing 0.7% of passion fruit peel-powder, fermented at 42°C by different probiotic cultures. Similar findings were reported using marine fish-oil in the yoghurt processing (Estrada et al., 2011).

After 14 days of storage at 4°C, viable cell counts was 8.65 ± 0.11 log cfu.100 g^{-1} on an average for *S. thermoplilus*, less than 6.0 log cfu.100 g^{-1} for *L. bulgaricus* and 8.96 ± 0.15 log cfu.100g^{-1} for *B. lactis*. The envisioned health benefits of probiotics can only be attained when the food contains the required minimum viable microorganism counts at the time of consumption, and a minimum recommended level of 10^6 CFU ml^{-1} has been suggested (TRIPATHI; GIRI, 2014); in this sense the values found in this study suggested the passion fruit peel-powder yogurt is in accordance with this criterion.

3.3.2. Instrumental firmness

Results of the firmness of the experimental yoghurts evaluated 14 days after cold storage at 4°C are shown in Figure 1. Considering the same process of temperature, the incorporation of vegetal oil emulsion and the presence of passion fruit peel-powder did significantly affect instrumental firmness of different yoghurts ($P \leq 0.05$).

According to Damin et al. (2008) firmness is higher in yoghurts produced with longer fermentation time. In the present study, despite yoghurts Y3 and Y4's longer fermentation time, they presented lower firmness. These results corroborates with those of Espírito-Santo et al. (2012) that also studied the effect of passion fruit peel-powder's addition in probiotic yoghurts.

In this study, as the main ingredient used in yoghurt processing was skim milk, is interesting to report that this raw material could be responsible to the firmness values observed. However, based on instrumental firmness results, it is possible to assure that vegetal-oil emulsion incorporation and passion fruit peel-powder caused alteration in the products' structure with substantial effects on firmness. Moreover, the reasons for this consequence need further investigation.

3.3.3. Sensory evaluation

Table 3.2 shows the mean sensory scores of the consumer test of probiotic yoghurts. Overall, the control yoghurt (YC) presented the best scores in all attributes evaluated, suggesting that the addition of the oil provided a decrease in the sensory performance. Indeed YC scores ranged from 7.70 (texture in spoon) from 7.09 (flavour) which are located in (I liked moderately) and 8.00 (I liked very much) at the 9-point hedonic scale. The mean value for texture (at spoon) was 7.44 ± 0.19 with no significant difference(s) between yoghurts ($P \geq 0.05$), which differ from the results obtained for instrumental firmness of the yoghurts (Figure 3.1). In the same way, creaminess (in mouth) showed to be similar, varying from 6.69 (Y1) to 7.67 (YC).

Table 3.2. Sensory attributes of yogurts incorporated with vegetal oil emulsion or milk fat and passion fruit peel powder after 14 days of cold storage at 4◦C

Yoghurt	Flavor	Texture (in spoon).	Consistency (at mouth)	Global Impression
Y1	5.31[c]	7.18[a]	6.69[b]	5.80[d]
Y2	6.06[b]	7.29[a]	6.71[b]	6.45[c]
Y3	6.46[b]	7.44[a]	7.04[ab]	6.77[bc]
Y4	7.05[a]	7.60[a]	7.26[ab]	7.12[ab]
YC	7.09[a]	7.70[a]	7.67[a]	7.39[a]
p-homogeneity of variables[a]	< 0.001	0.56	0.14	< 0.001
P-ANOVA [b]	< 0.001	0.19	0.01	< 0.001

[a] Values of probability obtained by Hartley test (F max) for homogeneity of variables; [b] Values of probability obtained by *one-way* ANOVA.

Different letters at same column represents results statistically different ($P < 0.05$). Y1, Y2, Y3, Y4, YC: see text.

The mean scores for flavour and global impression which were significantly different ($P \leq 0.05$), can be assembled in three distinct groups: (i) represented by the lowest score, Y1 (yoghurt prepared with vegetal-oil emulsion at 42°C); (ii) intermediate scores for flavour and global impression, yoghurts Y2 and Y3 containing milk fat and vegetal-oil emulsion, respectively, fermented at 42 and 37°C, and (iii) highest scores, Y4 and YC yoghurts prepared with milk fat and the control one at 37 and 42°C. On the other hand, it was also observed that the yoghurt Y3 presented similar scores towards YC in all sensory attributes ($P > 0.05$), except flavour and overall liking, which presented 6.46 and 6.77, respectively for these attributes, suggesting that the addition of vegetal oil emulsion caused an off-flavour in the food matrix, resulting in decreased sensory scores, and this effect can be highlighted by its interaction with the passion fruit peel powder, and the flavour compounds produced by the probiotic bacteria (KARIMI et al., 2012). The link between flavor and overall liking has been reported previously in probiotic cheese (GOMES et al., 2011), and this emphasizes the importance of the attributes in the consumer´s mind to build a global opinion of the product which affects the purchase and the repeated consumption. In the same way, creaminess (in mouth) showed to be similar varying from 6.69 (Y1) to 7.67 (YC).

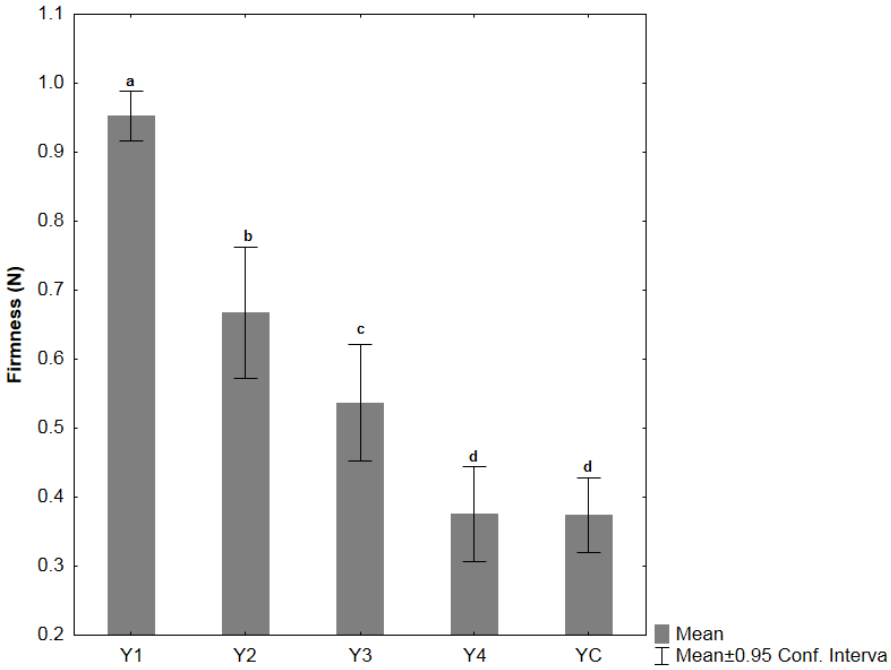


Fig. 3.1. Instrumental firmness of yoghurt incorporated with vegetal oil emulsion (VOE) or milk fat (MF), and passion fruit fiber. Means (n=6) with different letters are significantly different ($P \leq 0.05$).

YC: control yoghurt; Y1: VOE yoghurt prepared at 42°C; Y2: MF yoghurt prepared at 42°C; Y3: VOE yoghurt prepared at 37◦C; Y4: MF yoghurt prepared at 37°C.

Overall, the findings of the present study suggest that sensory acceptance of probiotic yoghurts with vegetal oil emulsion added is caused by an interaction among several factors: presence/absence of passion fruit peel powder, the use of vegetal-oil, and fermentation temperature. Another factor that deserves comments is the lack of familiarity of the presence of vegetable-oil in the yoghurt suggesting the occurrence of food neo-phobia, as observed in a similar way in the acceptance of savor-flavoured yoghurt enriched with n-3 lipids (ROGNLIEN et al., 2012).

Using linear correlation of Pearson (Table 3.3), it was observed that the age of consumers did not correlate with the degree of liking/disliking sensory attributes as flavour, texture, creaminess and not even with global impression of yoghurt samples. On the other hand, it was noticed that creaminess (at mouth) is significantly correlated ($P \leq 0.001$) with flavour (r = 0.49), texture (in spoon) (r = 0.44) and global impression (r = 0.64), corroborating with results obtained in previous studies covering other functional foods, as soy desserts added with oligofructose and prebiotic desserts (GRANATO et al., 2010).

Table 3.3. Simple linear correlation analysis between response variables

Variable	Age	Flavor	Texture–in-spoon	Creaminess	Global Impression
Age	1.0000	-0.0212	-0.1223	0.0148	-0.0338
	P= ---	*P*=0.751	*P*=0.066	*P* =0.825	*P* =0.612
Flavor	-0.0212	1.0000	0.2400	0.4907	0.885
	P =0.751	*P* = ---	*P* =0.000	*P* =0.000	*P* =0.000
Texture–in-spoon	-0.1223	0.2400	1.0000	0.4389	0.3851
	P =0.066	*P* =0.000	*P* = ---	*P* =0.000	*P* =0.000
Consistency	0.0148	0.4907	0.4389	1.0000	0.6398
	P =0.825	*P* =0.000	*P* =0.000	*P* = ---	*P* =0.000
Global impression	-0.0338	0.885	0.3851	0.6398	1.0000
	P =0.612	*P* =0.000	*P* =0.000	*P* =0.000	*P* = ---

Differences between participants with respect to age did not seem to affect the evaluated sensory attributes within each sample. From the paired comparison between men and women, it was noticed that there were no statistically different degree of liking flavor (P = 0.06) and creaminess (P = 0.11). On the contrary, mean scores for liking texture (at spoon) and global impression significantly different between men and women were observed, with P values of 0.01 and 0.04, respectively (data not shown).

During mapping, consumers answered were grouped in ten terms in order to describe the products (Table 3.4). The most cited terms used by consumers were “viscous”, “pink colour”, “sweet tasty” and “strawberry flavour” with 110, 70, 53 and 51 citations, respectively, while “bittersweet taste” and “natural yoghurt-flavour” were less cited terms. The overall liking can be related to the sensory attributes, strawberry aroma, and minor extension to sweet taste, strawberry flavour and the presence of grains which can be considered drivers of liking for the yoghurts. The sensory descriptor generated is very similar to that mentioned by a recent study that utilized a trained panel in commercial strawberry-yoghurts available in Brazilian market (MORAES; BOLINI, 2010). Moreover, the performance of sensory methodologies based on consumers' perception (CRUZ, 2013a) which demonstrates the sensory methodologies based on consumer perception, as the projective mapping technique utility can be useful to

generate a preliminary vocabulary of food product, in the absence of financial resources that would suffice in contacting a trained panel.

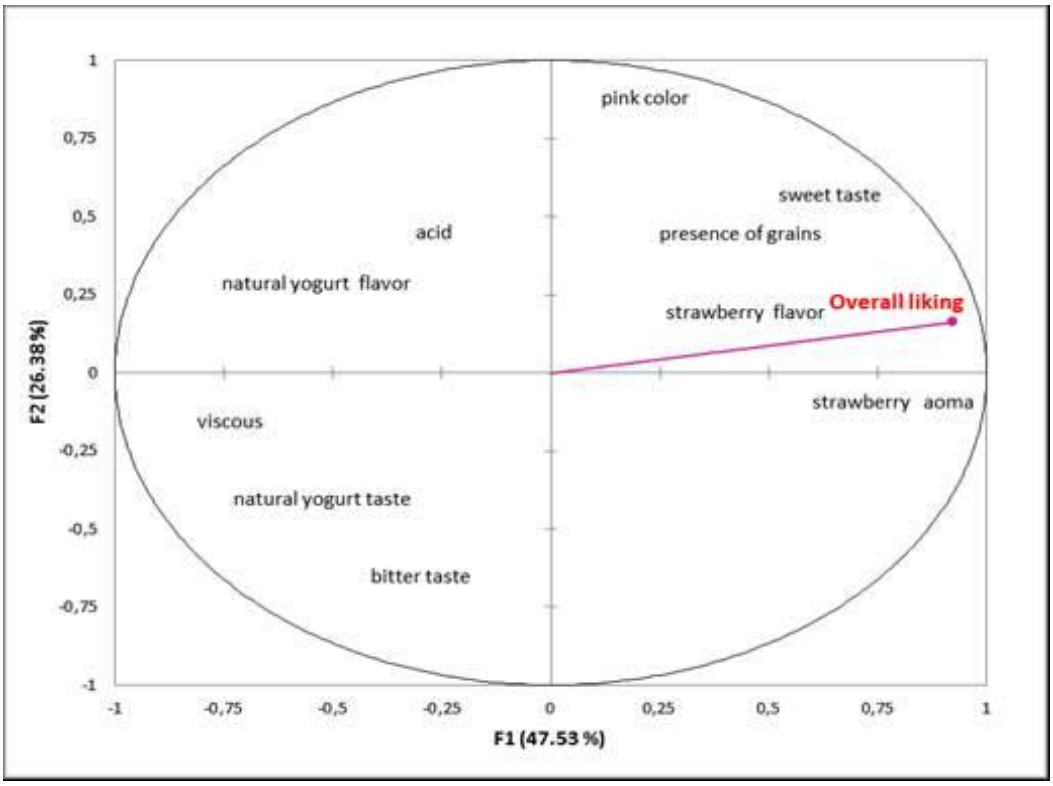
Table 3.4. Terms utilized by panelists during mapping and number of citations

Term	Number of Citations
Viscous	110
Pink color	70
Sweet Tasty	53
Strawberry flavor	51
Acid taste	46
Natural strawberry taste	36
Presence of grains	33
Natural yoghurt taste	18
Natural yoghurt flavor	15
Bittersweet taste	9

The elevated citations index to viscous term suggests that texture was a valorised attribute for consumers, being verified two times the number of citations related to flavour, which indicates that the influence of vegetal-oil emulsion or milk fat as well as exopolysaccharide produced by the probiotic bacteria have an effect on yoghurt formulation. However, it was possible to note that several other terms were used by the consumers to express the attribute texture as "cremosity" and "firmness" confirming previous studies, that reported the existence of such assorted words to express this attribute (ANTMANN et al., 2012; ANTMANN et al., 2011a, b). Furthermore, consumers' attitude towards sensory evaluation depends on their memory structure, which is definitely related to their personal characteristic (VARELA et al., 2013).

Multiple factor analysis with the representation of consumers' attributes descriptors and probiotic yoghurts showed that the first two principal components were able to explain up to 73.9% of the total variability (Figures 3.2A and 3.2B), representing 47.53 and 26.38% of the variance, respectively.

A)



B)

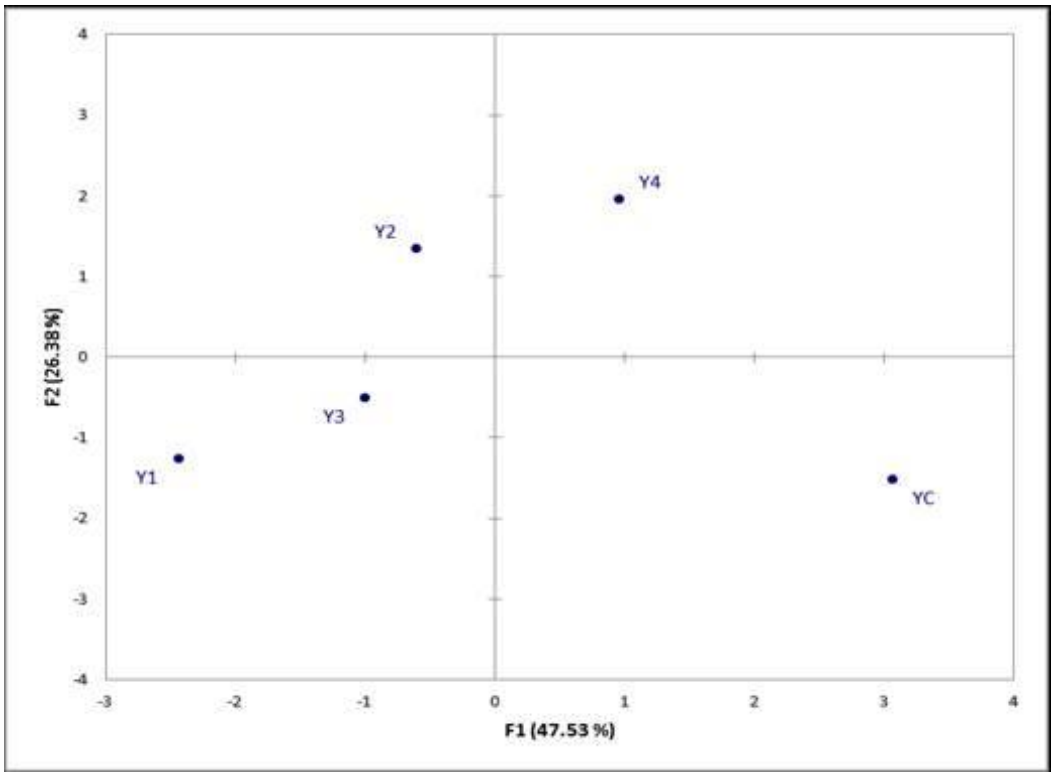


Fig. 3.2. Representation of the data of projective mapping at the first two dimensions. The terms used to describe the samples (A) and the probiotic and conventional yoghurts (B).

YC: control yoghurt; Y1: VOE yoghurt prepared at 42°C; Y2: MF yoghurt prepared at 42°C; Y3: VOE yoghurt prepared at 37◦C; Y4: MF yoghurt prepared at 37°C.

Overall liking scores were positively correlated to the sensory descriptors strawberry flavour, strawberry aroma, presence of grains and sweet taste which can be considered drivers of liking and should be taken in consideration at the development of the probiotic yogurts vegetal-oil emulsion and passion fruit peel-powder. In addition, this finding agrees with the number of citations. Being the sensory descriptors used to characterize the samples Y4 and YC, which are the samples with best performance at the consumer test. Finally, the sensory descriptors viscous, natural yoghurt taste, bitter taste, natural yoghurt flavour and acid are negatively correlated with the first dimensions of the MFA, and can be considered drivers of disliking.

Figure 3.4 shows the resulted dendrogram obtained by HCA. It could be observed that yoghurts were grouped in three categories: (i) represented by the highest score, YC, yoghurt prepared, respectively, with milk fat and the control one at 42 °C, (ii) Y4 and Y2, yoghurts prepared, respectively, with milk fat and the control one at 37 °C and milk fat and vegetal oil emulsion, fermented at 42 °C and (iii) Y3, yoghurt containing, respectively, milk fat and vegetal oil emulsion, fermented at 37 °C. Overall, this finding indicates the addition of vegetal oil emulsion did not change the intrinsic operational parameters used in yoghurt processing.

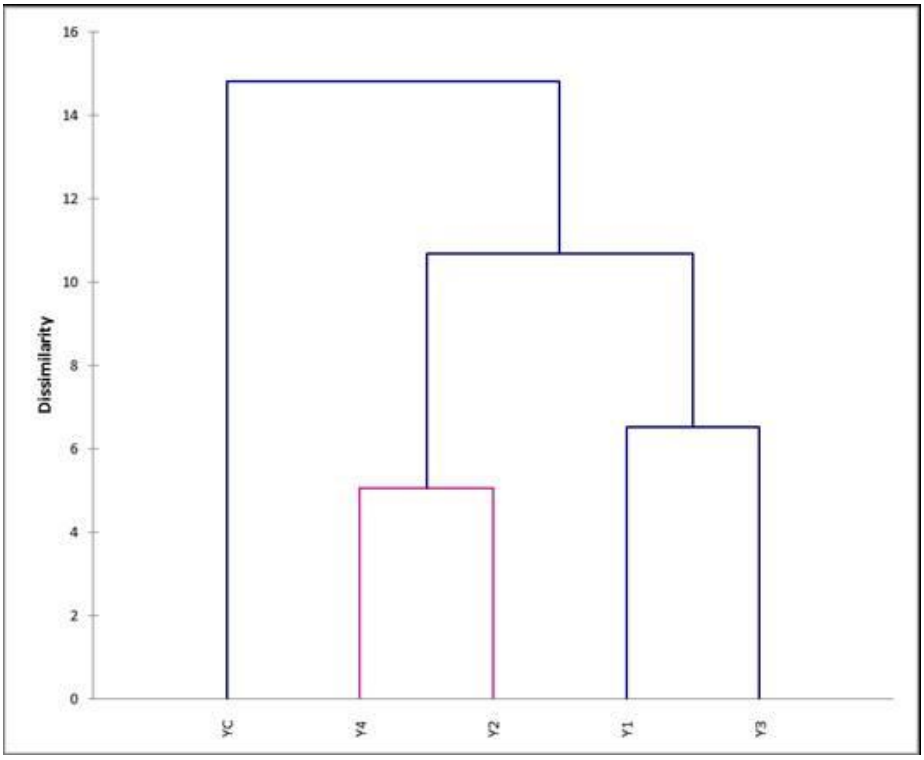


Fig.3.3. Dendogram of the hieraquical cluster analysis (HCA) of samples assessed by projective mapping

YC: control yoghurt; Y1: VOE yoghurt prepared at 42°C; Y2: MF yoghurt prepared at 42°C; Y3: VOE yoghurt prepared at 37◦C; Y4: MF yoghurt prepared at 37°C.

Further studies should consider the sensory profiling, established by a trained panel, to obtain a complete and detailed study of the sensory characteristics of the yoghurts (CADENA et al., 2013; PIMENTEL et al., 2013). Indeed, it has been reported that the limitations of the projective mapping in academic areas is related with the holistic characteristics of the procedure in which the consumers pay attention just in the principal and relevant attributes presented in the products. Besides, the possibility of sample reposition before a final configuration of the products has a decisive effect on the results; sometimes, the little participation of panellists, as well as their low-interest in performing the activity, compromising its efficiency (VEINAND et al., 2011). Recent study covering prototype and commercial flavoured-strawberry probiotic yoghurt indicates that projective mapping presented a configuration totally different from other sensory methodologies, using consumers as *check all that apply* (CATA), sorting and intensity scale (CRUZ et al., 2013b) suggesting that the method was not adequate.

3.4. CONCLUSIONS

In the present study, vegetal-oil emulsion and passion fruit peel-powder were concomitantly incorporated to milk. Considering the same fermentation temperature, and even though some observed differences in sensory properties issued from the hedonic scale and projective mapping, absence of significant differences in consumer's acceptance between supplemented and conventional yoghurts were distinguished. Overall, the insertion of the vegetal oil and the peel-powder did not provide positive results for the samples mainly for affecting the firmness, which is an attribute of considerable importance for yogurts. In this sense, the supplementation of vegetal-oil emulsion and passion fruit peel-powder in yoghurt, although can be an attractive for consumers, with potential benefits to the health, besides the intrinsic role of nutrition, should be carefully evaluated.

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3.6. APPENDIX

3.6.1. HEDONIC SCALE

Name:_____ Date: _____

SAMPLE _____

1. Appearance

|_____|

Disliked very much Liked very much

2. Flavor

|_____|

Disliked very much Liked very much

3. Texture

|_____|

Disliked very much Liked very much

3.6.2. MAPPING – DESCRIPTIVE TEST

Name: _____ Date: _____

You are receiving 4 probiotic fermented milk samples. Taste each sample separately and write at maximum five words that, in your point of view, better describe them. Then design the samples in the blank sheet according to the similarities and differences between them using your own criterion. There is no right or wrong. For clarification purposes, samples that are perceived as similar should be positioned close together, and perceived as different samples should be placed far away.

SAMPLE	WORDS USED TO DESCRIBE EACH SAMPLE
_____	_____
_____	_____
_____	_____
_____	_____

CHAPTER 4

4. STREPTOCOCCUS THERMOPHILUS TA040 AND BIFIDOBACTERIUM LACTIS (BIFIDOBACTERIUM SPECIES 420) IN YOGHURT SURVIVE UPON SIMULATED DYNAMIC GASTROINTESTINAL CONDITIONS

This work is the result of collaboration with Daniel Picque, from INRA / AgroParisTech, Grignon, France and with Tomás Cattenoz, a tecnitian of the laboratory taht is specialist in the digestor system.

As will be discussed in this chapter, conventional yogurt's bacterias were never considered capable of providing benefits after ingestion because they were expected to die after digestion process in human gut; however, our study shows that reality can be a little bit different, with the survival of one of the classical yoghurts bacteria's, *Streptococcus thermophilus*, after the digestion process in an human gastrointestinal tract simulator. This result changes the overview of yogurts benefits and can help our better understanding about gut microbiota.

4.1. INTRODUCTION

Probiotics are live microorganisms (mainly lactobacilli, bifidobacteria or yeast) which, when ingested in sufficient quantities, results in health benefit to the host (FAO/WHO, 2002). Numerous species are considered as probiotics and some of them had already been used in commercial applications (FOLIGNÉ et al., 2013; KHAN; ANSARI, 2007; VASILJEVIC; SHAH, 2008). Nevertheless, the main starter cultures *Streptococcus thermophilus* (St) and *Lactobacillus delbrueckii sp. bulgaricus* (Lb) are

not included in this category because they may not reach all the essential requirements desired (FOLIGNÉ et al., 2013; GUARNER et al., 2005).

Although there are a lot of uncertainties in considering the yoghurt starter cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii sp. bulgaricus as* probiotics they cannot be definitely excluded from this classification once there are numerous recent studies showing that they can provide health benefits to the host (GUARNER et al, 2005), and reach the gastrointestinal tract alive (ELLI et al., 2006; GARCÍA-HERNÁNDEZ et al., 2012; GUARNER et al, 2005; MATER et al., 2005;).

This heavily debated prospect of applying the term "probiotic" to the starter cultures *S. thermophilus* and *L. delbrueckii* subsp. b*ulgaricus* (GUARNER et al., 2005) is based not only on its historically benefits on human health (ELLI et al., 2006) but also on the strong possibility showed by then in surviving during the passage through gastrointestinal tract (GARCÍA-HERNÁNDEZ et al., 2012; GUARNER et al, 2005), remembering that these are key factors for considering a bacteria as a probiotic (BEZKOROVAINY, 2001; VAN LOVEREN et al., 2012).

In this respect, the bioavailability or the proportion of ingested bacteria that is made available i.e. that is delivered to the colon, for its intended mode of action is more relevant than the total amount present in the original fermented food. Disruption of the natural matrix or the microstructure created during digestion may influence the release of microorganisms. Alternatively, beneficial microorganisms may be protected during their transit in the digestive system to the absorption sites by designed matrices as they can improve the stability of probiotics during storage, increase the effectiveness at the absorption site, and ensure optimal dosage (RANADHEERA et al., 2010).

The addition of fat from milk cream to a matrix base formulated by skim milk is already a traditional practice in the industry (TAMIME; ROBINSON, 2007). Although

this addition of fat to the skim milk base is a frequent method of producing yoghurt and its importance in providing a more rigid network gel (XU et al., 2008), and improve yoghurts viscosity (SHAKER et al., 2000) is well established, few is known about its importance to the microorganism viability. In this perspective, a study from (VAN BRANDT et al., 2011) evaluating the survival of *Mycobacterium avium* ssp. *Paratuberculosis* in yoghurts with different fat contents showed no consistent protective effect promoted by the enhanced fat content, yet, nothing was said about the viability of the starter cultures utilized (St and Lb) in the fermentation.

Simulation of digestion during passage of dairy products in the digestive tract requires particular methodology. A range of *in vitro* static models of digestion have been developed for the evaluation of probiotic survival in the gastrointestinal tract (MADUREIRA et al., 2011; ORTEGA et al, 2009; TAO et al., 2009), however once the digestion products aren't removed during the digestion process and they may possibly promote a potential inhibitory effect on enzyme activities and probiotic survival, this is an significant limitation (PITINO et al., 2010).

There are some methods/equipment's used in simulating conditions in the large intestine (MINEKUS et al., 1993; MINEKUS et al., 1999; RAJILIC-STOJANOVIC et al., 2010). Nevertheless, only few types of equipment - Dynamic Gastric Model (DGM), worldwide are available for in vitro dynamic approaches in the upper GI tract (LO CURTO et al., 2011). The DGM can not only process a food or meal 'as eaten' but it also replicates the additions, residence time, mixing and shear experienced by the digesta in the upper part of the stomach and the antrum.

In order to enrich the discussion around the probiotic effect of the traditional starter cultures - St and Lb - this study aimed to evaluate the survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340, *Streptococcus thermophilus* TA040 and

Bifidobacterium lactis (Bifidobacterium species 420, DuPont, Vaasa, Finland) upon simulated dynamic gastrointestinal conditions in two different yoghurt matrices, with and without fat addition.

4.2. EXPERIMENTAL SECTION

4.2.1. Materials

***Ingredients*.** Skim milk powder (SMP) (EPI ingredients, ZI de l´Hermitage, France) was used to both matrices. Milk cream (Auchan, France) was used to enrich milk´s fat content at 2.7% according to previous work (PERINA et al., submitted).

Lactic starter cultures. Three strains of pure commercial starter cultures: (i) St - Streptococcus thermophilus strain TA040 (DuPont, Vaasa, Finland), (ii) Lb - Lactobacillus delbrueckii subsp. bulgaricus strain LB340 (DuPont, Vaasa, Finland), and (iii) Bl - Bifidobacterium lactis (Bifidobacterium species 420, DuPont, Vaasa, Finland) were used for the preparation of yoghurts. The first two were pre-cultured in M17 and MRS broth (BIOKAR Diagnostics, Baden, Switzerland), respectively, initially for 17 hours than replicated and activated for more 7 h (totalizing 24 h) and the last one, B. lactis, was pre-cultured in Reinforced Clostridial Agar (RCA) broth (Oxoid, Basingstoke, UK) for 24 h and stored in a -80 ^{o}C with glycerol (10 %) before being added to the processed milk. The former two organisms are the ‘classical’ yoghurt starter cultures, whilst the bifidobacteria is a probiotic one.

4.2.2. Yogurt preparation

In order to study bacteria survival upon simulated dynamic gastrointestinal conditions two different probiotic yoghurts denoted df-y and w-y were prepared considering respectively defatted milk base or milk base supplemented with milk cream.

Milk bases were heated at 100°C for 5 min in a Thermomix TM31 (Vorwek & Co. KG, Wuppertal, Germany), divided into four flasks, then cooled to 10°C in ice water bath and stored overnight under refrigeration at 4°C. On the following day, each milk base was tempered to 42 °C and inoculated with the previously defrosted 'classical' yoghurt starter culture plus the bifidobacteria one, either pre-activated for approximately 10 min before inoculation. Each batch of milk was incubated at 42°C in a thermostatically controlled water bath until the pH reached 4.7. The acidification profile of microbial blends was monitored by using the Cinac system (Ysebaert Frépillon, France). After reaching pH 4.7 each yoghurt was quickly cooled in an ice bath. When yoghurts reached the temperature of 10 °C each sample was agitated manually using a stainless steel plunger (i.e. consisting of a rod and perforated disc) that was moved upwards and downwards for 60 s, and stored at 4 ºC until required for analysis after one day (d1) and 21 days (d21). The trials were replicated twice on different days.

4.2.3. Dynamic simulation of gastrointestinal conditions

DIDGI ("DIgesteur Dynamique Gastro-Intestinal") (MÉNARD et al., 2014) is a system that simulates the digestion in stomach and in the small intestine. The system consists of separate serial compartments simulating the stomach, the duodenum and the jejunum-ileum, an electronic box with peristaltic pumps and a computer with STORM (Stomach Regulation Monitoring) software. The system was kept at 37°C due to a glass

jacket filled with water pumped using a temperature-controlled water bath that surrounded each compartment in order to mimic the physiological human body temperature. The peristaltic movements were simulated by stirring.

The system is equipped with temperature, pH and redox sensors (Electrode InPro 4801i/SG/120, reference 52003581, Metler Toledo, France) variable speed pumps (Verder, France) to control substrates flow, a drain for each compartment and a Teflon membrane with 2 mm holes placed before the transfer pump between the gastric and the intestinal compartment mimicking the sieving effect of the pylorus in humans (KONG; SINGH, 2008).

In each compartment, physiological conditions of digestion were applied. The substrate mixed with artificial saliva was introduced in the gastric compartment. Pepsin, lipase, HCl (for stomach compartment), Na_2CO_3, bile, pancreatin, (for duodenum compartment) were introduced by computer controlled pumps. Beyond that, nitrogen was introduced by a tube connected at a nitrogen cylinder in the duodenum compartment to simulate the anaerobic conditions of the small intestine. Kinetic models were applied to simulate the pH evolution in the gastric compartment. To control the transit of the chime between the different compartments, a power exponential formula was used to monitor and control the gastric and intestinal delivery (ELASHOFF at al., 1982) and thus the times of delivery and the kinetics were chosen according to the wished type of digestion. Concentrations of the solutions, the flow rates and transit were programmed to reproduce gastrointestinal conditions according *in vivo* experiments described previously (BLANQUET et al., 2004; MINEKUS et al., 1995). Samples were collected at 10, 30, 60, 100, 140 and 210 min of digestion at the specific compartment according to the schedule of analyses determined in preliminary tests. During dynamic simulation of gastrointestinal conditions enumeration of viable bacteria was conducted.

Trials for dynamic simulation of gastrointestinal conditions were replicated twice independently for all yoghurts 24 hours after fermentation (d1) and after 21 days of storage at 4°C (d21).

4.2.4. Experimental design

Before each experiment the equipment was decontaminated by steaming at 121ºC for 20 min. All digestive juice compoments were purchased from Sigma (Saint Quentin-Fallavier, France) and diluted in MRD (BD, Difco - Sparks, USA). The tested yoghurts (150 mL) were introduced by a computer controlled pump into gastric compartment after dilution (2:1, vol/vol) in a sterile solution prepared with α-amylase from *Aspergillus oryzae*, 10065, and mucin from porcine stomach, type II, M2378 (to simulate the in vivo dilution by saliva.

After rehydration, pepsin plus lipase were kept on ice bath throughout the experiment in order to avoid autolysis. Bile salts were slightly heated to better dilution and the other enzymes were kept in room temperature.

In the stomach, the pepsin from porcine gastric mucosa P6887added to lipase from *Rhizopus oryzae* 80612 was added at constant flow rate of 0.25 mL/ min. In the small intestine compartment, a 1% solution of porcine bile extract B8631 and a 10% solution of pancreatin from porcine pancreas P1750, were added at constant flow rate of 0.5 mL/ min and 0.25 mL/ min, respectively.

The pH curve in the stomach was computer controlled following a linear regression [pH= -0.011*t + 5.4, where t is time] to reproduce the values found in humans after yoghurt consumption (MOUGHAN et al., 1992): pH 4.5 at initiation, pH 3.8 at 20 min, pH 2.9 at 40 min, pH 2.0 at 60 min, and pH 2.0 at > 80 min. In the small intestine pH was kept constant in a neutral condition (6.5 ± 0.5).

Samples were collect during digestion in stomach at 10, 30, 60 and 100 min after beginning of the process (introduction of the yoghurt in the first compartment), at 30, 60, 100 and 140 min in the small intestine, and at 100 and 210 min in the final intestinal portion.

4.2.5. Enumeration of viable cells

Samples (1 mL) were diluted with 0.1% sterile peptone water (9 mL). Afterwards, serial dilutions were carried out, and bacteria were counted, applying the pour plate technique. *S. thermophilus* colonies were enumerated in M17 agar (Biokar Diagnostics, Baden, Switzerland)), while those of *L. delbrueckii* subsp. *bulgaricus* in MRS (BIOKAR Diagnostics, Baden, Switzerland) at pH 5.4, both under aerobic incubation at 37°C for 48 hours. *Bifidobacterium animalis* subsp. *lactis* were enumerated in RCA (Oxoid, Basingstoke, UK) plus 100 μL mL^{-1} of dicloxacilin incubated at 37°C for 72 h under anaerobic conditions (SACCARO et al., 2011). The antibiotic allowed selective growth of the probiotic bacteria. Bacterial enumerations were carried out after 1 (d1) and 21 (d21) days of cold storage in at least two replicates of each batch. Cell counts were expressed as log $CFU.mL^{-1}$ of yoghurt and average values were calculated. The accounts of viable cells during the simulated digestion were corrected according to the dilution in each digestion time / compartment.

4.2.6. Statistical analysis

Multifactor analyses of variance and multiple comparison tests were done using Statistica 15.0 (Statsoft, Tulsa, USA) in order to determine statistical significance of differences among samples. Mean values were compared using the Newman Keuls test

at $P < 0.05$. All results were expressed as mean of two replicates, including two independent experiments.

4.3. RESULTS AND DISCUSSION

4.3.1. Survival of bacteria during simulated dynamic gastrointestinal conditions

Initial bacteria counts, in average, one day after fermentation (d1) were 9.20 ± 0.01, 7.12 ± 0.21 and 9.04 ± 0.69 log CFU mL^{-1} for *S. thermophilus*, *L. bulgaricus* and *B. lactis*, respectively. After 21 days of storage, St average counts remained stable (9.24 ± 0.06 CFU mL^{-1}) whilst a small decrease was noted in average counts of Lb (6.93 ± 0.40 log CFU mL^{-1}). But nevertheless, Bl counts slightly decreased reaching, in average, 8.51 ± 0.65 log CFU mL^{-1}. This behaviour was reported before by Florence et al (2014) for other Bifidobacteria strains, and could be mainly due to milk fat composition.

In order to standardize bacteria counts, percentage of viable bacteria cells after the simulated dynamic digestion was considered even though cell counts were enumerate as log CFU.mL^{-1} of yoghurt. Then the initial counts (before digestion) obtained at day 1 and day 21 was noted as total viable cells (100 %). Based on this value, the percentage of survivability of the microorganisms from the counts obtained at each time in each compartment (stomach, duodenum and intestine) was calculated. Total initial counts in duodenum and intestine were not considered 100 % because the microorganisms had already suffered damages in previous compartments and were at that moment more susceptible. Results were chosen to be expressed in percentage in order to facilitate comparisons with other studies and other microorganisms, once it permits the observation of their individual resistance even with different initial counts.

Lb behaviour during simulated digestion. Regarding *Lb* it is possible to observe a potential matrix protection of the microorganism during stomach digestion, and also that the storage in a milk base brought a sort of adaptation for the bacteria that survived in a greater number throughout all simulated digestion when present in yoghurt stored at d21 than in product after 1 day of fermentation (Figure 4.1), once it is known that dairy products are able to enhance the transit tolerance of probiotics (LO CURTO et al., 2011). Besides, it is possible that the bacteria may had achieved more resistance due to the cold adaptation caused by the storage period before inoculation and during the shelf life as some morphological and physiological may occur toward cold temperatures (LORCA; DE VALDEZ, 1998).

The product not added by milk cream (df) showed a survival rate of 39 % at d1, and 70.4 % at d21 in stomach. On the other hand, the enriched yoghurt (w) showed 54.9 % of *Lb* survival at d1, statistically higher ($P \leq 0.05$) than the df yoghurt. On the other hand, whole yoghurt stored at d21 showed only 62.7 % of Lb survival. These 21 days of cold storage seemed to reduce the protective effect of the enriched matrix upon Lb when compared with df yoghurt.

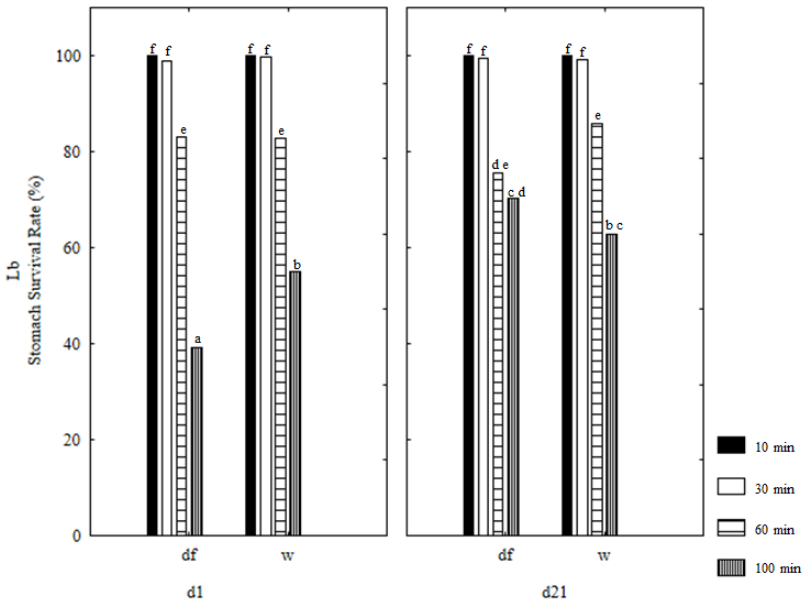


Fig. 4.1. Survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340 fermented in co-culture with *Streptococcus thermophilus* TA040 and *Bifidobacterium lactis* (Bifidobacterium species 420) in skimmed milk (df) and whole milk (w) at 42°C until pH 4.7 after 24 h and 21 days of cold storage.

During dynamic simulated digestion, samples were collected before digestion (10 min), after 10, 30, 60 and 100 min at stomach. Mean values ± standard deviation (n = 4) with different letters are considered significantly different; $P \leq 0.05$.

Unfortunately, even with the dynamic approach employed in the present study we could not identify or cultivate viable Lb cells after duodenum and intestine compartments passage. This results indicate a possible resistance of Lb to acidity (pH 2.0 at stomach) and to gastric enzymes, but not to bile salts and pancreatine. The first 30 min at duodenum compartment was sufficient to cause the death of all Lb cells that have survived throughout the stomach digestion. Lb survival data during dynamic digestion are divergent to those from García-Hernández et al. (2012) and Marteau et al. (1997) that found Lb to be more sensitive to gastric juices.

St behaviour during simulated digestion. Figure 4.2 (A,B and C) shows the results of St's survival after passing the three compartments that simulated the gastrointestinal tract - stomach, duodenum and ileum. Time of storage (after one and 21

days of cold storage) didn't affect the acid resistance of St, contradicting some data published until now (GARCÍA-HERNÁNDEZ et al., 2012; MARTEAU et al., 1997). There were no differences in St counts after the stomach digestion between the product with (w) or without fat (df), showing that enriched matrix wasn't imperative factor to promote better protection against acidity.

Counts reduction of St during gastric digestion was of 39 % for df yoghurt and 35 % for the fat enriched product (w), resulting in a survival rate of approximately 60 % for both products during their whole shelf-life (Figure 2A). This result opposes García-Hernández et al. (2012), whose work found a final survival percentage of 10.9 % for *Streptococcus thermophilus* CECT 801. Maybe this difference in survival rate of St between both studies is associated with the time of exposure to acidity, the pH, the protocol utilized or even the strain. The total gastric exposure time in our study was of 110 min, from pH 4.5 to pH 2 in dynamic approach with a maximal exposure time at pH 2 of 50 min whilst their study used a static protocol with 180 min of gastric juice (pepsin) exposure at pH 2. Besides, these authors have observed the survival of *Streptococcus thermophilus* CECT 801, even in small amounts. Intriguingly, a study published by Marteau et al. (1997), employing dynamic approach, showed also a tiny resistance of St strain ST20 in the gastric compartment, under 1 % within 70 and 110 min.

In the Figure 4.2B it is possible to notice that the initial viability of both products at both storage times were about 80 %. It is important to notice that because of the dynamic approach of our digestion protocol, "chime" reached duodenum compartment 30 minutes after the beginning of digestion (introduction of the product added by saliva at stomach), this explains why initial microorganism's counts at duodenum were greater than the final counts at stomach. The action time/contact of

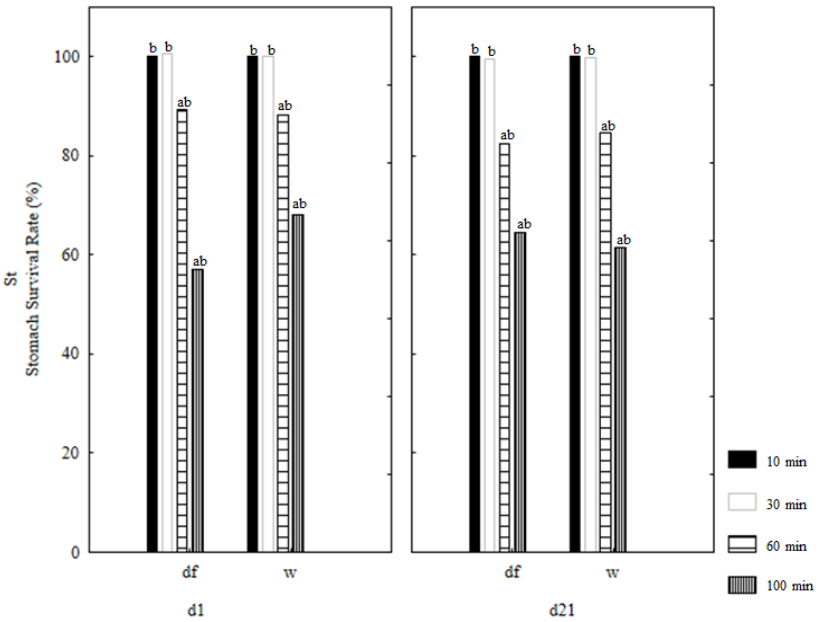
digestion enzymes (pepsin and lipase) and HCl with bacteria was of 30 minutes rather than 100 min (total time to stomach emptying). At 30 min, the pH was 5 and the level of St near 100% in the stomach compartment

Yoghurts showed a slightly decrease in St's viability after bile and pancreatine action (duodenum simulation). A greater reduction in St counts, with statistical relevance, was saw for the product supplied by milk cream (w) at the end of duodenum digestion simulation (140 min) after 21 days of cold storage (4ºC). This reduction was only observed in this point of digestion. The time of storage and the presence of fat can't be considered a cause for this, because it would have affected other points of the digestion. There's no result like this published before, and more research is needed to elucidate this behaviour.

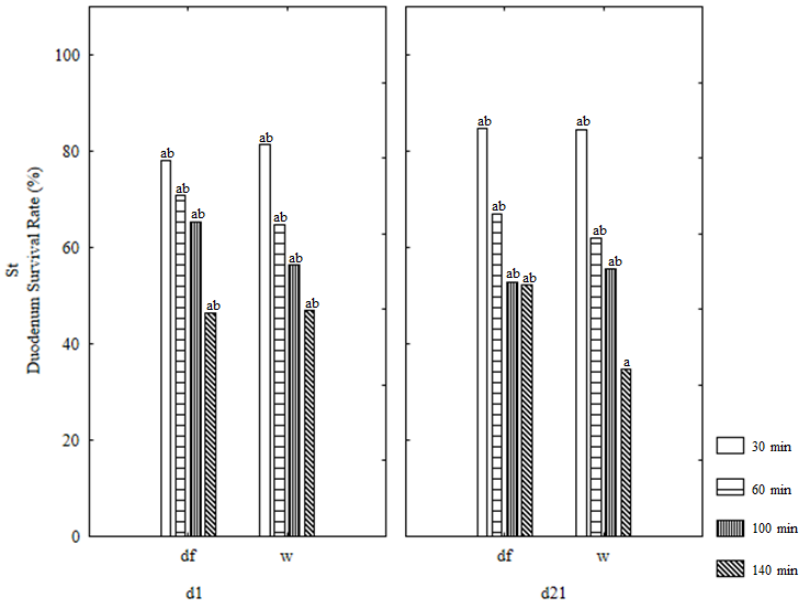
At the end of simulated dynamic digestion (Figure 4.2C) there is a significant reduction of St viability when compared to initial counts, thus, not greater than de reduction observed at stomach alone, demonstrating that St is sensible to acid digestion (low pH or gastric enzymes) and resistant to bile or pancreatic salts.

Finally, St counts are maintained unaltered in intestine compartment during the whole process considering both products at both storage periods as there were no additional enzyme action in this compartment. After 210 min of simulated dynamic digestion there were a reduction on St counts of 43 % and 51 % for no fat and whole fat yoghurts, respectively, showing no protector effect of the fat in the matrix. Apart from that, results of the present study demonstrate that the survival of St corroborates with Mater et al. (2005) that recovered viable St cells in human faeces after a 12-day period of fresh yoghurt consumption three times daily.

(A) Stomach



(B) Duodenum



(C) Intestine

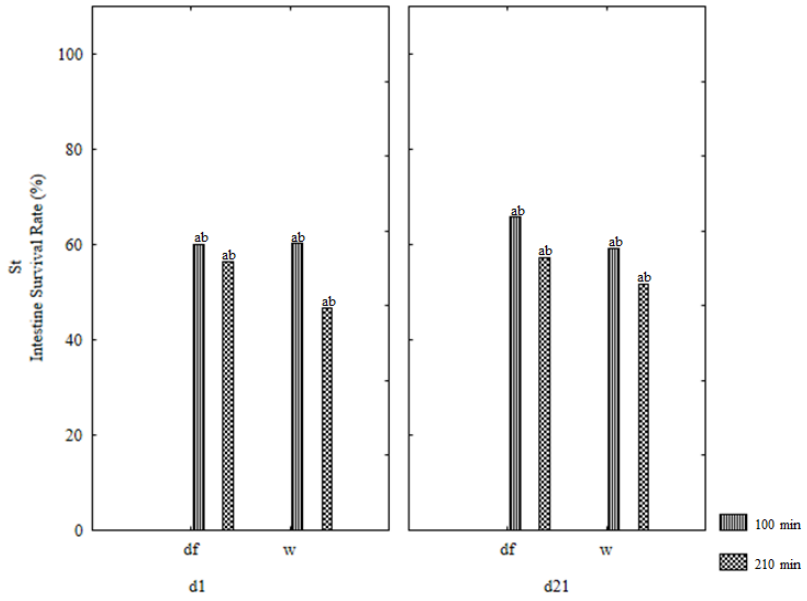


Fig. 4.2. Survival of *Streptococcus thermophilus* TA040 fermented in co-culture with *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340 and *Bifidobacterium lactis* (Bifidobacterium species 420) in skimmed milk (df) and whole milk (w) at 42°C until pH 4.7 after 24 h and 21 days of cold storage. (A) Stomach, (B) Duodenum and (C) Intestine.

During dynamic simulated digestion, samples were collected before digestion (10 min), after 10, 30, 60 and 100 min at stomach, 30, 60, 100 and 140 min at duodenum and 100 and 210 min at intestine compartment's. Mean values ± standard deviation (n = 4) with different letters are considered significantly different; $P \leq 0.05$.

Bl behaviour during simulated digestion. For the probiotic culture, *Bifidobacterium lactis*, it is possible to observe a probable protector effect of the matrix (Figure 4.3), mostly in fat enriched yoghurt, cold stored for 21 days. Bl seems to be strongly resistant to low pH and to gastric enzymes in the product at d1 (Figure 4.3A). From the beginning of digestion until the end (100 min), a total viable cell of Bl in stomach was similar for both products ($P \leq 0.05$).

Nevertheless, after 21 days of cold storage it was noticed an increase in the sensibility of Bl to stomach digestion with a reduction of 30 % in the survival rate at 100 min for the no fat yoghurt (df). Besides, the milk cream enriched matrix was

capable to protect the surveillance of probiotic bacteria keeping a survival rate greater than 90 %.

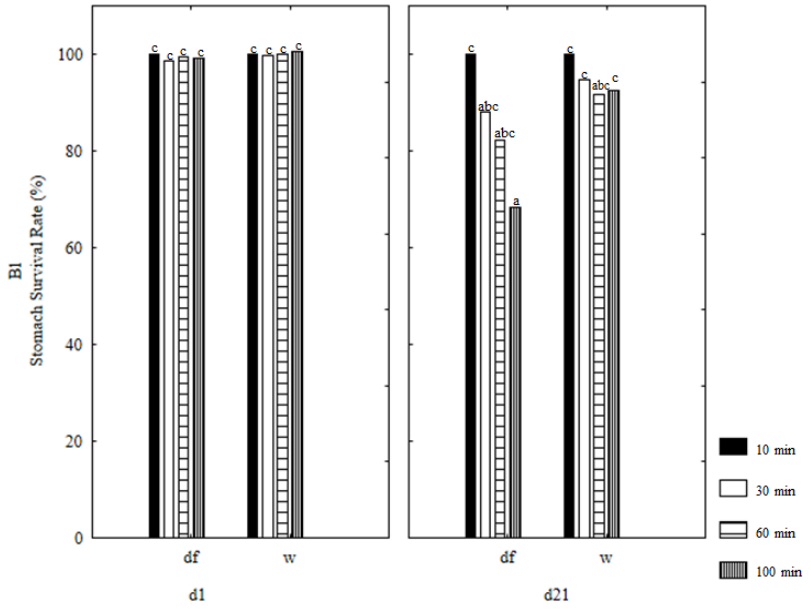
At duodenum compartment the result was slightly different. Bl resistance was significantly higher in yoghurt at d1 reaching the end of duodenum digestion (140 min) with a survival rate of 96 % and 94 % to defeated and whole yoghurts, respectively (Figure 4.3B). Nevertheless, Bl stays stable in no fat yoghurt (df) at d21, but slightly decreases, without statistical significance, after 140 min in duodenum for whole yoghurts (w).

Thus, the presence of fat in the matrix is capable to protect the probiotic culture *B. lactis* against acidity in stomach after 21 days of cold storage. However, it didn't affect its survival against bile salts and pancreatine in duodenum.

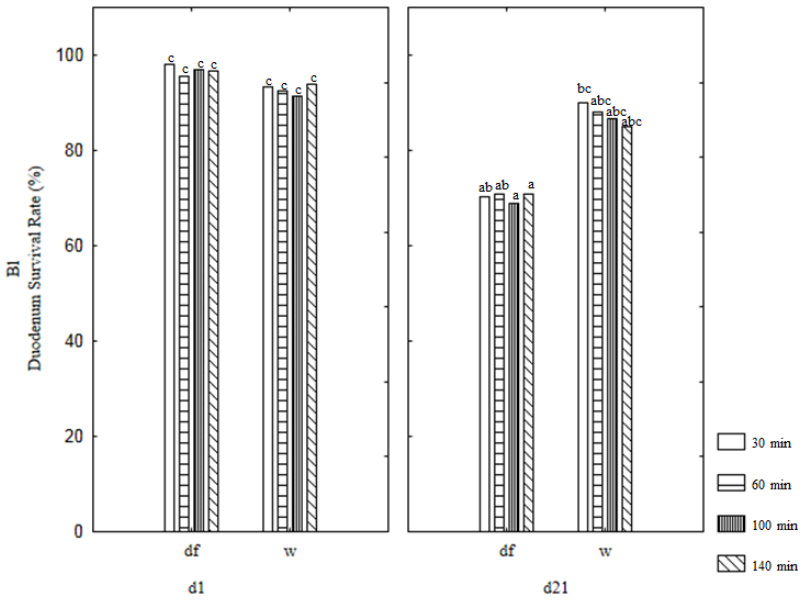
In the intestine, Bl counts remain stable in both yoghurts at d1 (Figure 4.3C). In yoghurts after 21 days of cold storage counts of Bl in whole yoghurt (w) were slightly higher than in non-fat yoghurt (df) due mainly to the greater survival rate observed in stomach that probably occurred by the presence of fat in the first product (w).

The model chosen in this study was appropriate to observe the sequential influence of gastric digestion / acidity, gastric emptying and bile / pancreatine effect at duodenum on the survival of different microorganisms. But it is important to notice that the results observed in the essays regarding the number of viable bacteria found might be underestimated due to the irregular distribution of microorganisms in the samples and the loss of cultivability as it was utilized the plate counting method instead of molecular techniques, highly sensitive and more specific for DNA bacterial detection (MATER et al., 2005).

(A) Stomach



(B) Duodenum



(C) Intestine

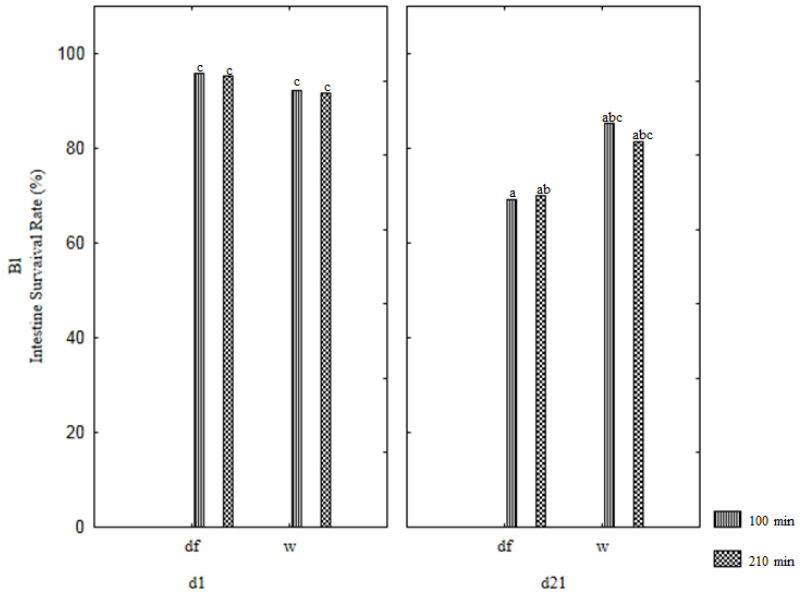


Fig. 4.3. Survival of *Bifidobacterium lactis* (Bifidobacterium species 420) fermented in co-culture with *Streptococcus thermophilus* TA040 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340 in skimmed milk (df) and whole milk (w) at 42°C until pH 4.7 after 24 h and 21 days of cold storage. (A) Stomach, (B) Duodenum and (C) Intestine.

During dynamic simulated digestion, samples were collected before digestion (10 min), after 10, 30, 60 and 100 min at stomach, 30, 60, 100 and 140 min at duodenum and 100 and 210 min at intestine compartment's. Mean values ± standard deviation (n = 4) with different letters are considered significantly different; $P \leq 0.05$.

Although the works of Del Campo et al. (2005), García de los Ríos et al. (2003), Marteau et al. (1997) and Yuste et al. (2003) affirmed that LAB in yoghurt are not able to survive the GI tract the essays presented here can only confirm this kind of comportment for *Lactobacillus delbruecki* strain *bulgaricus*, but not for *Streptococcus thermophilus*. *St* survival trough GI tract is in agreement with Drouault et al. (2002), García-Hernández et al. (2012), Lick et al. (2001) and Mater et al. (2005). The techniques presented by all these works are not mandatorily the same. Intriguingly the results of the present study were exactly the opposite of Alvaro et al. (2007) and Elli et al. (2006) that observed the survival of Lb but not of St.

4.4. CONCLUSIONS

Inappropriately, even with the dynamic approach employed in the present study it was not possible to identify or cultivate viable *L. bulgaricus* cells after duodenum and intestine compartments passage. At the end of simulated dynamic digestion there is a significant reduction of *S. thermophilus* viability mainly due to the low pH and gastric enzyme's action in the stomach. To the best of our knowledge, it is the first time that the survival of *Bifidobacterium lactis* strain 420 is described in the literature, confirming the stronger digestion resistance of *Bifidobacterium* spp, which are increasingly used as probiotics. As a final point, the survival of St strain TA040 in this study associated with the potential benefits provided by this starter culture strengthens the argument in considering this bacteria as a probiotic.

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CHAPTER 5

5. FERMENTED MILK CONSUMPTION, FOOD INTAKE AND WEIGHT CONTROL IN OVERWEIGHT AND OBESE ADULTS – A PROSPECTIVE, RANDOMIZED, DOUBLE-BLIND TRIAL

This work was developed in collaboration with the doctors Ricardo Barbuti, Jaime Eisig and Thomaz Navarro-Rodriguez of the Gastroenterology Department of the Clinical Hospital of University of São Paulo School of Medicine. The team of the processing plant of BRF S.A. have also an important role in manufacturing the products so we could distribuit them to the patients of the study.

In this chapter we investigated the influence of increasing fermented milk consumption in food intake and weight control in overweight adults. The fermented milks here studied i.e. those submitted to the clinical trial were different from those assessed earlier in chapters 2, 3 and 4. Based on the results achieved formerly and regarding the industrial conditions to manufacture high quantities of products required to the clinical trial, new products were designed keeping the main ingredients and microrganisms.

5.1. INTRODUCTION

It is a consensus that overweight and obesity has become a global epidemic. Obesity has doubled since 1980 and 35 % of adults aged 20 and over were overweight in 2008 (11 % were obese) (WHO, 2014). A increasingly number of studies have shown strong

associations between obesity and deep changes in metabolic function and composition of the gastrointestinal microbiota allowing this "obese microbiota" to take out more energy from the diet (TILG, 2010).

The human gastrointestinal tract has a vast number of bacteria that are diverse, complex and dynamic (TEITELBAUM and WALKER, 2002). Evidences suggest that this trillion of resident bacteria affects nutrient achievement and energy expenditure, as well as storage. It is also suggested that lean and obese people have distinctive microbiota (DIBAISE et al., 2008; TILG, 2010).

Gastrointestinal microbiota can be seen as a metabolic organ (WOLF, 2006) finely adjusted to human physiology performing functions that our body would not be able to do by itself, as the ability to digest otherwise indigestible diet components, heavily affecting energy harvest from food and energy storage in the host (BÄCKHED et al., 2004). Modifications in diet may, likewise, promote significant and potentially long-lasting changes in the gut microbiota (WU et al., 2011; KRAJMALNIK-BROWN et al., 2012). These findings raise the important role of gastrointestinal microbiota in controlling body weight, shows its possible role in obesity development (SBEM, 2007), explains in part the growing interest on probiotics - live microorganisms which, when ingested in sufficient quantities, results in health benefit to the host (FAO/WHO, 2002) and justifies the public health importance of developing foods or identifying food ingredients that are capable to reduce total energy intake, absorption or storage (BÄCKHED et al., 2004).

Besides the influence of the microbiota on energy harvest from diet, indigenous gastrointestinal microbes may have an effect on host’s metabolic and neuroendocrine functions, altering the production of digestion sub products, such as SCFA (short chain fatty acids), stimulating the expression of peptide hormones involved in appetite (leptin

i.e.) and, therefore, eating behavior (SANZ et al., 2010). Alterations in satiety hormones, for example, may influence food intake, as shown by Forssten et al. (2013).

Apart from probiotic use there are several other natural compounds already described to promote beneficial effects against obesity. As natural ingredients the use of so-called "natural bioactive substances" seems to be safer since they occur mainly in foods. Great examples of natural bioactive compounds are the dietary fibers and prebiotics (TORRES-FUENTES et al., 2015), which are well known to produce qualitative and selective alterations in the gastrointestinal' microbiota through fermentation of determined non-digestible carbohydrates by resident bacteria, what may lead to benefits for the host (ROBERFROID et al., 2010), and even blunt ghrelin, the hunger hormone, response to meal (PARNELL; REIMER, 2012). Dietary fibers, soluble and insoluble ones, have also the beneficial effect of increasing satiation and satiety (KRAJMALNIK-BROWN et al., 2012).

Therefore the present clinical trial had the objective to evaluate the effect of the consumption of four different fermented milks, fermented by *Streptococcus thermophilus* – TA040 and *Bifidobacterium lactis* – B420 (except for the FM1 product that was used as a no functional control fermented milk), enriched or not with fiber from passion fruit peel and / or a commercial vegetal oil emulsion (Fabuless™) on feed intake, ghrelin and leptin secretion – two hormones directly related to hungry and satiety –, as well as its influence on weight, BMI and % body fat.

5.2. EXPERIMENTAL SECTION

Members of the randomized controlled trial registered as "The effect of a probiotic compound in dyspeptic patients" (ISRCTN22923997) (Appendix 5.7.1) were

included in these analyses (208 participants with approximately 2212 observations). At each visit, dietary intake was assessed by a 24 h food questionnaire (Appendix 5.7.2) and weight was obtained following standardized procedures. Blood sampling was collected at the begging and after three months of treatment for biochemical analyses.

All patients should not be in a weight loss program, wake up and have breakfast after 9:00 h, frequently skip breakfast, lunch or dinner, simultaneously participate in other clinical studies, be in use of any prescription drug and women should not be pregnant or breastfeeding. Patients should have Body Mass Index (BMI) greater than 25 kg/m² to characterize the overweight or obese status. Prior to participation all patients had to give their informed written consent.

This study had the approval of the Ethic Committees of the School of Medicine Clinical Hospital from the University of São Paulo School of Medicine, protocol number 0602/11 (Appendix 5.7.3; Appendix 5.7.4) and the Pharmaceutical Sciences Faculty of São Paulo's University, protocol number 69572 (Appendix 5.7.5; Appendix 5.7.6).

5.2.1. Fermented milk production

Four test products (fermented milks) were specially designed as follows: (i) FM1: *Streptococcus thermophilus* strain TA040; milk fat, designed as a no functional fermented milk control; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

All products were prepared at BRF S.A. – Carambeí-PR, Brazil accordingly to preliminary tests (PERINA et al., 2015). The total amount of passion fruit peel powder used was the necessary to reach 1 % of fiber in the final product. Fat from milk cream (Carambeí, Paraná, Brazil) and vegetal oil emulsion - Fabuless™ - which is formulated from palm-oil and oat oil fractions (DSM Nutritional Products, Basel, Switzerland) were used to adjust the fat content of yoghurts at 2 %.

To mask any different appearance that the ingredients could provide a yellow natural dye annatto (A-260-WS, CHR-Hansen Brasil) and a natural passion fruit flavoring were utilized (Mane do Brasil, Jacarepaguá, Brazil).

The designed formulas had the objective to guarantee that the four products had the same energetic content and macro-nutrients composition and also similar aspect, consistency and flavour.

5.2.2. Fermented milk analyses

The total solid, fat and protein contents of the milk bases were determined by theoretical calculation.

Enumerations of viable cells were carried out in 4 out of 16 lots of products, applying the pour plate technique, accordingly to Saccaro et al. (2011), at day 7 (d7) and day 28 (d28) of cold storage to guarantee that all products had the minimal bacteria counts to promote the probiotic effect during the whole shelf life period; cell counts were expressed as log CFU mL^{-1} of yoghurt, and average values were calculate.

5.2.3. Experimental Protocol

Patients were randomly divided into four groups. Each group received one of the four fermented milks in enough quantity for one month of consumption in thermal bags

to maintain the products chilled. During 90 days from the first clinical evaluation all the patients had to drink 100 g portion size of the respective fermented milk twice a day (one portion in the middle of the morning and the other portion two to three hours after lunch). During this period, they were instructed to write down any undesirable symptoms caused by the consumption of the product.

Anthropometric measurements: Height and weight were measured at the first visit and at the end of the treatment period, with the participant standing, shoes off, and wearing light clothes. BMI was then calculated in kg/m². Body fat mass was measured with Inbody 720 multifrequency impedance plethysmograph body composition analyzer (Biospace, Korea) as a percentage.

Food questionnaire: Total calorie, carbohydrate, protein and fat intake were calculated by the Nutriquanti© online monitoring system of food consumption (version 2011).

Measurements of other variables: Fasting (≥ 8 h) blood samples were drawn for assessing the levels of ghrelin and leptin. Both hormones were measured by Elisa kits (Human Ghrelin (Total) Elisa, EZGRT-89k, Genese, São Paulo, Brazil and Human Leptin "Dual Range" Elisa, EZHL-80Sk, Genese, São Paulo, Brazil).

5.2.4. Statistical analysis

All analyses were conducted separately for each variable considered.

Comparisons of initial and final mean values of all participants were tested by paired t-test. Statistical analyses of variance, covariance and multiple comparison tests were done using IBM SPSS software for Windows version 13.0 (IBM, USA) in order to determine statistical significance of differences among samples.

To perform the descriptive analysis of ghrelin, leptin, weight, BMI and body fat percentage per group at each visit (V1 and V4), average, standard deviation, minimum, median, maximum and number of valid observations were calculated. To compare data variation of ghrelin, leptin, weight, BMI, body fat percentage and feed intake (calories and macronutrients) between visits V1 and V4 per group, considering the visit V1 as a covariate, the model analysis of covariance (ANCOVA) was used. When the covariate was not statistically significant in the model, analysis of variance (ANOVA) was used. To compare the responses of IMC between V1 and V4, per group, in the categories: Increase, Decrease, or maintained, the chi-square test or, when necessary, the likelihood ratio test was used.

A significance level of 5 % (p-value ≤ 0.05) was used for all analysis.

5.3. RESULTS

Table 1 shows participants' characteristics and dietary patterns at the beginning and after the treatment period. The mean (± s.d.) age of the participants at baseline was 40.9 (± 9.6) years (range 23-66 yr). Participants were in majority overweight and obese, with mean BMI of 31.8 (± 6.1) kg/ m². Because of exclusions by pregnancy or absence in the final exam the survey finished with 173 participants. Other exclusions were made due to problems with measures and laboratory analyses errors.

Table 5.1. Characteristics of participants and dietary patterns (means ± s.d. or percentage)[a]

	Visit 1	*Visit 4*
Number of participants	208	143
Age (years)	40.9 ± 9.6	
Men (%)	7.2	5.8
BMI (kg/ m²)	31.8 ± 6.1	31.5 ± 5.6
% Body fat	40.4 ± 7.0	39.4 ± 6.8[b]
Dietary Intake		
Total calorie (kcal)	1663.8 ± 570.6	1453.0 ± 484.9[b]
Carbohydrate (g)	203.6 ± 74.4	175.1 ± 60.0[b]
Total fat (g)	63.6 ± 28.2	54.6 ± 24.0[c]
Protein (g)	73.1 ± 34.3	67.3 ± 42.0
Dairy Intake		
Fermented milk (servings per day)	0.15 ± 0.4	1.3 ± 0.9[b]
Total calcium intake (mg)	578.8 ± 285.6	566.0 ± 317.5

Abbreviations: BMI, body mass index. [a] Comparisons between exams (visit 1 and visit 4) were tested by paired t-test. [b]*P-value* < 0.01 for comparing with first exam (visit 1). [c]*P-value < 0.05* for comparing with first exam (visit 1).

5.3.1. Fermented milk characterization and viable cell counts

All products presented the same calorie and macronutrients (carbohydrate, lipids and protein) content as well as calcium and lactose content (data not shown).

The mean of viable cell enumerations of the four lots analyzed were as follows: at d7 viable cell counts ranged from 8.81 to 9.21 log cfu.100 g^{-1} for *S. thermophilus*, and from 8.55 to 8.64 log cfu.100g^{-1} for *B. lactis*. During the 28 days of storage *S. thermophilus* and *B. lactis* counts were stable and ranged as an average from 8.68 to

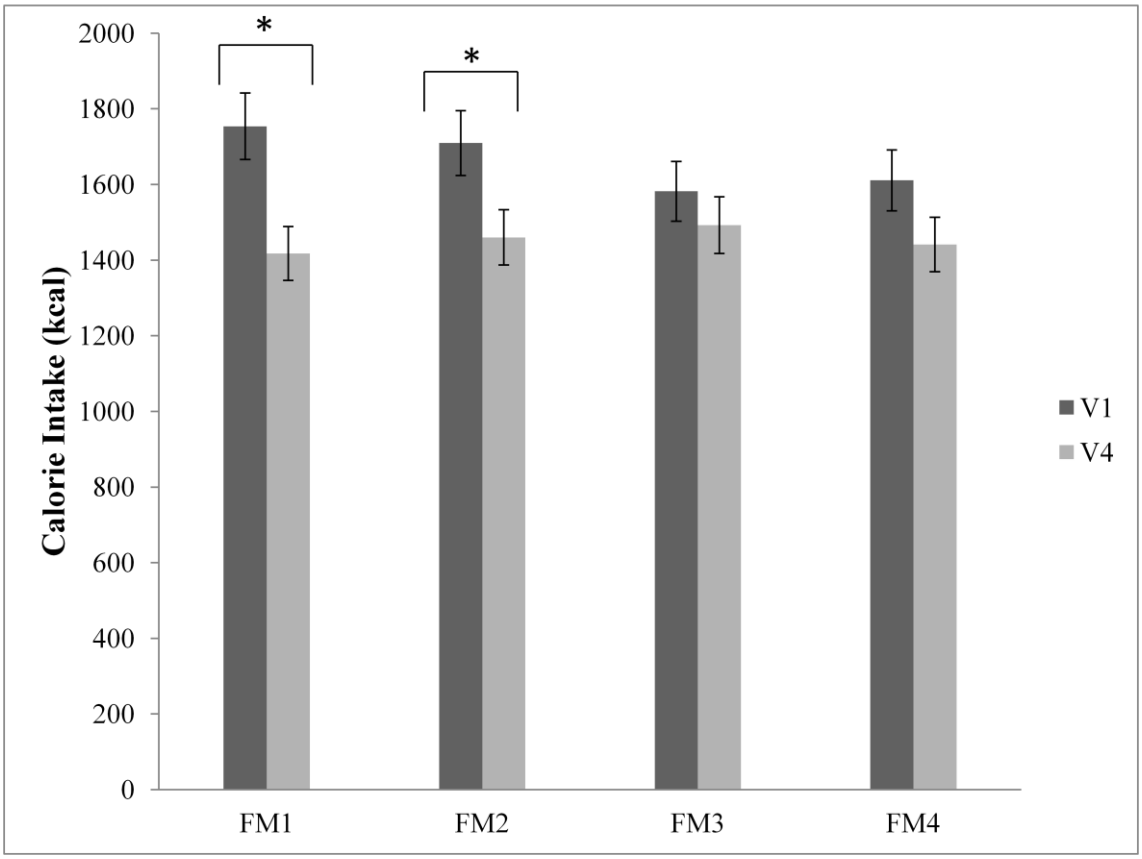
9.21 log cfu.100g^{-1} and from 8.62 to 8.79 log cfu.100g-1, respectively. These values are in accordance with those established by ANVISA for probiotic foods (ANVISA, 2002).

5.3.2. Food questionnaire (24 h)

Total calorie consumption tended to be higher at baseline, despite it was not made any guidance on energy restriction.

Although fermented milk servings consumption had significantly increased ($P < 0.01$), total calcium intake remained unaltered (Table 5.1). It can be observed that this boost in fermented milk consumption promoted a generalized propensity of lowering caloric ingestion after the treatment. Although it has not been observed any difference in calorie intake between the groups (Figure 5.1), there was an intra-group reduction in calorie intake for people that consumed FM1 and FM2 products ($P < 0.05$). The same trend of reduction in intake was observed for the consumption of carbohydrates, proteins and fat (Figure 5.2), but again, with no significant difference between the groups. Participants of FM1 and FM2 groups presented, respectively, a significant reduction of carbohydrate ($P < 0.05$) and total fat ($P < 0.01$) intake. There was no significant decrease in protein intake neither between nor within the groups.

No side effects were observed with the ingestion of any of the products tested in this survey.

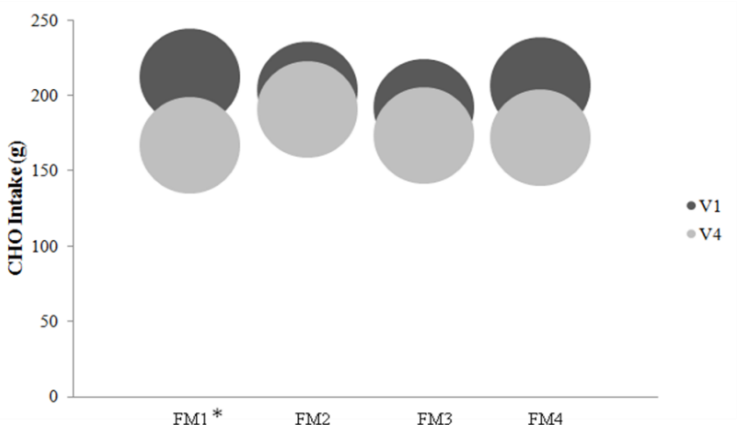


** P < 0.05*

Figure 5.1. Average calorie intake (kcal) before (V1) and after (V4) treatment.

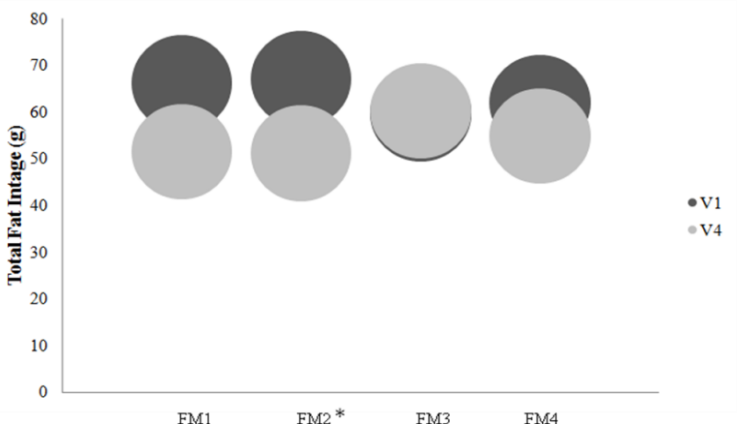
(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

a)



** P < 0.05*

b)



** P < 0.01*

c)

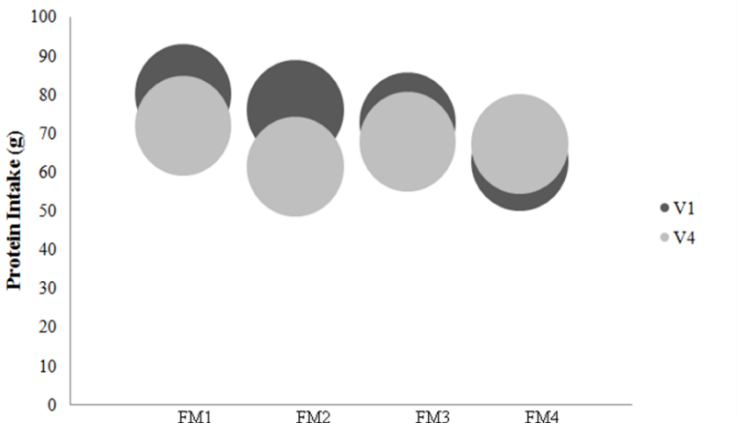


Figure 5.2. Average consumption of macronutrients before (V1) and after (V4) treatment. (a) Carbohydrate; (b) Total fat; (c) Protein

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

5.3.3. Body weight, BMI and Body Fat

For body weight measures, shown in Figure 5.3, it can be observed a significant difference between visit 1 (before beginning the treatment) and visit 4 (at the end of the treatment period) for people that consumed the product FM1 (no probiotic, no additions).

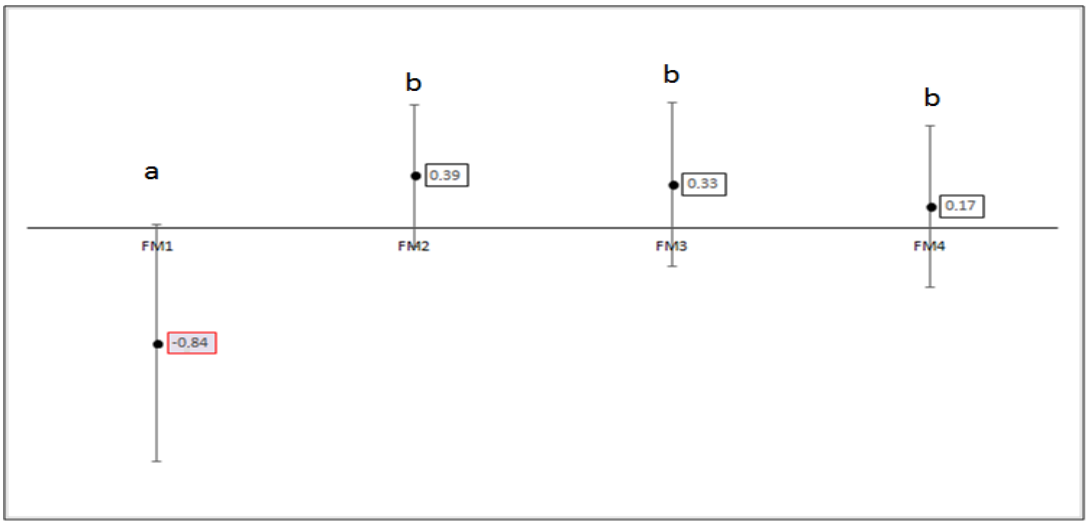


Figure 5.3. Variation of body weight according to groups before (V1) and after (V4) treatment.

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

Mean values ± standard deviation (n = 152) with different letters are considered significantly different; $P < 0.05$

For BMI measures, there were no significant differences when analyzing the whole groups' variation before and after the survey (Figure 5.4). However, when analyzing BMI response in the categories: increase, decrease or maintained it is possible to observe a significant decrease in most of the people in the group FM1 (59.5 %) while a lot of people in FM2 and FM3 groups presented a greater increase of this variable, reaching, respectively, about 66.7 and 61.0 % people. Group FM4 didn't differ from the other 3 groups (Figure 5.5).

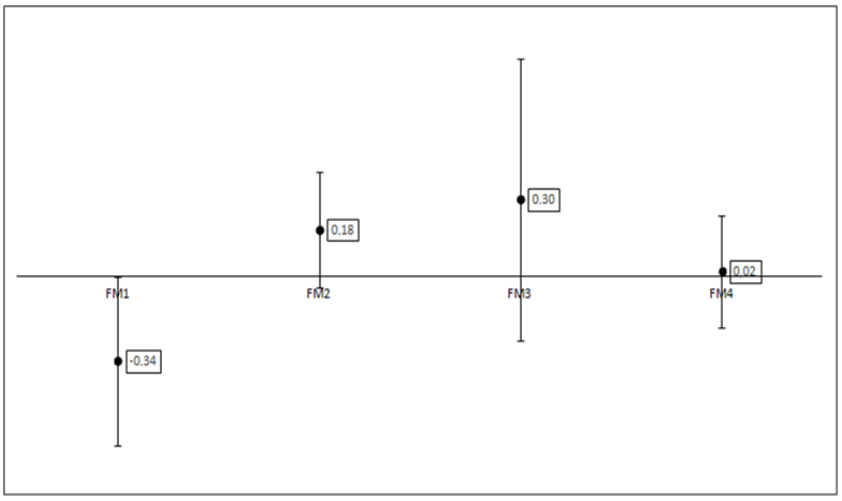


Figure 5.4. Variation of BMI according to groups before (V1) and after (V4) treatment.

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion; *P < 0.05*

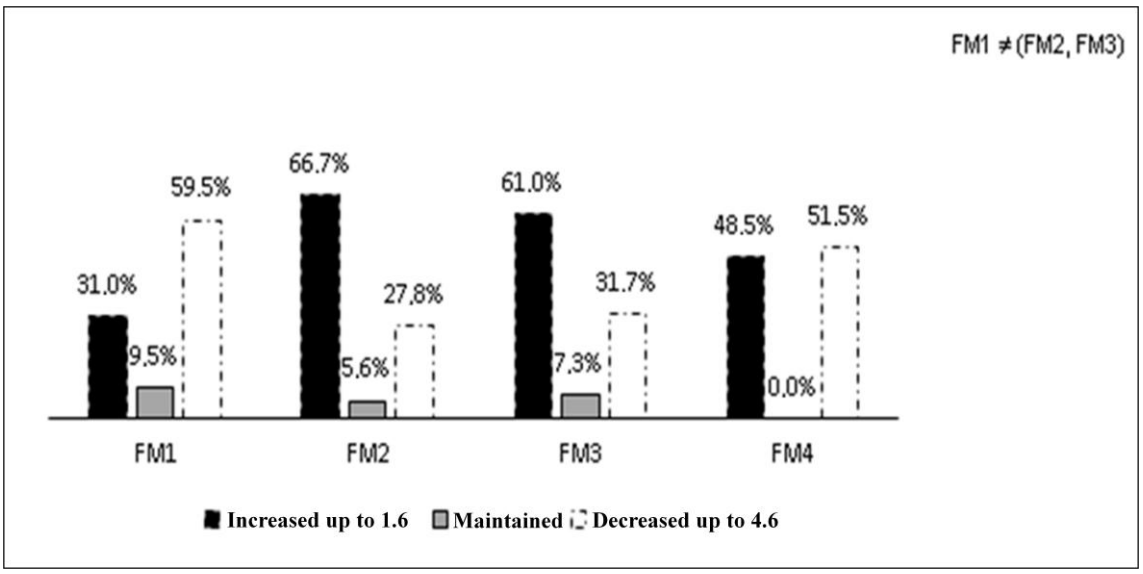


Figure 5.5. BMI response in the categories: increase, maintained or decreased, according to groups after treatment.

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion; *P < 0.05*

There was a decrease of percentage of body fat in all groups between visits 1 and 4, with no differences between the groups (Figure 5.6).

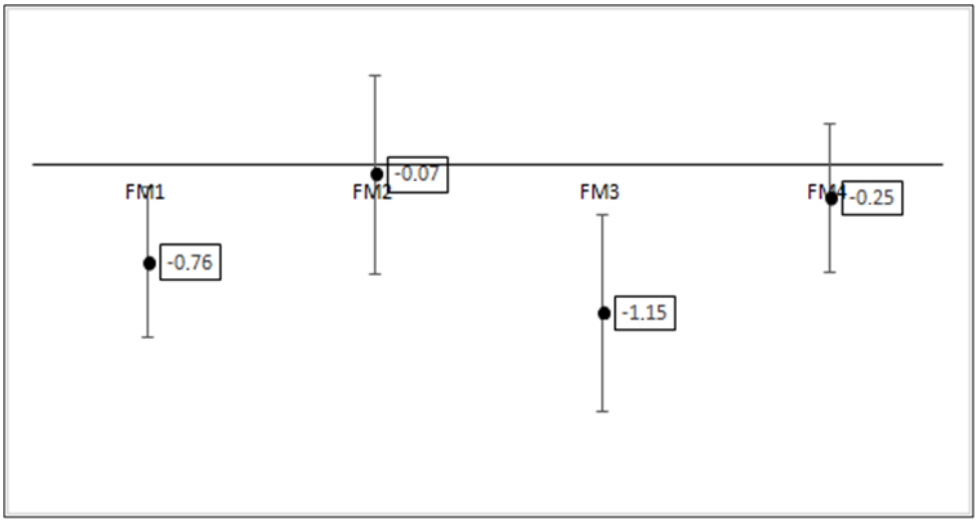


Figure 5.6. Variation of % Body fat according to groups before (V1) and after (V4) treatment.

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion; $P < 0.05$.

5.3.4. Ghrelin and Leptin

In spite of satiety hormones variation, although it wasn't observed significant differences in ghrelin levels between the groups it is remarkable that people in all groups tended to present an increase in this hormone level after the treatment period (Table 5.2).

No significant differences were noted between the products at the visits 1 and 4 for leptin variations, correcting the values by data of visit 1 (Table 5.3).

Table 5.2. Comparison of ghrelin (pg/ ml) variation between visits 1 and 4 by product (Covariance analyses model - ANCOVA*).

Ghrelin by group	**FM1**	**FM2**	**FM3**	**FM4**
GHRELIN (pg/ ml) (V4 – V1)				
Means (Standard Deviation)	72.5 (201.8)	141.6 (205.5)	98.6 (224.4)	42.1 (173.3)
Median (Min – Max)	27.9 (-510.3 -674.7)	92.5 (-207.8 - 716.5)	28.1 (-473 - 606.5)	2.1 (-373.8 - 432.9)
Total	47	38	47	40
p-value	0.144			

*For ANCOVA the co variable Ghrelin, at visit 1, presented statistical significance (p-valor < **0.001**).

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

V1: Visit 1; V4: visit 4

Table 5.3. Comparison of leptin (ng/ ml) variation between visits 1 and 4 by group (Covariance analyses model (ANCOVA*).

Leptin by group	**FM1**	**FM2**	**FM3**	**FM4**
LEPTIN (ng/ ml) (V4 - V1)				
Means (Standard Deviation)	0.2 (17.7)	5.1 (23.3)	9.4 (21.5)	2.7 (18.4)
Median (Minimum - Maximum)	2.5 (-41.5 - 62.2)	4.6 (-53.4 - 64.8)	2.8 (-53.9 - 73.7)	1.6 (-40.4 - 64.9)
Total	42	38	44	37
p-value	0.397			

*For ANCOVA the co variable Leptin, at visit 1, presented statistical significance (p-valor < **0.001**).

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

V1: Visit 1; V4: visit 4

5.4. DISCUSSION

Body weight typically fluctuates over time, tending to increase with age (WU et al., 2009) what corroborates to the increasing of the actual obesity epidemic (WHO, 2014).

Emerging data from randomized controlled trials (RCTs) evince modest, however, facilitated weight loss by the consumption of dairy products in energy-restricted trials (DOUGKAS et al., 2011; PFEUFFER; SCHREZENMEIR, 2006; WANG et al., 2014). Besides, accumulating evidence also suggests that dairy products present some specific components, mainly proteins and peptides, which affect satiety, regulating food intake, and consequently, body weight (ANDERSON; AZIZ, 2006).

As it was observed in our study, weight loss was confirmed with the increase in fermented milk consumption, unfortunately, this effect was only noted for the regular fermented milk (FM1) (Figure 5.3), produced by the fermentation with *Streptococcus thermophillus*, and not for the other formulations, added by probiotic bacteria (FM2) and also passion fruit peel powder (FM3) or even with the substitution of milk fat by an vegetal oil emulsion (FM4) that was expected to enhance the satiety. Still, it is important to note that intervention trials with no restriction in energy intake, as ours, may have more difficulty to prove robust outcomes such as significant differences in body weight or in abdominal obesity, due to possible lacks of statistical power. That's why this kind of study needs to be interpreted carefully (DOUGKAS et al., 2011).

As it was expected, the weight reduction of FM1 participants was accompanied by energy and carbohydrate intake decrease (Figure 5.1 and 5.2) and also by a diminution in BMI (Figure 5.5) and % body fat (Figure 5.6). Unfortunately, we cannot affirm if these positive results obtained through the FM1 consumption were due to a

change in the gut microbiota or by the components formed during milk fermentation by St040.

Contrary to what was expected, even the significant decrease in fat ingestion, with consequent reduction in total energy intake, by people in group FM2 (Figure 5.1 and 5.2) it was not observed any effect on weight. Actually, 66.7 % of the participants of this group showed an increase in BMI of 1.6 units against 27,8 % that presented a decrease of up to 4.6 kg/m², while 5,6 % of people maintained the initial BMI.

Surprisingly, despite all groups have presented an augment in circulating ghrelin levels (Table 5.3), a hormone with orexigenic effect (KLOK et al., 2007), it was observed a big trend in reducing energy intake by the four groups, with significant reduction for groups FM1 and FM2 (Figure 5.1). The probable causes for that are the well-known augment of plasma ghrelin levels in response to fasting and, least likely, this increase in ghrelin levels should be a compensation mechanism for weight loss, already observed in obese patients after lose weight (KLOK et al., 2007), remembering that, all groups, on average, presented decrease in % body fat (Figure 5.6), with no differences between the groups what could be caused by the probiotic bacteria, BL420, that had already shown a total fat lowering effect in mice, in addition to its effect in reducing insulin resistance and tissue inflammation (LAHTINEN et al., 2010).

On the other hand, circulating leptin levels remained unaltered (Table 5.3). A probable reason for that could be due to its long-term action, related to body adiposity and regulating energy balance (KLOK et al., 2007). In addition, leptin levels have been shown to be disturbed in obese humans, with influences in its diurnal variation (YILDIZ et al., 2004). Thus, we do not believe that the reduction in food intake and % body fat was influenced by any alteration in satiety hormones, as already proposed by Forssten et al. (2013).

There are many mechanisms by which dairy ingestion may influence body weight, mostly related to enhanced calcium consumption (ABETE et al., 2011). Nevertheless, we do not believe that this is the case in this trial, even because it was not observed any differences in calcium intake during the treatment period (Table 5.1). Despite the augment in fermented milk consumption by all the participants (Table 5.1), total calcium intake remained unaltered, maybe due to a substitution of sources of milk and cheese consumed at snack time and also by the lower calcium content of all four fermented milks utilized in this survey, correspondent to the calcium content of about 1/3 cup of milk, fluid, reduced fat (2 % milk fat), and 1/5 of the calcium present in 100 g of mozzarella cheese (USDA Food Search for Windows, version 1.0, database version SR22, Beltsville, USA).

5.5. CONCLUSIONS

There is still no consensus on the effect of fermented milk intake on weight control, although numerous observational studies have found inverse associations between dairy consumption and body weight (PFEUFFER; SCHREZENMEIR, 2006).

Further longitudinal and interventional studies are warranted to confirm the beneficial role of consuming probiotic fermented milk. Nevertheless, we strong recommend fermented milk consumption, added or not by probiotic cultures and other functional ingredients, combined with diet and exercise (ORZANO; SCOTT, 2004) for the long-term prevention of weight gain.

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5.7. APPENDIX

5.7.1. - "CLINICAL TRIALS" REGISTRATION

ISRCTN22923997 - The effect of a probiotic compound in dyspeptic patients [Eficáci... Page 1 of 3

Welcome

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14 Fevereiro 2012

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The effect of a probiotic compound in dyspeptic patients [Eficácia terapêutica de composto probiótico em pacientes dispépticos]

ISRCTN	ISRCTN22923997
ClinicalTrials.gov identifier	
Public title	The effect of a probiotic compound in dyspeptic patients [Eficácia terapêutica de composto probiótico em pacientes dispépticos]
Scientific title	Therapeutic efficacy of a probiotic compound in dyspeptic patients: a randomised controlled trial
Acronym	N/A
Serial number at source	N/A
Study hypothesis	Functional dyspepsia is the most common functional disease of the upper gastrointestinal (GI) tract., its prevalence is around 20-40% in the eastern population of Brazil. Functional dyspepsia is a disease whose physiopathology is dependent of gastric motility as well as gut microbiota. Probiotics can interfere with both. The chronic use of such supplements can improve dyspepsia.
Lay summary	Lay summary under review 3
Ethics approval	University of São Paulo Ethics Committee, 31 August 2011
Study design	Prospective randomized double-blind placebo controlled study
Countries of recruitment	Brazil
Disease/condition/study domain	Functional dyespesia
Participants - inclusion criteria	1. Must have diagnosis of functional dyspepsia (Rome III criteria) 2. Signed informed consent 3. Aged between 18 and 80
Participants - exclusion criteria	1. Abdominal surgery 2. Major commorbidities that lead to use of drugs which can interfere with symptoms or modify gastric or bowel motility 3. Gastroesophageal reflux disease (GERD) 4. Active peptic ulcer disease (PUD)

	5. Use of non steroidal anti inflammatory drugs (NSAIDs) or antibiotics 6. Gastrointestinal (GI) tract neoplasia 7. Pregnant women 8. History of yogurt intolerance or allergy
Anticipated start date	15/01/2012
Anticipated el1d date	31/12/2012
Status of tri<li	Ongoing
Patiel1t il1formaticm material	Not available in web format, please use the contact details below to request a patient information sheet
Target number of participants	150
Interventions	150 patients with functional dyspepsia will be divided in three groups: 1. Probiotic 2. Probiotic + lipid 3. Placebo They will receive the products for 3 months, symptoms and biochemistry will be achieved before the study, in the end of the products supplementation and 3 months after stopping the products.
Primary outcome measure(s)	1. The Short-Form Leeds Dyspepsia Questionnaire 2. Biochemestry of ghrelin and leptin levels
Secondary outcome measure(s)	1. Adverse events 2. Compliance 3. Bowel habit 4. Body Mass Index (BMI)
Sources of funding	Brazil Foods (Brazil)
Tria! w€!bsite	
Public<ltiofl5	
Contact name	**Dr Ricardo Barbuti**
Address	Eneas Carvalho de Aguiar, 255 Department of Gastroenterology Instituto Central do Hospital das Clínicas (ICHC)
City/town	Sao Paulo
Zip/Postcode	05403-000
Country	Brazil
Email	rbarbuti@terra.com.br
Sponsor	Brazil Foods (Brazil)
Address	Rua Hungria 1.400 - Edifício Quadra Jardim Europa
City/town	São Paulo
Zip/Postcode	01455-000
Country	Brazil

Email	susana.santos@brasilfoods.com
Sponsor website:	http://www.brasilfoods.com/
Date applied	**28/12/2011**
Last edited	**31/01/2012**
Date ISRCTN ass,ign1ed	**31/01/2012**

5.7.2 – 24 h Food Questionnaire

Visit: _____

Name: _____

Date of birth: _____ Gender: M ☐ F ☐

24 HOURS FOOD QUESTIONNAIRE

Wake up at: _____ **Go sleep at: _____**

Breakfast Time: Hunger:	
Snack Time: Hunger:	
Lunch Time: Hunger:	

Afternoon snack 1 Time: Hunger:	
Afternoon snack 2 Time: Hunger:	
Dinner Time: Hunger:	
Supper Time: Hunger:	

Observations: _____

_____

_____

5.7.3 - HC/FMUSP Ethics Committee Approval

Hospital das Clínicas da FMUSP
Comissão de Ética para Análise de Projetos de Pesquisa
CAPPesq

Protocol No.: 0602/11

TITLE: "PROBIOTIC THERAPEUTIC EFFICACY IN DYSPEPTIC PATIENTS"

Investigator in Charge: Ricardo C. Barbuti

Co-authors: Dr. Fernando Marcuz SilvaJaime Natan Eisig, Tomás Navarro-Rodriguez, Dr. Decio Chinzon, Dr. Joaquim Prado Pinto de Moraes Filho, Prof. Dra. Maricê Nogueira de Oliveira (Pharmacist), Dr. Cristina S Bogsan (Pharmacist), Dr. Natalia Pratis Perina (Nutritionist)

Department: GASTROENTEROLOGY

The Ethics Committee for the Review of Research Projects - CAPPesq of the Clinic Directory of Hospital das Clínicas of Faculdade de Medicina da Universidade de São Paulo, has **APPROVED** the above protocol in the session dated 08/31/2011.

In compliance with Resolution CNS 196/96, CAPPesq has requested the Investigator to prepare an interim and final reports.

In the case of the Interim Report, please inform the time expected until conclusion of the protocol and provide a short summary of the results obtained so far.

CAPPesq, September 1st, 2011
PROF. DR. EUCLIDES AYRES DE CASTILHO
Coordinator
Ethics Committee for the Review of Research Projects - CAPPesq

Rua Dr. Ovídio Pires de Campos, 225 - Prédio da Administração - 5° andar - CEP 05403-010 - São Paulo - SP.
Fone: 55 11 3069-6442 ramais 16, 17, 18 e 20 - e-mail: cappesq@hcnet.usp.br

5.7.4 - HC/FMUSP Ethics Committee Aproval (Addendum)

Hospital das Clínicas da FMUSP
Comissão de Ética para Análise de Projetos de Pesquisa
CAPPesq

N° Protocolo: 0602/11
Título: EFICÁCIA TERAPÊUTICA DE PROBIÓTICO EM PACIENTES DISPÉPTICOS
Pesquisador Responsável: Ricardo C. Barbuti
Pesquisador Executante: o mesmo
Departamento: GASTROENTEROLOGIA

A Comissão de Ética para Análise de Projetos de Pesquisa – CAPPesq da Diretoria Clínica do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, **APROVOU / TOMOU CIÊNCIA** na sessão datada de 17/04/2013, do(s) documento(s) abaixo mencionado(s):

•Carta datada de 27/02/2012 – 1° Adendo ao protocolo especificamente no tópico 3.6.2 – 2° Adendo ao protocolo especificamente ao tópico 3.6
•Carta datada de 23/03/2012 – 1° Adendo ao TCLE – 2° Adendo ao protocolo solicitando a formação de um 4° grupo de sujeitos de pesquisa
•Carta datada de 12/03/2013 – Protocolo de pesquisa versão 2.0 de 2013 em reposta ao parecer pendente de 11/04/2012

A CAPPesq em obediência à Resolução CNS 196/96, solicita ao pesquisador (a) s elaboração de relatório parcial e final.

No caso de relatório parcial é necessário informar o tempo previsto para a conclusão do protocolo e breve resumo dos resultados obtidos.

CAPPesq, 18 de Abril de 2013

PROF. DR. LUIZ EUGÊNIO GARCEZ LEME
Coordenador
Comissão de Ética para Análise de Projetos de Pesquisa - CAPPesq

Rua Dr. Ovídio Pires de Campos, 225 - Prédio da Administração - 5º andar - CEP 05403-010 - São Paulo - SP.
55 11 2661-7585 - 55 11 2661-6442 ramais: 16, 17, 18 | marcia.carvalho@hc.fm.usp.br

5.7.5 - FCF/USP Ethics Committee Aproval

Plataforma Brasil - Ministério da Saúde

Faculdade de Ciências Farmacêuticas da Universidade de São Paulo - FCF/ USP

PROJETO DE PESQUISA

Título: EFICÁCIA TERAPÊUTICA DE COMPOSTO PROBIÓTICO EM PACIENTES DISPÉPTICOS.

Área Temática: Área 4. Equipamentos, insumos e dispositivos para saúde novos, ou não registrados no país.

Pesquisador: Maricê Nogueira de Oliveira **Versão:** 2

Instituição: Faculdade de Ciências Farmacêuticas da Universidade de São Paulo **CAAE:** 01749712.1.0000.0067

PARECER DO COLEGIADO

Número do Parecer: 68537

Data da Relatoria: 25/06/2012

Apresentação do Projeto:

Conforme apresentado pela pesquisadora, o presente projeto é parte do projeto "Eficácia terapêutica de composto probiótico em pacientes dispépticos" sob a responsabilidade do Prof. Dr. Ricardo Barbuti do Hospital das Clínicas da Faculdade de Medicina da USP aprovado pelos Comitês de Ética do HC/FM/USP e da DuPont conforme documentos anexados. Este projeto refere-se à Tese de Doutorado de Natália Perina, é de caráter confidencial e é financiado através do Convênio 24679 entre a USP e a BRF.

Introdução: O trato gastrointestinal de humanos é rico em microrganismos, que podem tanto ser benéficos para a saúde do hospedeiro, prevenindo e/ ou tratando a intolerância à lactose, constipação intestinal, síndrome do intestino irritável, entre outras, quanto podem prejudicá-lo, afetando a aquisição de nutrientes e produção de mediadores inflamatórios. Estes distintos papéis da microbiota intestinal são tão marcantes, que podem inclusive, influenciar no desenvolvimento da obesidade em algumas pessoas, podendo levar até mesmo à Síndrome Metabólica.

O presente projeto apresenta os seguintes objetivos:
Objetivo Primário: Avaliar o efeito metabólico do uso de leite fermentado simbiótico, produzido com gordura láctea ou emulsão vegetal, em pacientes obesos e /ou com
sinais indicadores de síndrome metabólica. Mais especificamente, avaliar a capacidade do produto em reduzir o tecido adiposo visceral e sua contribuição para o emagrecimento e redução de fatores de risco cardiovascular, associados à SM (circunferência abdominal, porcentagem de gordura corporal, HDL colesterol, triglicérides, glicemia e pressão arterial).
Objetivo Secundário:
Caracterizar sensorialmente os produtos desenvolvidos pelos métodos: (i) teste de aceitação e (ii) mapa projetivo.

Material e Métodos: Para esta pesquisa serão utilizados três produtos, dois deles inoculados com uma cultura comercial de probiótico, adicionado de casca de maracujá em pó (prebiotico), preparados por indústria de renome, sendo o primeiro feito com gordura láctea, o segundo com emulsão de óleo vegetal; e o terceiro, o produto placebo. Os leites fermentados teste e placebo serão formulados de acordo com os seguintes ingredientes: leite em pó, gordura láctea ou emulsão de óleo vegetal, polpa de fruta de maracujá, casca de maracujá em pó, edulcorantes e aromatizante, cujas quantidades foram definidas em testes preliminares. A fabricação dos produtos será realizada nas instalações da BRF em Carambeí, Paraná e armazenados em câmara fria em São Paulo. Os produtos serão analisados, segundo métodos convencionais, quanto à sua composição química, propriedades viscoelásticas, microestrutura, contagem de células viáveis,teste de resistência às
condições gastrintestinais in vitro e controle microbiológico.

Análise Sensorial:serão realizados dois testes para avaliação sensorial do produto: (i)
teste de aceitação e (ii) mapa projetivo. Teste de aceitação sensorial dos leites fermentados teste e placebo, armazenados à 4°C, será realizado por 150 voluntários - 50 por produto, adultos de ambos os sexos, com idade entre 20 e 60 anos, habituados ao consumo freqüente de produtos lácteos como leite fermentado, iogurtes e queijos. Leite fermentado(50mL) serão servidos em copos de plástico, codificados com números aleatórios de três dígitos de acordo com um delineamento experimental. Será servida uma amostra em cada sessão de análise, exceto para o mapa projetivo, em que todas as amostras serão
entregues simultaneamente (3 produtos).

Ensaio Clínico:
A intervenção será de 12 semanas, período no qual os voluntários deverão consumir 100 mL do produto, duas vezes ao dia. A avaliação dos voluntários será feita antes do início do ensaio e também aos 30, 60 e 90 dias. O ensaio clínico será monocêntrico, randomizado, duplo-cego, em três grupos de adultos com dispepsia funcional consumindo leite fermentado probiótico. Os voluntários serão avaliados antes e aos 30, 60 e 90 dias nos quais será realizada: consulta clínica, medidas de peso, circunferência da cintura e pressão arterial, porcentagem de gordura corporal por bioimpedância e exames bioquímicos ¿ dosagem de triglicérides, colesterol total e frações, glicemia de jejum, leptina e grelina. As avaliações serão repetidas 90 dias após o término do período de consumo do produto, para avaliar se as alterações provocadas pelo consumo do produto foram
mantidas, considerando que haverá alguma alteração benéfica ao consumidor.

Objetivo da Pesquisa:

Objetivo Primário:
Avaliar o efeito metabólico do uso de leite fermentado simbiótico, produzido com gordura láctea ou emulsão vegetal, em pacientes obesos e /ou com sinais indicadores de síndrome metabólica. Mais especificamente, avaliar a capacidade do produto em reduzir o tecido adiposo visceral e sua contribuição para o emagrecimento e redução de fatores de risco cardiovascular, associados à SM (circunferência abdominal, porcentagem de gordura corporal, HDL colesterol, triglicérides, glicemia e pressão arterial).

Objetivo Secundário:
Caracterizar sensorialmente os produtos desenvolvidos pelos métodos: (i) teste de aceitação e (ii) mapa projetivo.

Avaliação dos Riscos e Benefícios:

Segundo a pesquisadora os riscos da pesquisa são mínimos. Quanto aos benefícios, relata-se que tais estudos poderão auxiliar na avaliação da caracterização funcional de um novo produto probiótico com possível efeito emagrecedor em obesos e portadores de síndrome metabólica. Estes dados contribuirão na compreensão do efeito do consumo do leite fermentado probiótico na saúde humana.

Comentários e Considerações sobre a Pesquisa:

Esta pesquisa envolverá a avaliação de três produtos, leites fermentados, dois deles inoculados com uma cultura comercial de probiótico, adicionado de casca de maracujá em pó (prebiotico), preparados por indústria de renome, sendo o primeiro feito com gordura láctea, o segundo com emulsão de óleo vegetal; e o terceiro, o produto placebo.
Estes produtos serão submetidos à análise sensorial e à avaliação da eficácia terapêutica ou do efeito metabólico dos mesmos em pacientes obesos e /ou com sinais indicadores de síndrome metabólica. Desta forma, o presente projeto envolverá 150 voluntários com diagnóstico de dispepsia funcional, obesidade e/ou síndrome metabólica. Estes pacientes serão selecionados dentro do grupo de pacientes do ambulatório de gastroenterologia do HC/FMUSP e deverão cumprir os critérios diagnósticos de obesidade e síndrome metabólica, além de estarem acostumados ao consumo de produtos lácteos e não apresentar alergia e/ ou intolerância aos componentes do leite. O ensaio clínico será realizado no HC e a análise sensorial / mapa projetivo será realizado no Departamento de Tecnologia Bioquímico-Farmacêutica da FCF/USP. Assim, os sujeitos da pesquisa para ambas etapas serão os mesmos.

Análise Sensorial:serão realizados dois testes para avaliação sensorial do produto: (i)
teste de aceitação e (ii) mapa projetivo. Teste de aceitação sensorial dos leites fermentados teste e placebo, armazenados à 4°C, será realizado por 150 voluntários - 50 por produto, adultos de ambos os sexos, com idade entre 20 e 60 anos, habituados ao consumo freqüente de produtos lácteos como leite fermentado, iogurtes e queijos. Leite fermentado(50mL) serão servidos em copos de plástico, codificados com números aleatórios de três dígitos de acordo com um delineamento experimental. Será servida uma amostra em cada sessão de análise, exceto para o mapa projetivo, em que todas as amostras serão
entregues simultaneamente (3 produtos).
Ensaio Clínico:
A intervenção será de 12 semanas, período no qual os voluntários deverão consumir 100 mL do produto, duas vezes ao dia. A avaliação dos voluntários será feita antes do início do ensaio e também aos 30, 60 e 90 dias. O ensaio clínico será monocêntrico, randomizado, duplo-cego, em três grupos

de adultos com dispepsia funcional consumindo leite fermentado probiótico. Os voluntários serão avaliados antes e aos 30, 60 e 90 dias nos
quais será realizada: consulta clínica, medidas de peso, circunferência da cintura e pressão arterial, porcentagem de gordura corporal por bioimpedância e exames bioquímicos ¿ dosagem de triglicérides, colesterol total e frações, glicemia de jejum, leptina e grelina. As avaliações serão repetidas 90 dias após o término do período de consumo do produto, para avaliar se as alterações provocadas pelo consumo do produto foram
mantidas, considerando que haverá alguma alteração benéfica ao consumidor.

Considerações sobre os Termos de apresentação obrigatória:

Os termos de apresentação obrigatória estão adequados.

Recomendações:

A pesquisadora apresentou todos os esclarecimentos e justificativas solicitadas no parecer anterior na forma de carta resposta. Recomendo que tais informações sejam descritas ou incluídas no protocolo.

Conclusões ou Pendências e Lista de Inadequações:

Diante do exposto, sou favorável a aprovação do referido projeto mediante as alterações sugeridas.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Sim

Considerações Finais a critério do CEP:

O CEP/FCF, em reunião de 31 de julho de 2012, aprovou o presente projeto.

SAO PAULO, 07 de Agosto de 2012

Assinado por:
Mariza Landgraf

5.7.6 - FCF/USP Ethics Committee Aproval (Addendum)

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Comitê de Ética em Pesquisa – CEP

Ofício CEP/FCF 12.2015

São Paulo, 26 de março de 2015.

Senhor Coordenador,

Nosso CEP analisou, em 23/03/2015, a documentação apresentada pela **Profa. Dra. Maricê Nogueira de Oliveira**, pesquisadora responsável pelo projeto de pesquisa **Eficácia terapêutica de composto probiótico em pacientes dispépticos** (CAAE 01749712.1.0000.0067), sob análise da CONEP desde agosto de 2012.

No entendimento dos membros do CEP, procede o argumento da pesquisadora, relativamente a erro próprio no momento de cadastramento do projeto na Plataforma Brasil, que repercutiu no envio automático e não necessário para análise da CONEP.

Este CEP ressalta, ainda, que o referido projeto de pesquisa foi aprovado pelos comitês de ética das instituições envolvidas, conforme documentação disponibilizada pela pesquisadora responsável. Assim, a exemplo da pesquisadora, este CEP solicita que a análise do projeto em tela pela CONEP seja dispensada.

Cordialmente,

Prof. Dr. Mauricio Yonamine
Coordenador do CEP/FCF/USP

Ilmo. Sr.
Dr. Jorge Alves de Almeida Venancio
Coordenador

CONEP/CNS/Ministério da Saúde
SEPN 510 Norte, Bloco A, 3º. andar, Unidade II
Brasília – DF
70750-521

Av. Prof. Lineu Prestes, 580 Bloco 13 A Cidade Universitária, CEP 05508-900, São Paulo, SP
Telefone (11) 3091 3622 - e-mail: cepfcf@usp.br

CHAPTER 6

6. FERMENTED MILK AND METABOLIC SYNDROME – A PROSPECTIVE, RANDOMIZED, DOUBLE-BLIND TRIAL IN OVERWEIGHT AND OBESE ADULTS

This work, as the previous in chapter 5, was done in association with the the team of the Gastroenterology Department of the Clinical Hospital of University of São Paulo School of Medicine, with the collaboration of the team of the processing plant of BRF S.A. with the manufacturing of the products utilized in the study.

In this chapter we discussed the metabolic effects of the increasing in fermented milk consumption, in special in the metabolic syndrome parameters such as waist circumference, blood pressure, levels of triglycerides, HDL-c and plasma glucose and we discuss also the production of bioactive peptides during fermentation and its possible hypotensive effect.

6.1. INTRODUCTION

In recent years, more attention has been given to the relation between intestinal microbiota and metabolic functions related do energy storage in the host (CANI; DELZENNE, 2009). Overweight and obesity had already become a global epidemic (WHO, 2014), and is associated with a group of metabolic disorders such as insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, hypertension and stroke (HOTANISLIGIL, 2006).

The human gastrointestinal tract is an exuberant microbe ecosystem holding about 100 trillion microorganisms (TSAI; COYLE, 2009). This microbiota is considered critical factor, together with lifestyle, in the energetic metabolism and obesity (SANZ et al., 2008, 2010; TILG, 2010; DELZENNE et al., 2011). Besides that, it has been proposed that an alteration in the development or composition of the microbiota - known as dysbiosis, has great participation in promoting obesity (CANI e DELZENNE, 2009; ANGELAKIS et al., 2012).

Among numerous studies regarding the changes in intestinal microbiota during obesity, several of them in humans, pointed for the reduction in the quantity of bifidobacteria as a probable cause (CANI; DELZENNE, 2009; PARNELL; REIMER, 2012). A recent study showed for the first time in humans that changes in gut microbiota may precede weight gain (KALLIOMÄKI et al., 2008). Probiotics also confer alterations in the microbiota properties, affecting the bacteria growth and their metabolism and use of nutrients, what may influence glucose and fat metabolism in the host (ARORA et al., 2013).

Other studies go beyond showing the role of dietary fibers, which are not digested in the gastrointestinal tract, in modulating gut microbiome in a short period of time (WU et al., 2011). As shown, probiotics and dietary fibers present an important role in modulating intestinal microbiota, with possible positive effects in obesity.

Apart from the benefits of probiotics in gut, they can also be very usefull producing benefitial coumpounds in foods, especially dairy food. The fermentation of milk, for example, can improve the bioavailability of nutrients (KORHONEN; PIHLANTO, 2006) and also produce bioactive peptides by the hydrolise of milk protein, mostly casein, that can promote a reduction on blood pressure (MURRAY; FITZGERALD, 2007). Nevertheless, few studies in humans were made in order to comprove this effect (PIHLANTO et al., 2010).

However, as data is still heterogeneous, more research is needed to amplify the knowledge about the function of pre and probiotics in preventing and treating chronic metabolic diseases, in special, metabolic syndrome. For this purpose, the use of dietary strategies aiming changes in gut microbiota as tool to control metabolic functions seems to be a more natural alternative.

The aim of the present study was to examine the association between the consumption of four different fermented milks, fermented by St – TA040 and *Bifidobacterium lactis* – B420 (except for the fermented milk used as a control product), enriched or not with fiber from passion fruit peel and / or a commercial vegetal oil emulsion (Fabuless) and the bioactive coumpounds resulted from milk fermentation with changes in body weight and the metabolic syndrome parameters - waist circumference (WC), glycaemia (GL), triglycerides (TGL), HDL-cholesterol (HDL-c), and blood pressure (BP) in overweight and obese adults with very low fermented milk consumption.

6.2. EXPERIMENTAL SECTION

Members of the randomized controlled trial registered as "The effect of a probiotic compound in dyspeptic patients" (ISRCTN22923997; Appendix 5.7.1) were included in these analyzes, in a total of 208 participants with about 2588 observations. At each exam weight, WC and blood pressure were assessed following standardized procedures, and at the first and third months (after 3 months of treatment) blood sampling was collected for biochemical analyzes. All patients should not be in a weight loss diet program, wake up and have breakfast after 9:00 h, frequently skip breakfast, lunch or dinner, simultaneously participate in other clinical studies, be in use of any prescription drug and women should not be pregnant or breastfeeding. Patients should have at least Body Mass Index (BMI) greater than 25 kg/ m² and also present waist circumference ≥ 94.0 and 80.00 cm (men and women respectively). The following indices were also desired, but not necessarily present: triglycerides ≥ 150 mg/ dl, HDL cholesterol < 50 mg/ dl (men) and < 40 mg/ dl (women), high blood pressure (systolic ≥ 130 mmHg; diastolic ≥ 85 mmHg), plasma glucose ≥ 100 mg/ dl. All patients gave their informed written consent prior to participation.

This study had the approval of the Ethic Committees of the School of Medicine Clinical Hospital, protocol number 0602/11 (Appendix 5.7.3; Appendix 5.7.4) and the Pharmaceutical Sciences Faculty of São Paulo's University, protocol number 69572 (Appendix 5.7.5; Appendix 5.7.6).

6.2.1. Ingredients and Fermented Milk production

Four test products i.e. fermented milks were specially designed as follows: (i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

All products were prepared by BRF S.A – Carambeí-PR, Brazil. The total amount of passion fruit peel powder used was the necessary to reach 1 % of fiber in the final product. Fat from milk cream (Carambeí, Paraná, Brazil) and vegetal-oil emulsion - Fabuless™ - which is formulated from palm-oil and oat-oil fractions (DSM Nutritional Products, Basel, Switzerland), were used to adjust the fat content of yogurts at 2 %. To mask any different appearance that the ingredients could provide a yellow coloring and an artificial passion fruit flavoring were utilized. The designed formulas had the objective to guarantee that the four products had the same energetic content and macro-nutrients composition and also similar aspect, consistency, and flavor.

6.2.2. Fermented Milk Characterization

Gross composition: the total solid, fat and protein contents of the milk bases were determined by theoretical calculation.

Bacterial counts: bacteria enumerations were carried out after 7 (d7) and 28 (d28) days of cold storage in two replicates of each batch to guarantee that all products had the minimal bacteria counts to promote the probiotic effect; cell-counts were expressed as log CFU mL^{-1} of yoghurt, and average values were calculated (SACCARO et al. 2011).

6.2.3. Bioactive peptides detection and identification

To prepare the samples, approximately 1 mL of each product were filtered through in 0.22 μm nylon filters (Biofil syringe filter – Microlab Scientific, China) and

freezed at -20°C for future analyses. Samples were prepared in duplicate. MS and MS/MS analyses were performed using ESI-UHR-Q-TOF Bruker Daltonics MaXis 3G spectrometer (Bremen, Germany). The peptides spectra were obtained in the positive mode using electrospray source (ESI). The ESI conditions were capillary 4.5 kV; dry heater 200 ^{0}C; dry gas 8.0 l/min; nebulizer 2.0 Bar; end plate -500V. Nitrogen was used as collision gas and evaluated CID energy was optimized from 5 to 40 eV. The samples were injected through spectrometer using a liquid chromatography HPLC Shimadzu CBM-20AD (Tokio, Japan) with a 150 × 0.3 mm Jupiter 4 u proteo column (Phenomenex, Torrance, CA). A binary gradient acetonitrile (B) and water (A) with 0.1 % of formic acid and flow rate of 0.20 mL/min was used. The gradient was as follows: (B) 4 % for the first 3 min, increased to 45% by 45 min, held for 5 min and increased to 100 % by 5 min. They were subsequently changed from 100 % to 4 % for 5 min and held for 5 min to re-equilibrate the column. The instrument was externally calibrated using ESI low concentration tuning mix over the *m/z* range of 100 to 2000. Bruker DataAnalysis software (version 4.0) was used for data acquisition and processing.

6.2.4. Clinical Trial

Experimental Protocol: Patients were randomly divided into four groups. Each group received one of the four fermented milks. During 90 days from the first clinical evaluation all the patients had to drink 100 g portion size of the respective fermented milk twice a day (one portion in the middle of the morning and the other portion two to three hours after lunch). During this period, they were instructed to write down any undesirable symptoms due to the consumption of the product, feces consistency, etc.

Anthropometric measurements: Height and weight were measured at the first exam with the participant standing, shoes off, and wearing light clothing. Body mass index (BMI) was then calculated in kg/m². Waist circumference (WC) was measured by a trained professional by applying anthropometric tape at the level of the umbilicus underneath the clothing.

Measurements of other variables: Sitting blood pressure was measured after a 5-min rest using a random-zero sphygmomanometer. Fasting (≥ 8 h) blood samples

were drawn for assessing the levels of glucose and lipids. Hexokinase-test method was used to measure serum glucose. Total cholesterol, high-density cholesterol, and triglycerides were measured by enzymatic colorimetric automated methods.

24 h food questionnaire: A 24 h food questionnaire was administered at baseline and at the end of the survey to estimate total calcium intake and the amount of fermented milk consumption as servings per day, whereas a serving corresponding to 100 g of food ready for consumption (Appendix 5.7.2).

6.2.5. Statistical analysis

Statistical analyzes of variance, covariance, and multiple comparison tests were done using IBM SPSS software for Windows version 13.0 (IBM, USA) in order to determine statistical significance of differences among samples.

For descriptive analyses of anthropometric variables by group in each visit (V1 and V4) frequency and percentage of categorical variables, means, standard deviation, minimum, median, maximum and number of valid numeric variables were calculated. To compare anthropometric variation of WC, lipidic blood tests and blood pressure data between visits V1 and V4 by group, considering V1 as the covariate, it was utilized the covariate model (ANCOVA). To compare the variation of plasma glucose data between visits V1 and V4 by group, considering V1 as the covariate, it was utilized the variance model (ANOVA) with repeated measures. Statistical significance was established at $P < 0.05$.

6.3. RESULTS

6.3.1. Fermented Milks Characterization

Total solid, fat and protein contents of the milk bases were determined by theoretical calculation. Products presented no composition (kcal, PTN, CHO, and LIP) differences (data not shown).

Bacterial enumerations were carried out after 7 (d7) and 28 (d28) days of cold storage in two replicates of each batch to guarantee that all products had the minimal bacteria counts to promote the probiotic effect; cell-counts were expressed as log CFU mL^{-1} of yoghurt, and average values were calculated (SACCARO et al. 2011). All fermented milks presented high probiotic (*Bifidobacterium lactis* – BL420) counts during all shelf life, from day 7 after production to day 28, ranging from 8.53 ± 0.19 log CFU mL^{-1} to 8.66 ± 0.27 log CFU mL^{-1} throughout the storage time. *Streptococcus thermophilus* (TA040) was also present in a great number, ranging from 8.93 ± 0.21 log CFU mL^{-1} to 8.85 ± 0.25 log CFU mL^{-1} during the storage time.

6.3.2. Bioactive peptides

Peptides present in the samples FM1, FM2, FM3 e FM4 were identified by ultra-high resolution mass spectrometry (UHR-MS) and MS/MS. The ions monitored are based on the study presented by Kunda et al., 2012 and were isolated and carry out for collision induced dissociation (CID). Peptides were recognized in the Smoothed base peak chromatograms (BPCs) showed in Figure 6.1 and they are summarized in the Table 6.1.

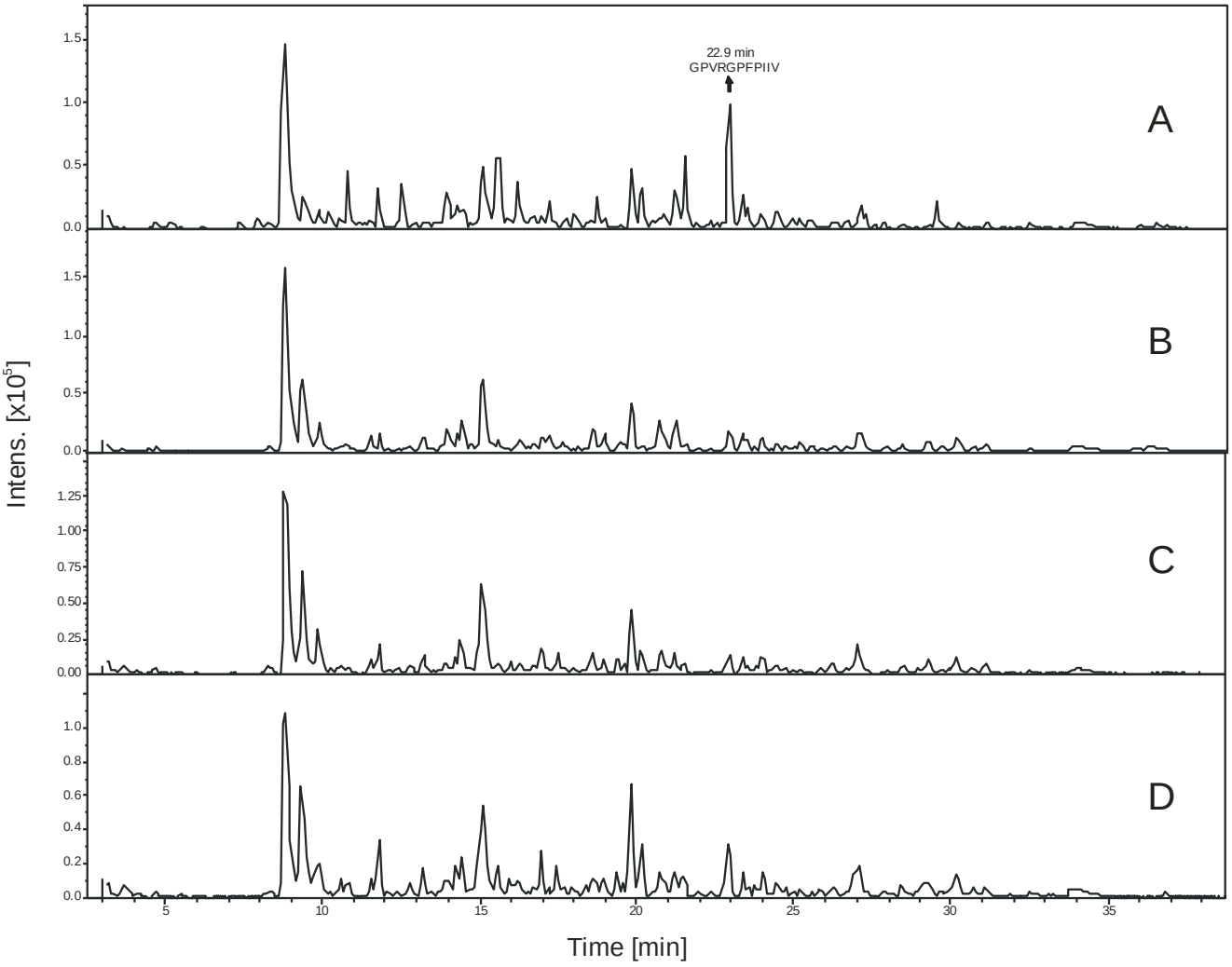


Figure 6.1. Base peak chromatograms (BPCs) of fermented milks. (A) FM1: Streptococcus thermophilus strain TA040; milk fat; (B) FM2: Bifidobacterium lactis - Bifidobacterium species 420 together with Streptococcus thermophilus strain TA040; milk fat; (C) FM3: Bifidobacterium lactis - Bifidobacterium species 420 plus Streptococcus thermophilus strain TA040; fiber from passion fruit peel and milk fat; (D) FM4: Bifidobacterium lactis - Bifidobacterium species 420 plus Streptococcus thermophilus strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

Table 6.1. Bioactive peptides identified in fermented milk using LC-MS and MS/MS spectrum.

Identified ion (m/z)	M_{teo} (Da)	Error (ppm)	Sequence	Protein Precursor*
263.1406 (+1)	262.1317	6.0	FP	BCN A1-A2
279.1351 (+1)	278.1267	4.3	YP	BCN A1-A2
312.1928 (+1)	311.1845	3.5	VPP	BCN A1-A2
313.1557 (+1)	312.1474	3.5	FF	AS1CN
326.2087 (+1)	325.2001	4.0	IPP	BCN A1-A2
392.2559 (+1)	391.2471	4.0	LLF	BLG
415.2347 (+1)	414.2267	2.0	PLW	AS1CN
433.2458 (+1)	432.2372	3.0	FVAP	AS1CN
513.2829 (+1)	512.2747	2.0	IHPF	BCN A1-A2
557.3671 (+1)	556.3584	2.5	TKVIP	AS2CN
568.2924 (+1)	567.2839	2.3	ALPMH	BLG
577.3199 (+1)	576.3119	1.4	VTSTAV	KCN
651.4036 (+1)	651.3955	1.3	VLPVPQ	BCN A2
748.3713 (+1)	747.3625	2.0	TTMPLW	AS1CN
748.3891 (+1)	747.3803	2.2	YQEPVL	BCN A1-A2
756.4056 (+1)	755.3966	2.3	DKIHPF	BCN A1-A2
780.4994 (+1)	779.4905	2.0	KVLPVPQ	BCN A2
428.7258 (+2)	855.4338	4.0	NVPGEIVE	BCN A1-A2
438.2842 (+2)	874.5501	4.3	RPKHPIK	AS1CN
438.7377 (+2)	875.4575	3.8	KTTMPLW	AS1CN
449.7803 (+2)	897.5436	3.0	RGPFPIIV	BCN A1-A2
453.2522 (+2)	968.5179	3.8	TVQVTSTAV	KCN
489.2486 (+2)	976.4800	2.8	RDMPIQAF	BCN A1-A2
499.3150 (+2)	996.6120	3.6	VRGPFPIIV	BCN A1-A2
540.3098 (+2)	1078.6022	2.6	NIPPLTQTPV	BCN A1-A2
550.7944 (+2)	1099.5702	3.8	VYPFPGPIPN	BCN A2
556.2761 (+2)	1110.5346	3.0	ALNEINQFY	AS2CN
576.3522(+2)	1150.6862	3.0	GPVRGPFPII V	BCN A1-A2
597.3518 (+2)	1192.6856	3.0	TPVVVPPFL QP	BCN A1-A2
599.3489 (+2)	1196.6805	2.3	LTQTPVVVP PF	BCN A1-A2

* lactoglobulin (BLG); αs1-casein (AS1CN); αs2-casein (AS2CN); β-casein (BCN) and κ-casein (KCN).

The peptide sequence was performed using MS/MS spectra using fragments –y e –b according to the nomenclature proposed by Roepstorff-Fohlmamn Biemann (ROEPSTORFF; FOHLMAN, 1984). These fragments are generated from the breakdown of the peptide bond, resulting from an energy transfer to the peptides. Ion –y refers to *C*-terminal whereas ion –b to *N*-terminal. As can be observed in Table 6.1,

trirty bioactive peptides were identified. There are ACE-inhibitors, enzyme involved in regulation of blood pressure. Some peptides are described as antihypertensive. Interestingly, was identified in the BPC a peptide with high intensity to sample FM1 (retention time 22.9 min – Figure 1A), fermented only by *Streptococcus thermophilus*. The same peptide was identified in other samples, fermented by both bacteria cultures *St* and *Bifidobacterium lactis*, however at lower intensities. This compound is the peptide GPVRGPFPIIV with m/z 576.7522 ($[M+2H]^{2+}$) described as an antihypertensive. Elucidation of sequence was performed from MS/MS spectrum shown in Figure 6.2. Fragmentation has led to total identification of the series b- and y-.

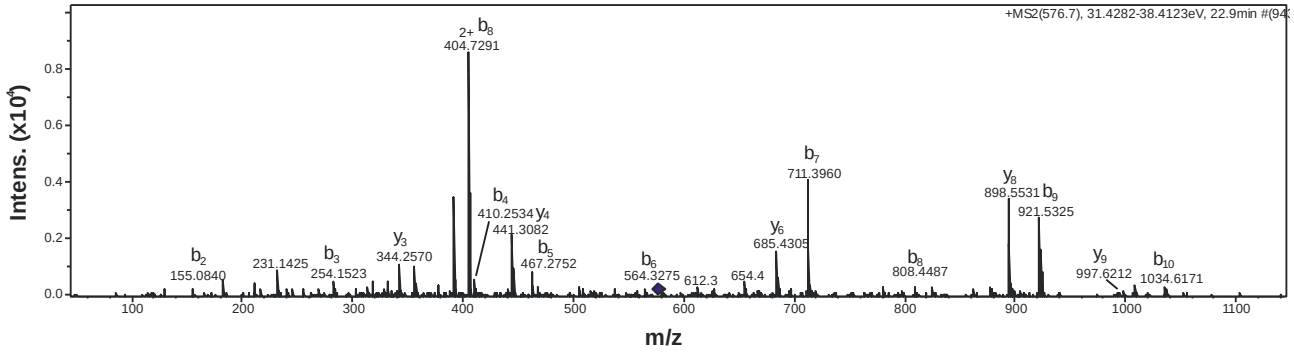


Figure 6.2. MS/MS spectrum from a parent ion of m/z 576.7522, which correspond to peptide GPVRGPFPIIV

Other peptides that were identified in the fermented milks utilized in this study have already been described as having hypotensive effects in spontaneously hypertensive rats (FITZGERALD et al., 2004). These peptides correspond to casein-derive peptides YP and TTMPLW, derived from α_{S1}-casein; TKVIP, derived from α_{S2}-casein; and IPP, TPVVVPPFLQP, VPP and KVLPVPQ, derived from β-casein.

6.3.3. Clinical Trial

From the initial participants at baseline (208), the present study excluded individuals who did not attend to the last blood sampling collection, and the women that got pregnant. Thus, a total of 143 participants finished the survey. The age of the participants at baseline ranged from 23 to 66 yr, initial BMI was 31.8 ± 6.06 kg/m²,

characterizing an overweight and obese population, and WC was in average 104.6 ± 12.42 cm for men and 94.94 ± 13.74 cm for women.

Fermented Milk Consumption

There were no reports referring to undesirable symptoms or side effects with the consumption of any of the products administered in this study. Average fermented milk consumption before the clinical trial beginning was of 0.15 per person. At the last day of the treatment period, this consumption had increased 8.6 times and was about 1.3 per person.

Although there was observed an increase in fermented milk consumption, there were no significant difference in total calcium intake, being about 578.8 mg at baseline and 566.0 mg at the end of the survey.

Waist Circumference

Accordingly to results showed in Figure 6.3, at 5 % of significance, there is a significant difference regarding de WC between the exams made at the end of the treatment (visit 4 – V4) and the first exam, before the beginning of the treatment (visit 1 – V1), and this difference is dependent of the product consumed (*P*-value of interaction between product and visit < 0.05).

Patients in the group FM1 conveyed a decrease in WC between visits 1 and 4 as well as those patients in group FM2 which presented a reduction of WC after the treatment. However, no statistical differences in WC of patients from groups FM3 and FM4 were observed. While there seemed to be a slight increase of WC measures for the group that consumed the FM3 product, there was a slight decrease in WC for the FM4 group.

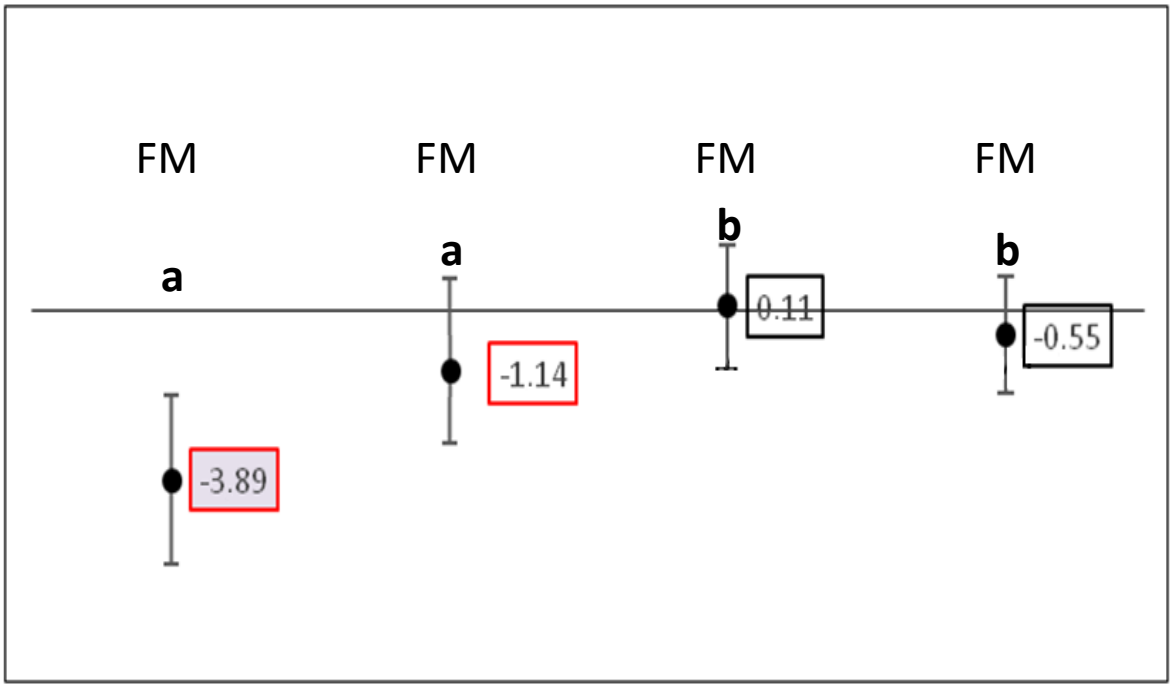


Figure 6.3. Comparison of waist circumference (WC) by product between visits 1 and 4*.

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

*Data correspond to average of difference between V4 and V1 in cm.

Metabolic syndrome marks

The incidence of metabolic syndrome at baseline was of 25.5% of the studied population but decreased to just about 9.1% of the participants who ended up the study. High BP (64.2%) was the most common MS component at baseline, followed by hypertriglyceridemia (58.5%), low HDL-c (52.8%) and high fast blood glucose levels (26.4%). However, at the end of the treatment period, this indices were a little different, with hypertriglyceridemia (84.6%) corresponding to me major MS component present, followed by low HDL-c (53.9%), high BP and high fast blood glucose levels affecting 38.5% of participants.

As presented in Table 6.2 there is no significant difference between product and visit and within the interaction product and visit for blood glucose measures. The same results were observed for total cholesterol (data not shown), with a slight reduction for groups FM1, FM2 and FM3, with a more noticeable for FM1 while it was observed a

higher increase for FM4. Likewise, the same lack of results was observed for low-density cholesterol. However, a decreasing trend for the groups that consumed the FM1 and FM3 products (data not shown) were evidenced; even though both tests aren't considered a MS marker they were measured aiming to evaluate cardiovascular disease (CVD) risks and to enrich the analyses.

Table 6.2. Comparison of plasma glucose (mg/ dl) variation between visits 1 and 4 by group (Covariance analyses model - ANOVA*).

Plasma glucose by group	**FM1**	**FM2**	**FM3**	**FM4**
PLASMA GLUCOSE (mg/ dl) (V4 - V1)				
Means (Standard Deviation)	0.13 (15.36)	0.47 (9.98)	4.84 (17.52)	3.39 (13.18)
Median (Min – Max)	3 (-57 - 30)	2 (-27 - 15)	2 (-22 - 85)	2 (-30 - 40)
Total	38	34	38	31
p-value	0.435			

* ANOVA was used as the covariate plasma glucose showed statistical difference in visit 1.

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

V1: Visit 1; V4: Visit 4

Fortuitously, results for HDL-c cholesterol were a little more encouraging (Figure 6.4). Patients at group FM3, the probiotic, milk fat plus fiber fermented milk, presented an increase on plasma HDL-c significantly higher than patients at group FM2, the probiotic product with no fiber and with milk fat, with no significant differences between the products FM1 and FM4 that seemed to be stable.

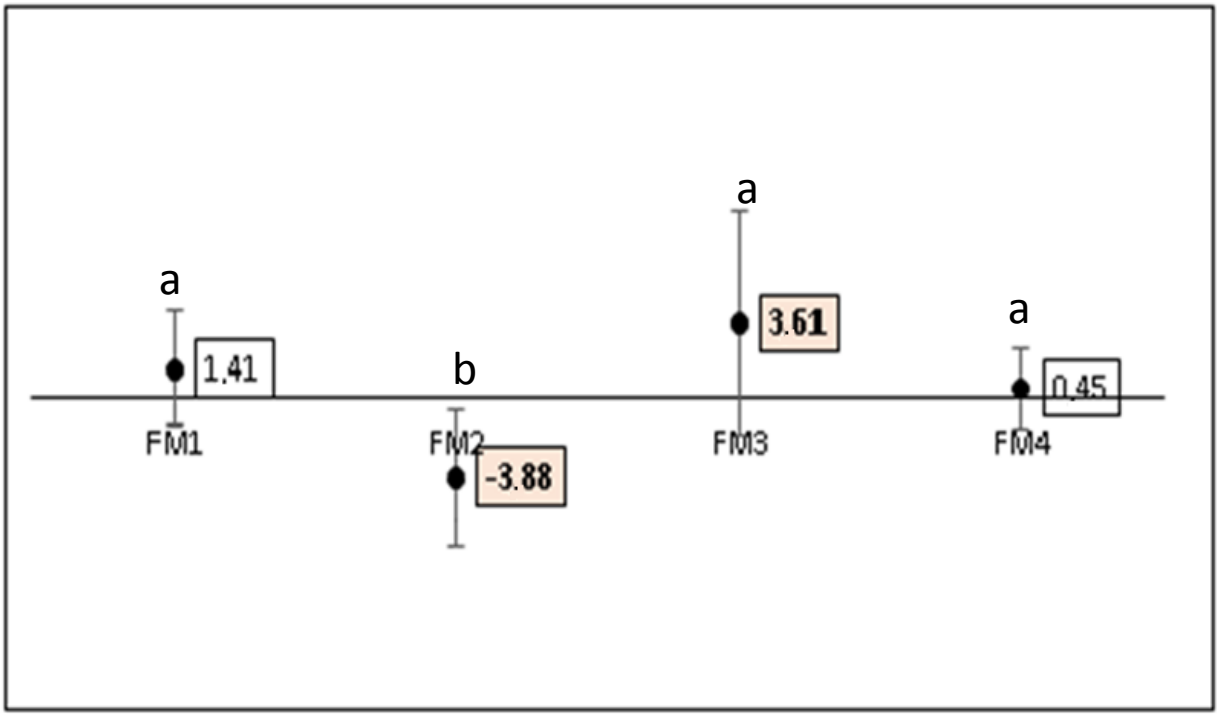


Figure 6.4. Comparison of high-density lipoprotein cholesterol (HDL-c) by product between visits 1 and 4*.

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

*Data correspond to average of difference between V4 and V1 in mg/ dL.

Concerning to TGL dosage (Table 6.3) the results were rather disappointing, although it was observed a reduction trend for the FM1 group, there was no significant difference between this and the other groups, probably due to the large deviation observed.

Figure 5 shows the results for BP. As it can be seen in Figure 6.5a, there is no significant difference between the measures of SBP between the products, neither within the interaction between products and visits (P-value ≥ 0.05 for all analyzes). Only a slight trend of BPS reduction for the groups FM1, FM2, and FM3, with no difference from FM3 that remained stable was observed. However, a significant difference was shown for DBP (Figure 6.5b) between the visits. This difference is not dependent of the product (P-value ≥ 0.05 for the interaction between product and visit). All products showed a decrease of DBP at the end of the treatment period of three months.

Table 6.3. Comparison of plasma triglycerides (mg/ dl) variation between visits 1 and 4 by group (Covariance analyses model (ANCOVA*)).

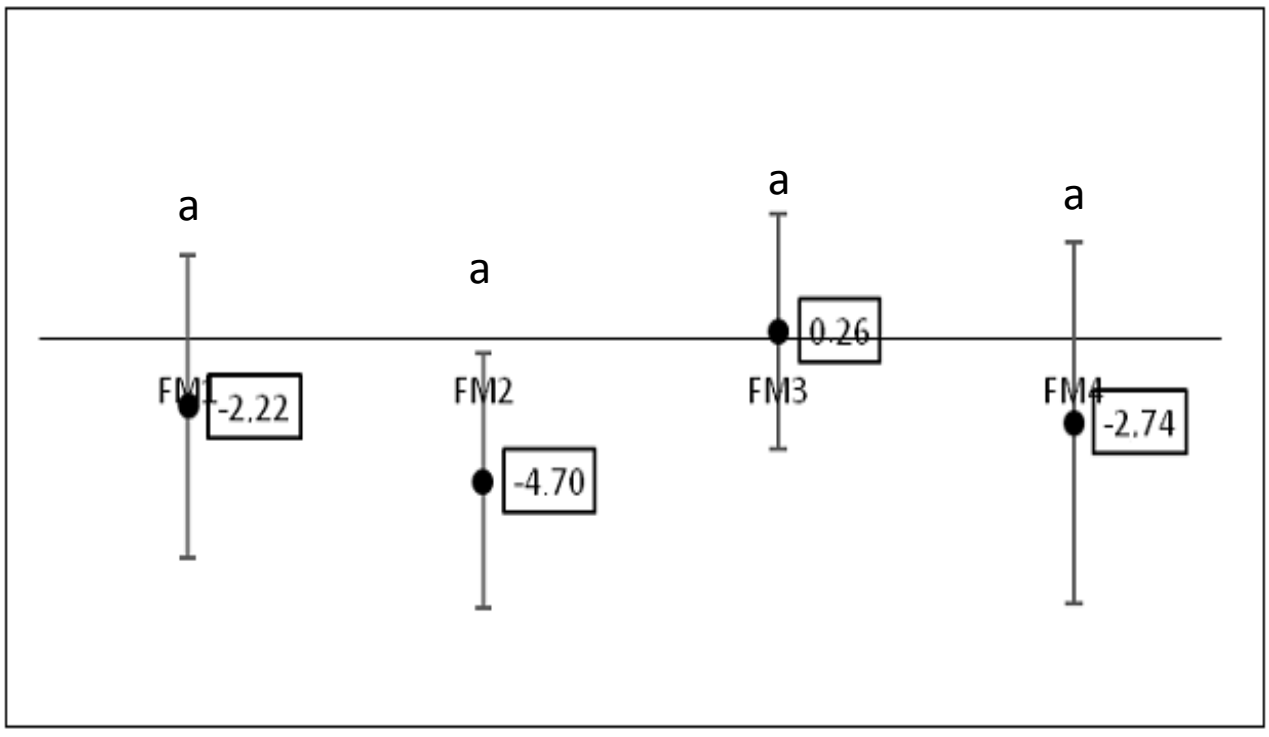
Triglycerides by group	**FM1**	**FM2**	**FM3**	**FM4**
TRIGLYCERIDES (mg/ dl) (V4 - V1)				
Means ± Standard Deviation	-24.9 (88.96)	4.91 (32.52)	4.95 (43.88)	-2.71 (34.65)
Median (Min - Max)	-11 (-495 - 57)	7 (-83 - 54)	8,5 (-171 - 82)	2 (-84 - 62)
Total	39	34	38	31
p-value	0.270			

*For ANCOVA the co variable plasma triglycerides (mg/ dl), at visit 1, presented statistical significance (p-valor < **0.001**).

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

V1: Visit 1; V4: Visit 4

A)



B)

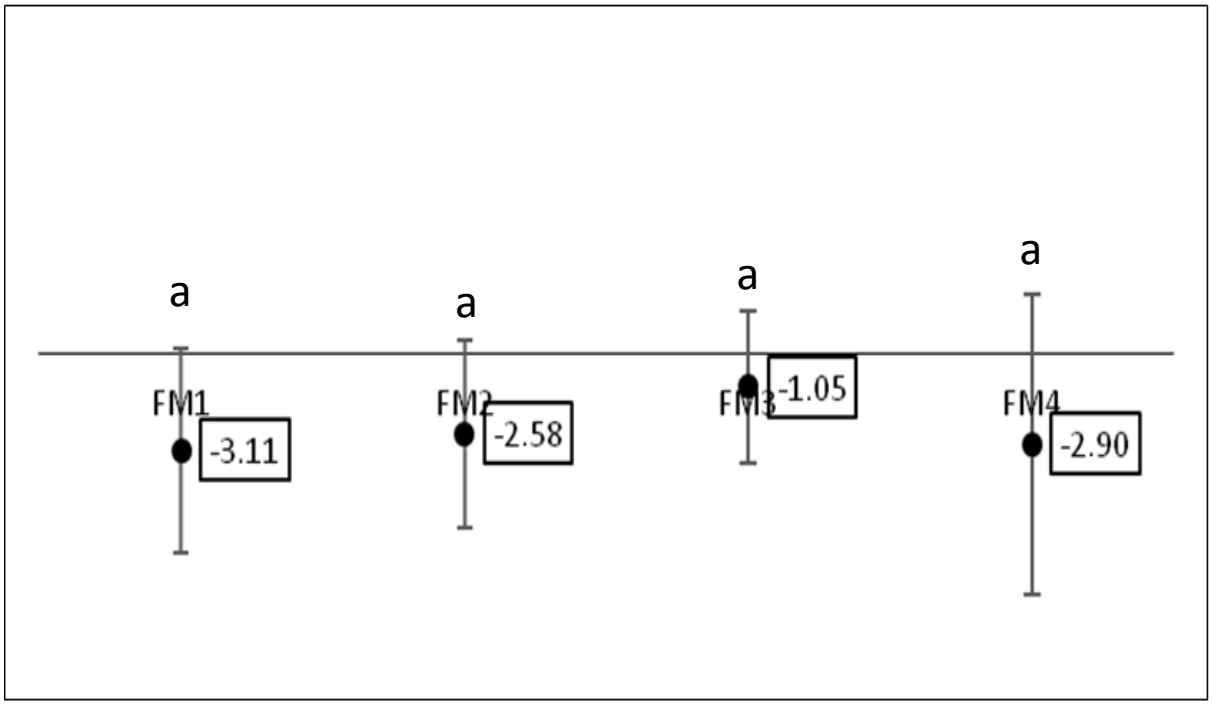


Figure 6.5. Comparison of Systolic (a) and Diastolic (b) Blood Pressure (mmHg) variation between visits 1 and 4 by group.

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

For a better overview of the results of this study a comparative table was made highlighting key results and possible benefits of consuming each product in mitigating the metabolic syndrome markers (Table 6.4).

Table 6.4. Overview of the results after fermented milk ingestion augmentation.

	FM1	**FM2**	**FM3**	**FM4**
Waist Circumference (cm) *	↓	↓↔	↔	↔
Plasma glucose (mg/ dl)	↑↔	↑↔	↑↔	↑↔
HDL-c (mg / dl) *	↔	↓	↑	↔
Triglycerides (mg/ dl)	↓↔	↔	↔	↓↔
Systolic Blood Pressure (mmHg)	↓↔	↓↔	↔	↓↔
Diastolic Blood Pressure (mmHg)	↓	↓	↓	↓
Total Cholesterol (mg/ dl)	↓↔	↓↔	↓↔	↑↔
LDL-c (mg/ dl)	↓↔	↑↔	↑↔	↑↔
Indicative of positive effects	6	3	4	3

* With statistical significance ($p < 0.05$)

(i) FM1: *Streptococcus thermophilus* strain TA040; milk fat; (ii) FM2: *Bifidobacterium lactis* - Bifidobacterium species 420 together with *Streptococcus thermophilus* strain TA040; milk fat; (iii) FM3: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and milk fat; (iv) FM4: *Bifidobacterium lactis* - Bifidobacterium species 420 plus *Streptococcus thermophilus* strain TA040; fiber from passion fruit peel and vegetal oil emulsion.

6.4. DISCUSSION

Several dairy components seems to present potential benefits in metabolic syndrome (ABETE et al., 2011; CRICHTON et al., 2011; PFEUFFER; SCHREZENMEIR, 2006), contributing to insulin sensitivity, blood pressure and lipid levels (PFEUFFER; SCHREZENMEIR, 2006). Besides, recent data suggests that BP can be improved by probiotics and their products of fermentation, with improvement of total and low-density cholesterol levels, reduction of blood glucose level and insulin resistance (KHALESI et al., 2014). Beyond that, the increase in dairy intake seems to decrease the risk of stroke (ELWOOD et al., 2004), and ischemic heart disease (ELWOOD et al., 2005).

As observed in the present study and previous research from other authors (SHIN et al., 2013; ABETE et al., 2011), dairy consumption, and more specifically, in

this survey, fermented milk consumption, may promote a protective effect on metabolic syndrome, principally by the WC reduction observed. Another positive result was the increase of HDL-c. However, the results of lipid profile observed here were a little perplexing, mainly due to the controversy over emerging evidence indicating that high fiber diets shows greater cholesterol-lowering effects than low fiber one's (ABETE et al., 2011). The results presented here showed more pronounced cholesterol reduction (with no statistical significance) for the people that consumed the product with milk fat and no fiber added (FM1) but an significant increase in plasma HDL-c for the product added by fiber, with the probiotic and milk fat (FM3). As this behavior were not observed for the product with just probiotic (FM2) nor for the product with fiber, probiotic and vegetal oil, it seems that this positive effect in increasing HDL-c are not related with the probiotic, but maybe the fiber has some effect, that may have been diminished by the vegetal oil emulsion.

Even not being able to prove an direct effect of fermented milk consumption and BP reduction, our results showed this tendency in both measures, systolic and diastolic blood pressures, which is in conformance with a recent systematic review and meta-analyses of randomized controlled trials (KHALESI et al., 2014) that have shown a positive effect of the consumption of probiotics in lowering BP. As ours, the results presented in this meta-analysis were modest but already important for public health and cardiovascular outcomes.

Contradicting other authors (ZEMEL, 2003; ZEMEL, 2009) we do not believe that the abdominal fat reduction, the change in lipid profile and the possible decrease in BP observed were due to an augmentation of calcium intake because, although there had been and augmentation in fermented milk ingestion, dietary calcium intake remained stable. So, we suppose that the positive effects observed may be milk fermentation resulting compounds and other bioactive peptides or branched-chain amino acids (KHALESI et al., 2014; PFEUFFER; SCHREZENMEIR, 2006).

It is very important to remark that the peptides IPP and VPP (Table 6.1) identified in our products are the best characterized ACE inhibitory peptides found in fermented milks and about twenty studies conducted in humans, despite still not being considered convincing, have already linked the reduction of both, SBP and DBP with the consumptions of products containing these two tripeptides (PIHLANTO et al., 2010).

Unfornutately, as the identified ACE inhibitory peptides were found in all products we cannot affirm if both bacteria utilized in the study are capable of producing them, but surely, Streptococcu thermophilus is, once FM1 was fermented only by this bacteria and presented all the peptides described.

Analyzing all results together, even with a possible benefit in metabolic syndrome markers from an increase in fermented milk consumption we do believe that the consumption of regular fermented milk, independently of their milk base composition, is satisfactory for providing health benefits. Markedly more research should be done to highlight this trend, particularly with regard to the antihypertensive effect that can be provided by the bioactive peptides indentified, confirming other human studies with fermented milk that already showed this important outcome (SEPPO et al., 2003; TUOMILEHTO et al., 2004).

6.5. CONCLUSIONS

Overall, although the results of our study weren't able to exhibit a concrete relation between the increase in fermented milk consumption and a reduction of the metabolic syndrome marks, it was observed a promising way to, at least, control some blood marks, such as HDL-c and also reducing the adipose fat, as observed by the WC measures. According to our knowledge it is the first time that Streptococcus thermophilus is described as an important productor of IPP and VPP ACE inhibitory peptides.

Future studies with greater statistical power are strongly recommended to further examine the possible blood pressure lowering effect of fermented milk consumption. As well as the identification of possible bioactive peptides formed during fermentation.

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CHAPTER 7

7. SUMMARY AND FUTURE WORK

7.1. THESIS SUMMARY

Chapter 2 discussed about designing a innovatively functional probiotic fermented milk with the enrichment of the milk matrix with passion fruit peel-powder and vegetal oil emulsion (Fabuless™) in six different formulations, fermented with the starter cultures *Streptococcus thermophilus* (TA040) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB340) and the probiotic bacteria *Bifidobacterium lactis* (BL420). It was observed that the passion fruit peel-powder's addition had a beneficial effect on the counts of *B. lactis* strains. Besides, an improved fatty acids' profile, due to the substitution of milk cream by the vegetal oil emulsion, with reduced saturated and increased mono-unsaturated and poly-unsaturated fatty acids as an additional beneficial and nutritional factor was noted. The results showed that the microstructure network may indeed affect probiotic viability and its survival in the intestinal tract. Moreover, probiotic yoghurt enriched with passion fruit peel powder and vegetal oil emulsion could, possibly, suppress appetite due to its technological behaviour on fermentation counts of viable bacteria and texture.

In chapter 3 the influence of milk supplementation with passion fruit peel powder and /or vegetal oil emulsion (Fabuless™) was investigated in instrumental firmness, and also about its sensory characteristics, such as flavour, texture (on spoon), creaminess (in the mouth) and overall liking. Milk without supplementation was used as control. The sensory evaluations were conducted by the application of two methods: hedonic scale, that is an affective test, and projective mapping, that is a descriptive test. Despite the observed differences in flavor and global impression there were no

significant changes in consumers' overall liking between supplemented and conventional yoghurts. Overall, the insertion of the vegetal oil and the peel-powder did not provide positive results for the samples mainly for affecting the firmness, which is an attribute of considerable importance for yogurts. In this sense, the supplementation of vegetal oil emulsion and passion fruit peel-powder in yoghurt, although can be an attractive for consumers, with potential benefits to the health, besides the intrinsic role of nutrition, should be carefully evaluated.

In chapter 4 the survival of the classical starter cultre *Streptococcus thermophilus* trought gastrointestinal system by the dynamic simulation of the human digestion was investigated. At the end of simulated dynamic digestion there had been observed a significant reduction of *S. thermophilus* viability mainly due to the low pH and gastric enzyme's action in the stomach. It was also investigated the survival of *L. Bulgaricus* and *Bifidobacterium lactis* strain 420. The first did not survivaled the intestine digestion, but the second reached the end of the GUT in great number, confirming its stronger digestion resistance.

Chapters 5 and 6 described the results of the clinical trial decelloped in collaboration with the team of doctors of the gastroenterology department of the Clinical Hospital of University of São Paulo School of Medicine. In these chapters the effect of the increase in fermented milk consumption was studied and a lot of benefits were observed. In chapter 5 specifically it was observed that, although there is still no consensus on the effect of fermented milk intake on weight control, its consumption, added or not by probiotic cultures and other functional ingredients, combined with diet and exercise may be a good strategy for the long-term prevention of weight gain. In chapter 6, in turn, the increase in fermented milk consumption, especially formulations FM1 and FM3, demonstrated to successfully attenuate metabolic syndrome symptoms

such as waist circumference and blood pressure, with an increase in high-density lipoprotein cholesterol for FM3 group. Some important ACE inhibitors peptides were identified, with possible antihypertensive effect; however, it was not confirmed.

The results indicate a positive effect of B420 on Syndrome Metabolic, when using the fermented milk with B420; the time of consuming the product (3 months) was small to verify a very significant effect. Nevertheless, the results showed a very clear tendency. The product containing only *S. thermophilus* TA040 has shown positive effects. It was observed also, when consuming this product, an effect on blood pressure, and a bioactive peptide production - antihypertensive peptide, in this product was detected. In this product we observed also a great production of exopolysaccharide – EPS that could justify the observed effects, and more investigation is required to attest this hypothesis. Finally, although very promising, more studies using the same methodology must be conducted to better understand the relation between fermented milk consumption and metabolic syndrome parameters.

7.2. FUTURE WORK

As it has been exposed the consumption of dairy food, specially, as showed in this study, the consumption of fermented milks, is a promissor nutritional strategy to prevent and even ease the metabolic syndrome parameters (ABETE et al., 2011; CRICHTON et al., 2011; PFEUFFER; SCHREZENMEIR, 2006), and also contribute to long-term weight control (ORZANO; SCOTT, 2004). One of the possible promoters of these benefits is the milk fermentation resulting compounds and bioactive peptides (KHALESI et al., 2014; PFEUFFER; SCHREZENMEIR, 2006).

Unfortunatelly, it is still not possible to confirm this hypotese and more research regarding the effect os fermented milk bioactive coumpounds is still needed to better understand its effect in humam health. Additionaly, the complete knowledge of the benefits of fermented milk consumption could be an important nutritional strategy for public health.

Intestinal microbiota must also be deeply investigates as it can be profoundly influenced by food intake, specially by probiotics, prebiotics and antibiotics intake, by lifestily, subjects country of origin and age, and the manipulation of it’s composition is intimately linked with obesity and associated deseases (ANGELAKIS et al., 2012).

7.3. REFERENCES

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8. APPENDIX

8.1. Informações para os Membros de Bancas Julgadoras de Mestrado/Doutorado

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Secretaria de Pós-Graduação

Informações para os Membros de Bancas Julgadoras de Mestrado/Doutorado

1. O candidato fará uma apresentação oral do seu trabalho, com duração máxima de trinta minutos.

2. Os membros da banca farão a argüição oral. Cada examinador disporá, no máximo, de trinta minutos para argüir o candidato, exclusivamente sobre o tema do trabalho apresentado, e o candidato disporá de trinta minutos para sua resposta.

2.1 Com a devida anuência das partes (examinador e candidato), é facultada a argüição na forma de diálogo em até sessenta minutos por examinador.

3. A sessão de defesa será aberta ao público.

4. Terminada a argüição por todos os membros da banca, a mesma se reunirá reservadamente e expressará na ata (relatório de defesa) a aprovação ou reprovação do candidato, baseando-se no trabalho escrito e na argüição.

4.1 Caso algum membro da banca reprove o candidato, a Comissão Julgadora deverá emitir um parecer a ser escrito em campo exclusivamente indicado na ata.

4.2 Será considerado aprovado o aluno que obtiver aprovação por unanimidade ou pela maioria da banca.

5. Dúvidas poderão ser esclarecidas junto à Secretaria de Pós-Graduação: pgfarma@usp.br, (11) 3091 3621.

São Paulo, 23 de maio de 2014.

Prof. Dr. Adalberto Pessoa Junior
Presidente da CPG/FCF/USP

8.2. Ficha do Aluno

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9133 - 4936280/1 - Natália Pratis Perina

Email:	naperina@usp.br
Data de Nascimento:	22/04/1984
Cédula de Identidade:	RG - 32.953.783-0 - SP
Local de Nascimento:	Estado de São Paulo
Nacionalidade:	Brasileira
Graduação:	Nutricionista - Faculdade de Saúde Pública - Universidade de São Paulo - São Paulo - Brasil - 2008

Curso:	Doutorado Direto
Programa:	Tecnologia Bioquímico-Farmacêutica
Área:	Tecnologia de Alimentos
Data de Matrícula:	02/02/2011
Início da Contagem de Prazo:	02/02/2011
Data Limite para o Depósito:	02/06/2015
Orientador:	Prof(a). Dr(a). Marice Nogueira de Oliveira - 02/02/2011 até o presente. Email: monolive@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 02/02/2011
Prorrogação(ões):	120 dias Período de 02/02/2015 até 02/06/2015
Data de Aprovação no Exame de Qualificação:	Aprovado em 06/11/2012
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 02/02/2011 Prorrogação em 30/07/2014

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).

Última ocorrência: Matrícula de Acompanhamento em 02/02/2015

Impresso em: 26/04/2015 17:32:32

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9133 - 4936280/1 - Natália Pratis Perina

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBC5719-2/2	Trato Gastrointestinal: Imunomodulação da Colonização e Infecção Bacteriana	04/03/2011	16/06/2011	90	6	98	A	N	Concluída
FBA5892-5/1	Processos Deteriorantes de Alimentos pelo Desenvolvimento de Microrganismos	14/03/2011	24/04/2011	60	4	90	B	N	Concluída
FBT5773-6/2	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	07/04/2011	16/06/2011	30	0	-	-	N	Pré-matrícula indeferida
FBT5785-3/4	Tecnologia de Produtos com Alto Teor Lipídico	10/05/2011	20/06/2011	60	4	100	A	N	Concluída
HNT5737-3/2	Ciência de Alimentos (Faculdade de Saúde Pública - Universidade de São Paulo)	11/05/2011	22/06/2011	60	4	100	A	N	Concluída
FBT5705-2/1	Tecnologia de Produtos Lácteos Funcionais	15/08/2011	25/09/2011	90	6	100	A	N	Concluída
EDM5008-4/1	Professor Universitário: Vida, Perfil e Formação (Faculdade de Educação - Universidade de São Paulo)	16/08/2011	07/11/2011	120	0	-	-	N	Pré-matrícula indeferida
FBT5773-6/3	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	26/09/2011	04/12/2011	30	2	80	A	N	Concluída
HNT5758-1/3	Doenças Crônicas não Transmissíveis do Espectro da Síndrome Metabólica: Fisiopatologia, Epidemiologia e Controle (Faculdade de Saúde Pública - Universidade de São Paulo)	13/03/2012	24/04/2012	30	2	87	A	N	Concluída
FBT5723-5/2	Conservação de Alimentos por Processos Térmicos	13/03/2012	15/05/2012	90	6	80	B	N	Concluída
MCM5716-5/4	Atualização em Diabetes Mellitus Tipo 1 e 2 e Síndrome Metabólica (Faculdade de Medicina - Universidade de São Paulo)	13/08/2012	16/09/2012	75	5	85	B	N	Concluída
EDM5102-3/1	Preparação Pedagógica PAE (Faculdade de Educação - Universidade de São Paulo)	14/08/2012	24/09/2012	60	4	80	A	N	Concluída
BMB5804-3/1	O Órgão Adiposo como Centro Regulador do Metabolismo (Instituto de Ciências Biomédicas - Universidade de São Paulo)	14/10/2013	25/11/2013	60	4	87	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	40	47
Estágios:			
Total:	0	40	47

Créditos Atribuídos à Tese: 167

Conceito a partir de 02/01/1997:
A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.
Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 02/02/2015
Impresso em: 26/04/2015 17:32:32