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" Production of a nutraceutical beverage fermented by lactic acid bacteria using as substrate "Mate Tea" (*Ilex paraguariensis*): probiotic aspects and bioactive compounds."

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ISABELA FERRARI PEREIRA LIMA

PRODUCTION OF A NUTRACEUTICAL BEVERAGE FERMENTED BY LACTIC ACID BACTERIA USING AS SUBSTRATE "MATE TEA" (*Ilex paraguariensis*): PROBIOTIC ASPECTS AND BIOACTIVE COMPOUNDS

CURITIBA
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TERMO DE APROVAÇÃO

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Tese aprovada como requisito parcial para obtenção do grau de Mestre em Bioprocessos Biotecnológicos, do Programa de Pós-graduação em Processos Biotecnológicos, da Universidade Federal do Paraná, pela seguinte banca examinadora:

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Curitiba, 23 de julho de 2007

UFPR

Programa de Pós-Graduação em Processos Biotecnológicos
Setor de Tecnologia

RELATÓRIO DE DEFESA DE DISSERTAÇÃO DE MESTRADO

Aos vinte e três dias do mês de julho de 2007, na Sala de Vídeo Conferência do CESEC, no Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pelo Prof Dr Carlos Ricardo Soccol, Coordenador do Curso de Pós-Graduação em Processos Biotecnológicos, a banca examinadora para a Décima Quarta Defesa de Dissertação de Mestrado, área de concentração: Agroindústria. Estiveram presentes no Ato, além do Coordenador do Curso de Pós–Graduação, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do colegiado do Curso de Pós-Graduação em Processos Biotecnológicos, ficou constituída pelos Professores Doutores Jean-Luc Tholozan (Université de Provence - França), Jean Lorquin (Université de Provence – França), Débora Brand (UFPR), e Carlos Ricardo Soccol (UFPR – orientador da dissertação).

Às 11:00 horas, a banca iniciou os trabalhos, convidando a candidata **Isabela Ferrari Pereira Lima** a fazer a apresentação da Dissertação intitulada: "Production of a Nutraceutical Beverage Fermented by Lactic Acid Bacteria Using as Substrate "Mate Tea" (*Ilex paraguariensis*): Probiotic Aspects and Bioactive Compounds". Encerrada a apresentação, iniciou-se a fase de argüição pelos membros participantes.

Tendo em vista a dissertação e a argüição, a banca composta pelos professores Dr Jean-Luc Tholozan, Dr Jean Lorquin, Dr José Angel Rodriguez-Leon, Drª Débora Brand e Dr Carlos Ricardo Soccol, declarou a candidata aprovada (de acordo com a determinação dos Artigos 32/33/34/35 da Resolução 13/96 de 23.07.96).

Curitiba, 23 de julho de 2007.

Prof Dr Jean-Luc Tholozan

Prof Dr Jean Lorquin

Prof Dr Carlos Ricardo Soccol

Profª Drª Débora Brand

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RESUMO

Neste trabalho, foram avaliados os aspectos sensoriais, microbiológicos e princípios bioativos de uma bebida probiótica elaborada a partir de extrato de *Ilex paraguariensis St.-Hill* adicionado de mel, e fermentado por *Lactobacillus acidophilus.* Foram analisados a quantidade de bactérias viáveis, pH, quantidade de biomassa, açúcares consumidos, ácidos orgânicos produzidos, conteúdo de cafeína e princípio antioxidante durante a fermentação e durante o tempo de prateleira de 28 dias. As características ótimas obtidas foram: Extrato de 15 g ± 1 g de Erva Mate tostada em 300 mL de água fervente; adição de 14% de mel comercial; Tempo de fermentação 10 horas; Temperatura de incubação 35^{o}C; condição microaerofílica; pH final 4,2; Acidez final 0,6 mL NaOH 0,1 N; Conteúdo total de bactérias 10 ^{8} UFC/mL; Biomassa total 2 mg/mL; Açúcares totais 81,91 g/L; Ácidos Orgânicos totais 2,14 g/L; Conteúdo de Cafeína 6,77 mg/100 mL; Atividade Antioxidante 56% de inibição de DPPH. Tanto o conteúdo de cafeína, bem como a atividade antioxidante desta bebida probiótica mantiveram-se inalteradas durante a fermentação e depois durante o tempo de prateleira.

A bebida produzida, por suas características probióticas adicionadas de propriedades antioxidantes, pode ser uma alternativa terapêutica para doenças intestinais.

ABSTRACT

In this paper, the development and the sensorial, microbiological and bioactive characteristics of a Probiotic Soft Drink made of *Ilex paraguariensis St.-Hill* extract and honey, fermented by *Lactobacillus acidophilus* are evaluated. Viable bacteria, pH, biomass, sugars consumed, organic acids produced, caffeine and antioxidant content are analyzed during fermentation and after, during 28 days of shelf-life. The optimal characteristics developed are: Mate Tea Extract: 15 g ± 1 g of Toasted Mate Leaves in 300 mL of boiling water; Concentration of Commercial honey: 14%; Time of fermentation: 10 hours; Temperature of incubation: 35°C; Condition: Microaerophylic ; Final pH: 4.2; Final Acidity: 0.6 mL NaOH 0.1 N; Total Bacterial content :10^{8} CFU/mL ; Total Biomass : 2 mg/mL; Total Sugars: 81.91 g/L ; Total Organic Acids: 2.14 g/L ; Caffeine content: 6.77 mg/ 100 mL; Antioxidant activity: 56% of inhibition of DPPH. Both caffeine content and antioxidant activity of Mate Tea infusion (measured in % of reduction of DPPH - 2,2 diphenyl 1 picrylhydrazyl) maintained the same during fermentation and shelf-life.

This work shows the possibility to produce a Probiotic Soft Drink with high antioxidant content, as an alternative beverage to prevent intestine diseases.

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LIST OF ABREVIATIONS

AGE	Advanced Glycation End-product
ATCC	American Type Culture Collection
BHT	Butyl-hydroxytoluene
CFU/mL	Colony-forming Unit per milliliters
DPPH	2,2 diphenyl 1 picrylhydrazyl
DIN	German Institute for Standardization
FDA	United States Food and Drug Administration
pH	Hidrogenionic potential
% R	Percentage of reduction or inhibition
GMO	Genetic Modified Organism
H_2SO_4	Sulfuric Acid
HPLC	High Performance Liquid Chromatography
MRS broth	De Man, Rogosa and Sharpe broth
N_2	Nitrogen
NaOH	Sodium Hydroxide
UFPR	Universidade Federal do Paraná

LIST OF SIMBOLS

atm	Atmospheres (Pressure)
^{O}C	Celcius Degrees (Temperature)
g	Grams (Mass)
mL	Milliliters (Dimension)
N	Normal (Concentration)
mM	Milimols (Mass)
µm	Micrometers (Dimension)
min	Minutes (Time)
X	Cell dry weight (mg/mL)
nm	Nanometers (Dimension)
mm	Milimeters (Dimension)
v/v	Volume/volume (Concentration)
µg	Micrograms (Mass)
µM	Micromols (Mass)
$\mu_{máx}$	Maximum grow rate
dX/dT	Increase of Biomass
T_D	Biomass duplication time
dLA/dT	Maximum and total productivity of lactic acid
$Y_{X/S}$	Overall yield of biomass formation
$Y_{P/S}$	Overall yield of substrate conversion into lactic acid

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1. Introduction

1.1. Nutraceutical Beverages

Nutraceuticals are often referred to as phytochemical and functional foods. They are considered natural, with bioactive compounds that have health promoting, disease preventing or medicinal properties.

Functional foods exert their actions on different systems, especially the gastrointestinal, cardiovascular and immunological ones, acting too as enhancers of development a differentiation and positively modulating nutrient metabolism, gene expression, oxidative stress and the psychic sphere.

Innumerable substances are known to have functional effects: soluble and insoluble fiber, phytosterols, phytoestrogens, monosaturated and polyunsaturated fatty acids, phenol derivatives, vitamins and other phytochemical compounds (RODRIGUEZ, MEGIAS and BAENA, 2003).

The leading functional foods, regarding which the soundest scientific evidence exist, are probiotics, live microbial food ingredients represented mainly by fermented dairy products. Prebiotics, such as inulin-type fructans, are the trophic substrate of probiotics and the potential intestinal microflora selectors. The combination of probiotics and prebiotics is termed symbiotic.

Probiotic foods are those which contain live microbiological culture either as a result of fermentation or as an intestinal addition to beneficially affect the host by improving the intestinal microbial balance (WAHIQVIST, 2002). Probiotics are culture of non-pathogen microorganisms, which resists the hydrochloric acid and digestive enzymes, so they can reach the colon viable alive form (VADENPLAS Y. et all, 2002).

Potential health benefits and mechanisms: It has been proposed that in the intestine these bacteria (WAHIQVIST, 2002):

1. Bind, block or remove carcinogens,
2. Inhibit bacteria which directly or indirectly convert procarcinogens to carcinogens by enzyme activity,
3. Activate the host's immune system to antitumorigenesis,
4. Reduce the intestinal pH, thereby altering microbial activity, solubility of bile

acids and mucus secretion.

One difficult with probiotic foods is the microorganism survival, both during the product shelf-life and after ingestion: it is difficult to guarantee. These microorganisms have a number of barriers to their survival such as the gastric acidity, bile secretions and competition over 500 resident bacterial species (WIN, 1999).

The choice of a strain of microorganism is important to avoid removal of micronutrients from the food, to avoid production of adverse components such as vasoactive amines and to avoid opportunistic acid bacterial pathogens. As an example of the wide range of strains that are available, the genus *Lactobacillus* contains up to 60 species (including *L. acidophilus* and *L. casei* – the ones most commonly added to yoghurts and drinks (TOMELIN and PEPLAU, 2005).

Probiotics must contain living organisms in appreciable numbers at the end of the product's shelf-life. To have any effect in the colon, the bacteria need to survive the passage through the digestive acids and enzymes. To have the desire effect, scientists believe that at least a million of each probiotic bacteria per gram of probiotic needed e.g. if a beverage contains three different types of probiotic bacteria, it should contain at least a million of each of them per gram. (WAHIQVIST, 2002)

MEDINA and JORDANO, cited by PARADA et al. (2003) recommend a minimum level of probiotic bacteria, in any dairy product, of 2×10^6 CFU/ml. As also cited by PARADA et all. (2003), culture manufacturers recommend approximately $10^6 – 10^7$ probiotic bacteria per gram of yoghurt at the end of shelf-life. The Brazilian Ministry of Agriculture recommends a minimum of 10^7 CFU/g when the probiotic product is consumed (BRASIL, 2000).

1.2. Mate Tea *(Ilex paraguariensis)*

1.2.1. Common names

The Mate Tea *(Ilex paraguariensis St. Hill*) - Spanish name "yerba", "yerba mate", and Portuguese name "erva mate" - is a shrub that grows naturally in the subtropical forest of NE Argentina, Southern Brazil and Paraguay, where it is also cultivated and has a great economic importance. It is used for making a hot beverage (infusions and decoctions, called "Chimarrão") and a refresh drink (called "Tererê").

The drink is so popular in Brazil, that some industries produce and sell all the country this soft drink in disposable bottles or cups, natural or flavored with lemon or peach, regular or light.

Figure 1.1 Samples of commercial Mate Tea, flavored and not flavored.
(Source: LIMA, Isabela, 2007)

1.2.2. Social and economic aspects

The habit of drinking is extended over Argentina, Uruguay, Paraguay and Southern Brazil and has an indigenous origin. In South America, approximately 30% of the population drinks more than 1 L/day of this beverage (GNOATTO, 2005).

The world production of Mate Tea is closely to 500,000 mil tons, as 260,000 mil tons in Argentina, 180,000 tons in Brazil and 30,000 tons in Paraguay, annually. The production of Mate Tea is one of the most important agricultural activities, realizing 80 million dollars each year. In Brazil, about 700,000 farmers and employees are involved in this agri-food chain, including 600 companies and 180,000 small farms at 450.000 km^2 of area (EMBRAPA, 2005).

From the environmental point of view, the mate tea has an important hole, because, it's a native shrub that doesn't need any chemical product; it is integrated, and belongs to its own environmental area. It's not a GMO (Genetic Modified Organism) and can have the claim as an organic tea (PASINATO, 2003).

Figure 1.2: Mate Tree in its natural environment. (Source: MAIA, 2001)

1.2.3. Chemistry and nutritional value of Mate Tea

The components of Mate Tea are: water, cellulose, gums, dextrines, glucose, pentoses, lipids, aromatic resins, albumin, caffeine, teofilin, caffearine, matetanic acid, folic acid, caffeic acid, chlorophyll and essential oil.

Table 1.1: Average constitution of Mate Tea (g/100g of tea)

Component	Average amount (g/100g)
Water	8.15 g
Protein	10.89 g
Carbohydrate	12.04 g
Starch	4.55 g
Glucose	3.84 g
Fiber	16.86 g
Ash	6.91 g
Calcium	0.668 g
Magnesium	0.337 g
Potassium	1.350 g
Sodium	0.002 g
Iron (mg)	59.9 mg
Copper (mg)	1.26 mg
Vitamin A	2.095 g
Thiamine (vitamin B)	222.7 g
Riboflavin (vitamin B2)	404.3 g
Ascorbic Acid (vitamin C)	11.9 g

Source: MAIA, 2001

1.2.4. Properties of bioactive compounds

The beneficial and therapeutic aspects of Mate Tea have recently been verified by a number of scientific studies. There are 196 active chemical compounds found in the Mate Plant (AVIVA Ltd, 2006), 11 are polyphenols.

Besides the substantial amounts of purine alkaloids and caffeoyl-quinic acid derivatives the leaves of *Ilex paraguariensis* contain also a significant amount of triterpenoid saponins. These bitter and highly water-soluble compounds are likely to be partially responsible for the taste of the beverage and also for foaming observed in the “mate” (GNOATTO, 2005).

The blood cholesterol-lowering properties of dietary saponins are of particular interest in human nutrition. Dr. Rene Malinow, at the Oregon Regional Primate Center, demonstrated the cholesterol-lowering properties of saponins. This desirable effect is achieved by the binding of bile acids and cholesterol by saponins. Bile acids form mixed micelles (molecular aggregates) with cholesterol, facilitating its absorption. Cholesterol is continually secreted into the intestine via the bile, with much of it subsequently reabsorbed.

Saponins cause a depletion of body cholesterol by preventing its reabsorption, thus increasing its excretion (DASHWWOD, 2003).

Mate Tea has significant antioxidant activity. Different papers account for the antioxidant activity of extracts of *Ilex paraguariensis,* strongly correlated to the total amount of phenolic components (FILIP, 2000), that is, the caffeoyl-derivatives present in the extracts of Mate Tea.

Also, BASTOS (2006) reports that the antioxidant efficacy of Mate Tea is the same as BHT (Butyl-hydroxytoluene), a well-known antioxidant used as food additive and LUNCEFORD (2005) showed that *Ilex paraguariensis* extracts has higher antioxidant effect than green tea, due to its capacity to inhibit the second phase of glycation reactions, namely the free radical mediated conversion of the Amadori products to AGE.

1.3. Substrates

As substrates for probiotic beverages, milk has been the most known, but its possible to work on other substrates for lactic fermentation using honey, malt extract, yeast extract and sugar cane molasse, for instance.

1.3.1. Honey

One of the better descriptions defines honey as a 'sweet, viscous fluid' elaborated by bees from the nectar of plants and stored in their combs as food. This definition will suffice under most circumstances but even this description fails to include honeys made from honeydew or fruit and plant juices.

Table 1.2: Composition of honey (%)

Component	Average
Moisture	17.2
Levulose (fructose)	56.2
Dextrose (glucose)	32.28
Sucrose	1.31
Higher sugars	1.50
Ash	0.169
Nitrogen	0.041

Source: Honey: Composition and Properties (2006)

Honey is composed primarily of sugars and water. The primary sugars are fructose (38.2%) and glucose (31.3%). These are 'simple', 6-carbon sugars that are readily absorbed by the body. Other sugars include maltose (7.3%) a 12 carbon sugar composed of 2 glucose molecules and sucrose (1.3%) a 12 carbon sugar composed of glucose and fructose molecules.

Honey also contains acids (0.57%), some protein (0.26%), a small amount of minerals (0.17%) and a number of other minor components including pigments, flavor and aroma substances, sugar alcohols, colloids and vitamins. This latter group of materials constitutes about 2.2% of the total composition.

1.3.2. Yeast extract

Yeast extract is a very important source of nitrogen and it's made from autolized yeast cells.

A regular yeast extract composition used to enrich broths is showed below.

Table 1.3 – Yeast extracts composition

Component/ physical characteristics	
Total Nitrogen	Min. 8.9 %
Water	6.0 %
Ash	14.0 %
pH	6.4 – 7.2

Source: Micromed – ISOFAR Ind. E Com. De Produtos Químicos, Ltda (2006)

1.3.3. Malt Extract

Malt Extracts, in general, are of a high nutritional value, with notable amounts of several of the vitamin B complexes and minerals. Malt extracts contain a wide range of amino acids due to conversion during extraction and so is readily absorbed into the body's bloodstream.

As defined by the FDA (U.S. Food and Drug Administration, 1996) Malt Extract is the product obtained by extracting malt, the partially and artificially germinated grain of one or more varieties of *Hoedeum vulgare Line* (Fam. Gramineae). The malt is infused with water at 60°C, preferably under reduced pressure. It contains dextrin, maltose, a small amount of glucose, and amylolytic enzymes.

Table 1.4: Standard Malt Extract Composition

General Composition (% dry weight)	
Carbohydrate	91 – 93 %
Protein	6 – 7%
Ash	1 – 2%

Protein Composition: All proteins are at least partially hydrolyzed Approximate Amino acid composition of polypeptides is:	
Glutamic Acid	21%
Proline	13%
Aspartic Acid	8%
Leucine	7%
Alanine	6%
Valine	5%
Serine	5%

Vitamin Content mg per 100g approx.	
B1	0.8
B2	0.3
B6	0.6
B12	0.005
Nicotinamide	7.2
Pantothenic Acid	5.5
Folic Acid	0.06
Biotin	0.006
Ascorbic Acid	0.10

Source: International DiaMalt Co. Ltd., 2005

1.3.4. Sugar Cane Molasse

The Brazilian Sugar Cane Molasse has in average composition, the elements showed in the table below:

Table 1.5: Average composition of Brazilian Sugar Cane Molasse.

Component	Average amount
Sucrose %	32.50
Reduced Sugars %	20.80
Total Sugars %	52.20
Protein (N x 6.25) g/100g	8.20
Ash %	8.20
Water %	19.05

Source: MORAES, 1996

2. Objectives

2.1. Main Objective

The main objective of this practical work is to develop and analyze a new nutraceutical beverage, as a soft drink, from substrates Mate Tea, Malt Extract, Yeast Extract, Sugar Cane Molasse and Honey, fermented by *Lactobacillus sp.* . The physical-chemical, bioactive content, microbiological and sensorial aspects are, then, studied and evaluated.

2.2. Specific Objectives

1. Produce and evaluate some different formulations using as substrates Mate Tea, Malt Extract, Yeast Extract Sugar Cane Molasses and Honey.
2. Conduct a screening, in order to choose the best fermented formulation, based on sensorial aspects.

For the chosen formulation, to measure the variables:

3. pH, acidity, sugars consumed and organic acids produced during fermentation.
4. Viable lactic bacteria and biomass produced during fermentation and shelf-life.
5. Bioactive compounds: caffeine and anti-oxidant content, after fermentation and during shelf-life.
6. Calculate the kinetics parameters.

3. Material and Methods

3.1. Mate Tea Extract

The extract was prepared by decoction: a quantity of 15 g ± 1 g (which was a optimal result of some pre-determined preparations) of roasted and ground commercial Mate Tea leaves was added to 300 mL of hot distilled water (95 ^{o}C), and still was boiled for 3 minutes. It was, then, filtered under vacuum using filter paper 1:11 µm (Watman, UK), at room temperature.

3.2. Substrate formulations

The broths for fermentation were made with 300 mL of Mate Tea Extract, added of honey, yeast extract, malt extract and sugar cane molasse, in proportions described in table below. The broths were sterilized at 1 atm during 20 minutes.

Table 3.1: Substrate formulations

FORMULATION	QUANTITY USED (w/v %)			
	HONEY	YEAST EXTRACT	MALT EXTRACT	SUGAR CANE MOLASSE
A	4	0.5	-	-
B	6	0.5	-	-
C	8	0.5	-	-
D	10	0.5	-	-
E	4	1.0	-	-
F	4	1.5	-	-
G	-	-	4	-
H	-	-	6	-
I	-	-	8	-
J	-	-	-	4
K	-	-	-	6
L	-	-	-	8
M	10	-	-	-
N	14	-	-	-

3.3. Strains

The strains used were *Lactobacillus acidophilus A*TCC 4356 and *Lactobacillus sakei* ATCC 43121*,* both frozen in liquid N_2, at –298^{o}C, gently donated by Prof. Dr. Soccol, from Bioprocess Engineering and Biotechnology Division Collection, UFPR. It was maintained at –18 ^{o}C, in a 20% glycerol solution. To activate, it was melted at room temperature and inoculated in MRS broth (De Man, Rogosa & Sharpe broth), during 24 hours at 35 ^{o}C. The sub-culturing was made at each three months.

Other strains were tested, as *L. rhamnosus* and *L. casei*, but the most interested ones were *L. acidophilus* and *L. sakei*.

3.3.1. The Inoculums Standardization

The inoculums standardization, were dropping down the inoculum in 100 mL of distilled water until reaching the optic density of 0,70 ± 0,2 , measured in 550 nm spectrofotometry, instrument DR/2000 Spectrophotometer (Hach Company). For this spectra band, it was found that 1 drop of inoculum in 100 mL of distilled water has, in average, 10^2 CFU/mL (quantified by inoculation of 1 ml of fermented sample in "pour plate" MRS medium for Lactobacillus). The methodology was based on Mc Farland scale principle, and adapted from CORDEIRO, 1999.

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3.4. Fermentation conditions

Aseptically, it was dropped down into 300 mL of substrate, 6 drops of standardized inoculum, that is 10^4 CFU/mL. It was, then, incubated for 12 hours at 35°C. (The fermentation period was determined after some longer fermentation that gave too high acidity). Both *Lactobacillus* strains were incubated in microaerophylic conditions (a Scotch flask with screw lid); see figure 4.1.

3.5. Fermentation Parameters analyzed

3.5.1 pH

The pH was determined through potentiometer Tecnal model TEC-3MP calibrated in pH 4.0 and pH 7.0.

3.5.2 Titrable Acidity

The determination of acidity was made by titrimetric analysis, using a sample of 1 mL of the fermented broth diluted in 49 mL of distilled water. This diluted solution was neutralized with NaOH 0.1 N, using as indicator alcoholic solution of phenolphthalein 1%.

3.5.3 Sugars Consumed and Organic Acids produced

The samples were diluted 1:10 in miliQ water, and filtered in a Millipore filter 0.22 µm. Sugars content (glucose and fructose) and organic acids produced (lactic and acetic)

were determined by HPLC, using an Aminex Column HPX 87 HI, mobile fase H_2SO_4 5 mM, flow 0.6 mL/min, 60°C and running time 18 minutes.

3.5.4 Quantification of viable lactic bacteria

The viable number of probiotic microorganisms (CFU/mL) was quantified by inoculation of 1 ml of fermented sample in "pour plate" MRS medium.

3.5.5 Quantification of Biomass (Cell Dry Weight) produced

Cell dry weight (X, mg/mL) was determined by washing tree times the samples, then centrifugation and filtration (0.45 µm, Whatman, Kent, UK) of 10 mL volume of fermentation and followed by drying at 80°C for 24 h. The centrifuge was Simplex/Super ITR Instruments.

Obs.: All the fermentation tests and respective analysis were made in triplicate.

3.5.6. Shelf-life Conditions

The samples were in Scotch flasks with screw lid and stored at refrigeration condition (temperature $4^0C \pm 1.0^0C$), during 28 days, and samples were taken out at 0 day, 7^{th} day, 14^{th} day, 21^{st} day, 28 th day.

3.6. Sensorial Evaluation

3.6.1. Pre-selection of fermented beverage

It was made 14 different formulation of substrate (A to N). As eliminatory step, those broths that have not been approved during the laboratory tests (precipitation formed, unpleasant flavor or odor), don't pass to next examinations.

3.6.2. Personnel/Panel

The group that evaluate the samples were from the labs, family and colleagues, that drink Mate Tea frequently. They were told about the aim of this research and have a little training about Qualitative Description Test. The Qualitative Description was run with 10 people.

3.6.3. Attributes

A group of five experienced people from this panel choose 8 attributes of the beverage that are: color, transparency, precipitation, sweetness**,** acidity, astringency, saltiness, distasteful aftertaste.

3.6.4. Sensorial tests: at the final of fermentation and during shelf-life

The samples are the beverage in development and 2 commercial Mate Tea with Lemmon flavor, because the probiotic beverage in development is a little acid due to organic acids produced during fermentation.

The samples were presented to each person, refrigerated at 5^{o}C in 50 mL plastic disposable cups.

For the ready beverage (at the final of the fermentation), the Qualitative Description was applied, as the same to shelf-life evaluation

3.7. Bioactive Compounds Evaluation

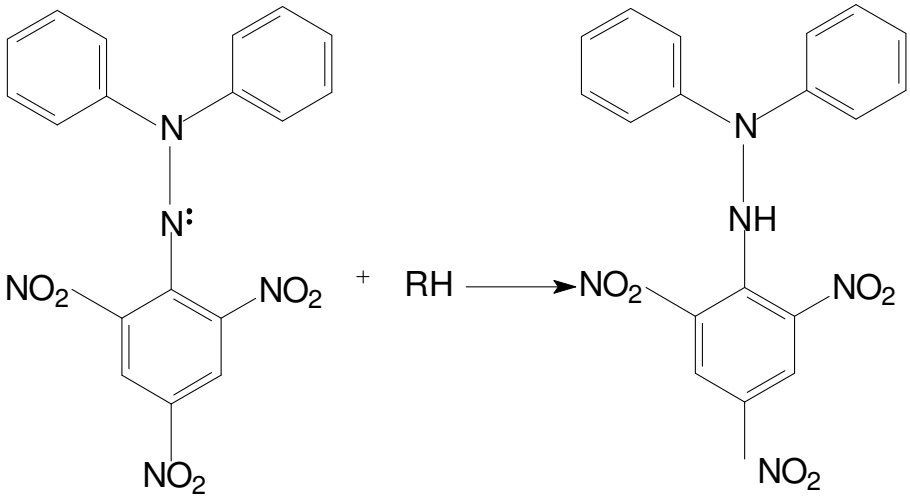
3.7.1 Caffeine content

The method used to detect caffeine was DIN 10777-2:1994 “Analysis of coffee and coffee products” (DIN, 1994). The samples were diluted 5 mL in 250 mL of MiliQ water, filtered in a Milipore Filter 0.22 µm. Caffeine was determined by HPLC, using as instrument HP 1100, detector DAD, 272 nm, and Zorbax RX C18 column 150mmx 4,6 and 5 µm of particles. The mobile phase was water: methanol (70:30), flow 1 mL/min, temperature 35^{o}C and running time 3 minutes.

3.7.2 Antioxidant activity

The method chosen was the reduction of DPPH (2,2 diphenyl 1 picrylhydrazyl) radical and it was determined according to DUARTE-ALMEIDA (2006) and MOLINEAUX (2004) with some modifications, based on work of SCHINELLA (2000). It was prepared an ethanolic solution of the fermented beverage (1:10 v/v) and ethanolic solution of Vitamin C (45 µg/mL). 5 mL of sample was added to 5 mL of ethanolic DPPH in tube covered with aluminum. Absorbance was spectrophotometrically determined after 30 minutes of reaction (“plateau”). The color turns from purple to yellow as the molar absorbance of the

DDPH radical at 517 nm reduces, when them odd electron of DPPH radical becomes paired with a hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. Vitamin C was used as reference compound. The instrument was a DR/2000 Spectrofhotometer (Hach Company) and 1 cm path length cuvettes. It was used mixtures of 2 ml solution of DPPH and 2 mL of the antioxidant.



Diphenylpycrilhydrazyl (free radical) Purple

Diphenylpycrihydrazyl (nonradical) Yellow

Figure 3.7.1 Reaction of hydrogen donation to DPPH

The equation used to calculate the percentage of inhibition (or reduction) of DPPH is **% R** , called, usually, “inhibition” or “quenching”, is:

$$\%\ R = \frac{A_0 - A}{A_0} \times 100$$

Eq. 3.7.1: Equation to calculate the percentage of reduction of DPPH.

Where $A_{0\ is}$ the initial absorbance and A is the value for added sample concentration. The value of A_0 is that one in the cuvette in the presence of DPPH without any reductor.

A calibration curve was prepared with 5, 15, 25, 35, 45 µM of Vitamin C (Ascorbic Acid) and the results were expressed in % reduction of DPPH. Also a comparison between the antioxidant activity of both Vitamin C and the probiotic beverage is presented.

4. Results and Discussion

4.1 Sensorial aspects during fermentation

An experimental and practical work must be submitted to a sequential of steps, beginning with the screening, optimization, measurement and verification of data.

So, the first step of this practical work was the screening of 14 formulation using as strains *L. acidophilus* and *L. sakei*, and observe the appearance, taste and odor of the fermented broth, then eliminate the ones are not acceptable at first.

The Aa to La *L. acidophilus* broths produced a precipitation during the fermentation. The same happened to As to Ls *L. sake* broths.

Table 4.1 Formulations fermented by *L. acidophilus* and aspects observed, from a practiced sensorial point of view.

FORMULATION	Aspects observed after fermentation
Aa	Low CFUgrowth. A little salty and bitter aftertaste. Precipitated bottom formation.
Ba	Low CFUgrowth. A little salty and bitter aftertaste. Precipitated bottom formation.
Ca	Good CFU growth. Salty and bitter taste. Precipitated bottom formation.
Da	Excellent CFU growth. Prominent salty taste. Precipitated bottom formation.
Ea	Good CFU growth. Salty and bitter taste. Precipitated bottom formation.
Fa	Good CFU growth. Salty and bitter taste. Precipitated bottom formation.
Ga	Good CFU growth. Salty and bitter taste. Precipitated bottom formation.
Ha	Excellent CFU growth. Prominent salty taste. Precipitated bottom formation.
Ia	Excellent CFU growth. Prominent salty taste. Precipitated bottom formation.
Ja	Good CFU growth. Astringent taste. Precipitated bottom formation
Ka	Good CFU growth. Astringent taste. Precipitated bottom formation
La	Excellent CFU growth. Prominent astringent taste. Precipitated bottom formation
Ma	Good CFU growth. Very sweet taste. Slightly acid.
Na	Good CFU growth. Sweet taste. Moderate acid.

Table 4.2 Formulations fermented by *L. sakei* and aspects observed, from a practiced sensorial point of view.

FORMULATION	Aspects observed after fermentation
As	Low CFUgrowth. A bitter aftertaste. Precipitated bottom formation.
Bs	Low CFUgrowth. A bitter aftertaste. Precipitated bottom formation.
Cs	Good CFU growth. Alcoholic flavor and bitter taste. Precipitated bottom formation.
Ds	Excellent CFU growth. Prominent alcoholic flavor. Precipitated bottom formation.
Es	Good CFU growth. Alcoholic odor and bitter taste. Precipitated bottom formation.
Fs	Good CFU growth. Alcoholic odor and bitter taste. Precipitated bottom formation.
Gs	Good CFU growth. Alcoholic odor and bitter taste. Precipitated bottom formation.
Hs	Excellent CFU growth. Prominent alcoholic odor. Precipitated bottom formation.
Is	Excellent CFU growth. Prominent alcoholic odor. Precipitated bottom formation.
Js	Good CFU growth. Astringent and alcoholic odor. Precipitated bottom formation
Ks	Good CFU growth. Astringent and alcoholic odor. Precipitated bottom formation
Ls	Excellent CFU growth. Prominent astringent and alcoholic odor. Precipitated bottom formation
Ms	Good CFU growth. Sweet and strong alcoholic odor.
Ns	Good CFU growth. Sweet taste and strong odor taste.

This first eliminatory step (screening) accomplishes to consider the best formulations, from the sensorial point of view, are those **named Na**, fermented by *L. acidophilus*, which have in its formulation only mate tea extract and honey as carbohydrate and nitrogen source. The figures 4.1 and 4.2 show the appearance of the formulation Na. **So, all the results presented in sequence are those referred to *Lactobacillus acidophilus* fermentations.**

Figure 4.1: Honey broths showing low precipitation (formulation Na, with 14% of honey). Samples in triplicate. (The tenuous precipitation is due to biomass).

Figure 4.2: Honey broths: at left, without inoculum, at right, with inoculum, at the final of fermentation. (Formulation Na, with 14% of honey)

4.2 Kinetic Curves

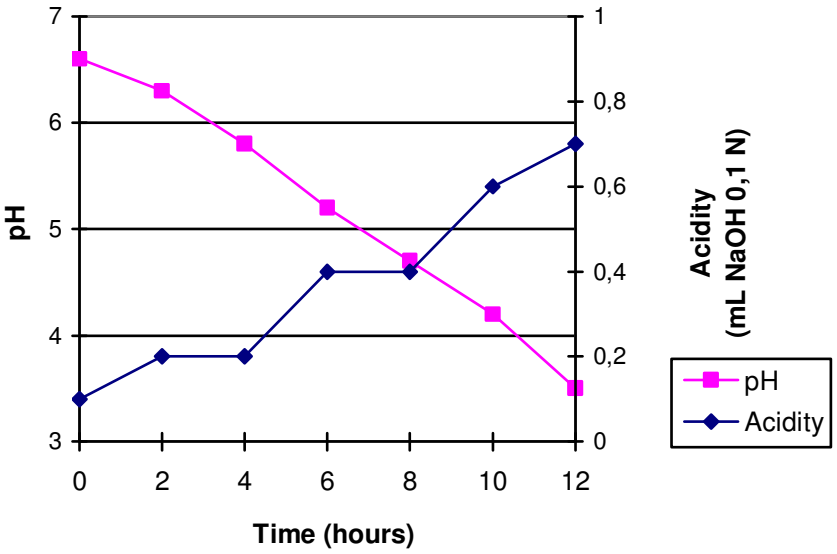


Figure 4.2.1: Kinetics of pH and Acidity

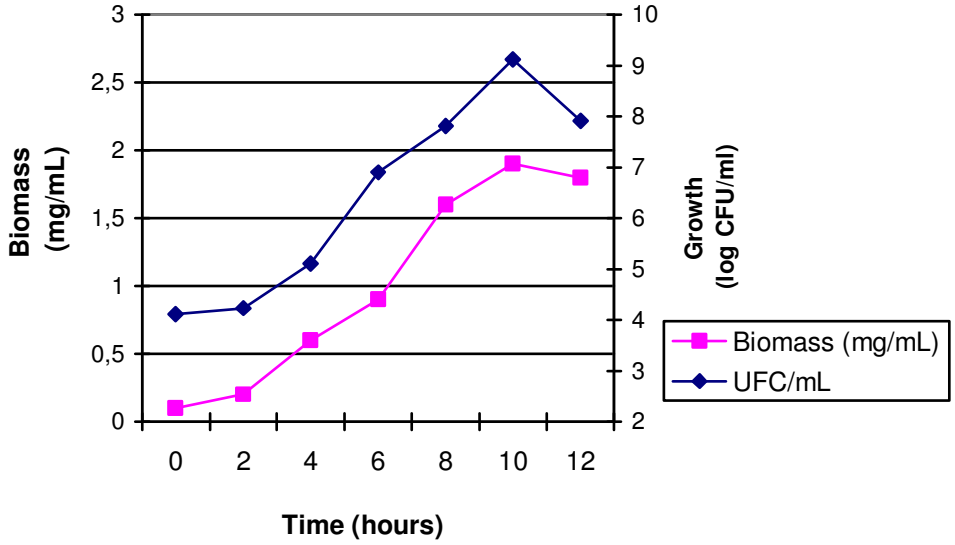
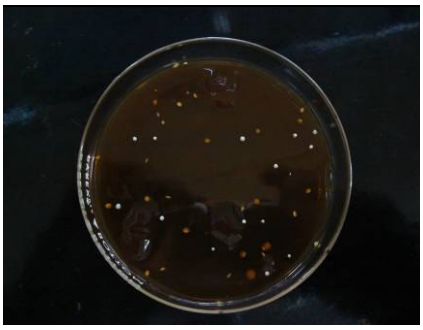
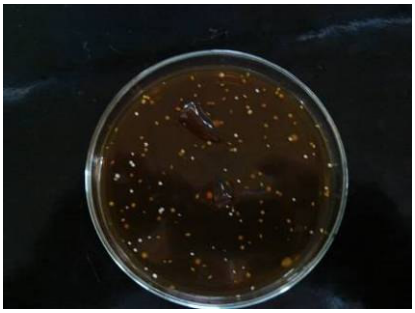


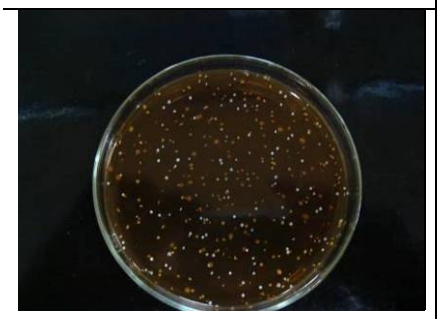
Figure 4.2.2: Kinetics of Cell Growth and Biomass produced



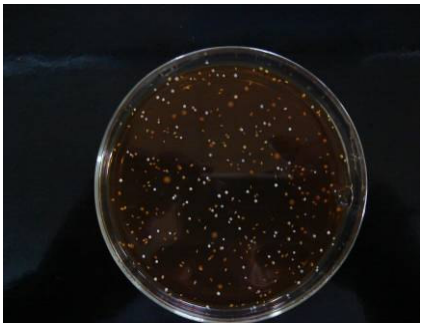
O h



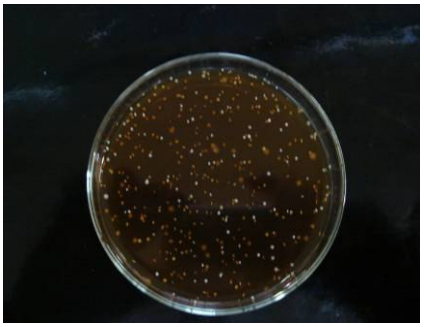
2 h



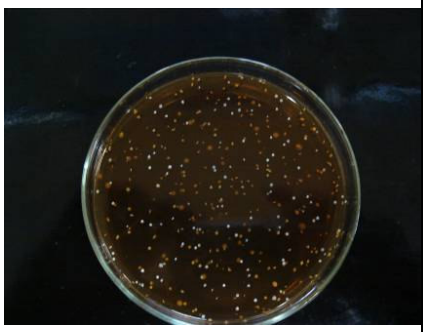
4 h



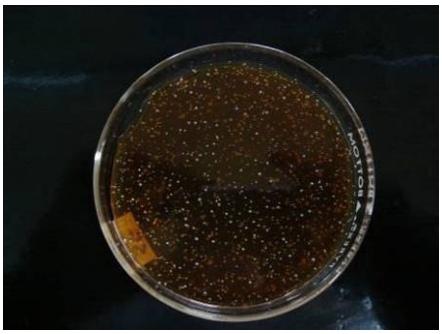
6 h



8 h



10 h



12 h

Figure 4.2.3: *Lactobacillus acidophilus* growth (MRS medium)

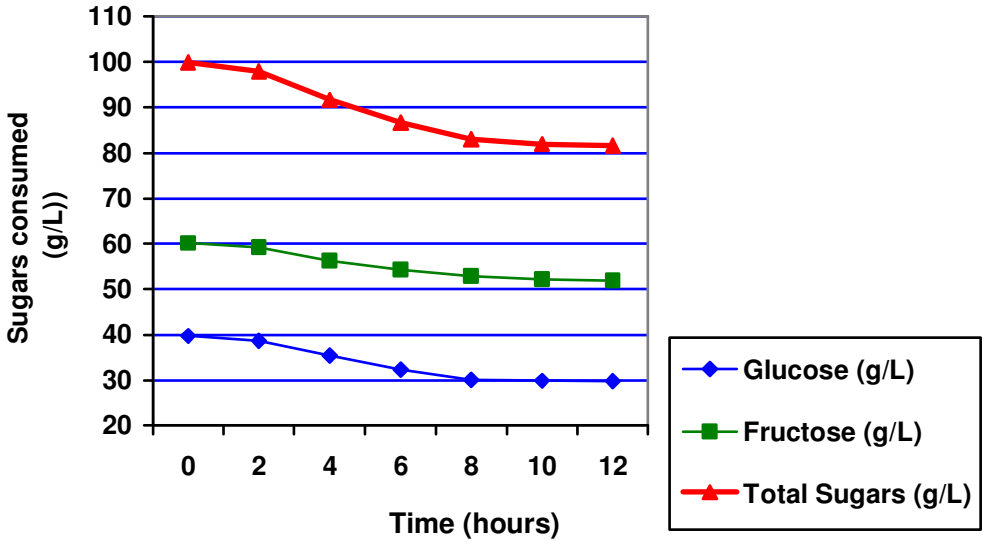


Figure 4.2.4: Kinetics of Broth Media glucose and fructose consumption

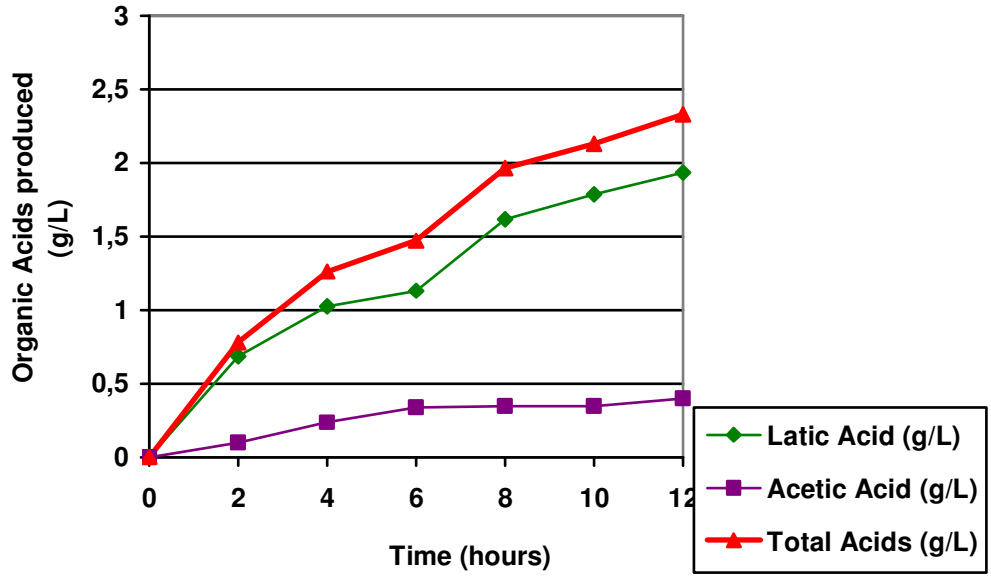


Figure 4.2.5: Kinetics of Broth Media Lactic and Acetic Acids produced

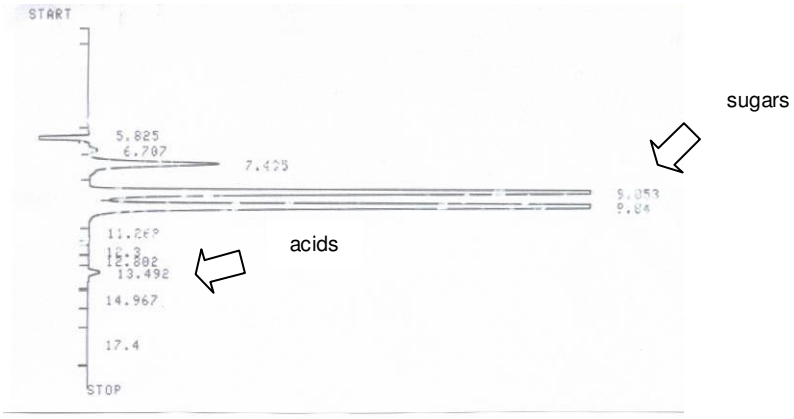


Figure 4.2.6 Sugars and acids chromatogram, for the beginning of fermentation

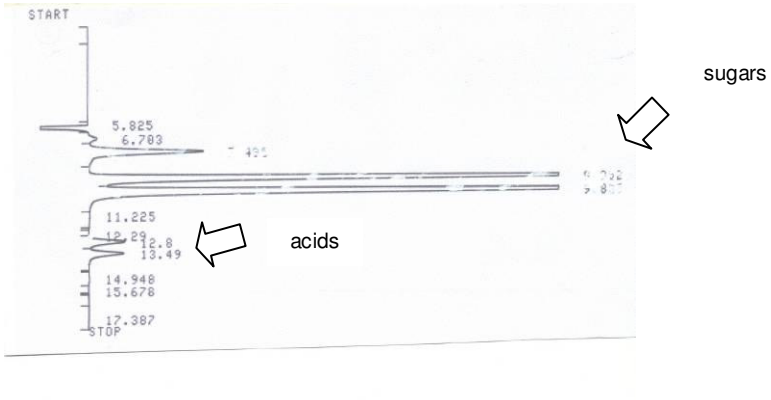


Figure 4.2.7 Sugars and acids chromatogram, for 6 hours of fermentation

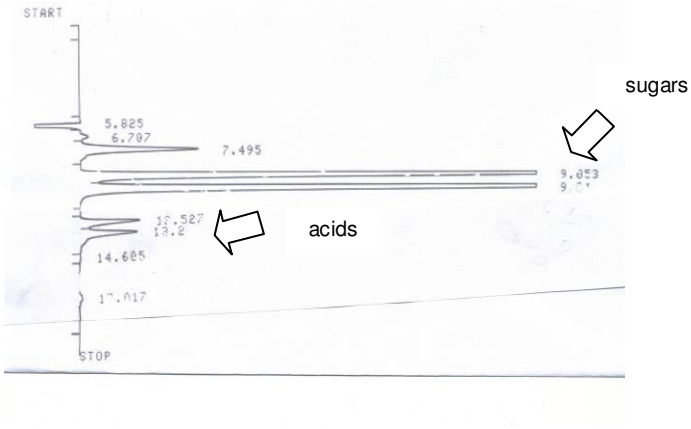


Figure 4.2.8 Sugars and acids chromatogram, for 12 hours of fermentation

4.3 Kinetic Calculation

Table: 4.3.1 Kinetics parameters of the fermentation
(substrate: mate tea extract, honey 14%, with *L. acidophilus*, during 12 hours)

Time	Biomass (X)	Total Sugars (S)	Total Acids (P)	Growth	Ln X	ΔS	Lactic Acid (LA)	ΔLA
(h)	g/L	g/L	g/L	Log UFC/L			g/L	
0	0.12	100	0	4.12	-2.12	-	0	-
2	0,23	97.92	0.78	4.23	-1.47	2.10	0.68	0.68
4	0.65	91.63	1.26	4.98	-0.43	6.31	1.03	0.35
6	0.92	86.64	1.47	5.84	-0.08	5.00	1.13	0.49
8	1.64	83.11	1.97	7.86	0.49	3.60	1.62	0.17
10	**1.92**	**81.91**	**2.14**	**8.32**	**0.65**	**1.10**	**1.79**	**0.15**
12	1.81	81.62	2.34	7.34	0.59	0.30	1.94	

4.3.1. Maximum grow rate (μ): between 4 and 8 hours

$\mu_{máx} = \ln X8 - \ln X4 = 0.49 - (-0.43) = 0.23\ h^{-1}$

4.3.2. Increase of biomass: until 10 hours

$$\frac{dX}{dT} = \frac{X_{10} - X_0}{T_{10} - T_0} = \frac{1.92 - 0.12}{10-0} = 0.18\ g/L.h$$

4.3.3. Biomass duplication time:

$$T_D = \frac{\ln 2}{\mu_{MAX}} = \frac{0.69}{0.23} = 3\ h$$

4.3.4 Maximum productivity of lactic acid: between 2 and 6 hours

$$\frac{dLA}{dT} = \frac{LA_6 - LA_2}{T_6 - T_2} = \frac{1.13 - 0.68}{6-2} = 0.1125\ g/L.h$$

4.3.5 Total production of lactic acid: until 10 hours

$$\frac{dLA}{dT} = \frac{LA_{10} - LA_0}{T_{10} - T_0} = \frac{1.79 - 0}{10 - 0} = 0.18\ g/L.h$$

4.3.6 Overall yield of biomass formation

$$Y_{X/S} = \frac{dX}{dS} = \frac{X_{10} - X_0}{S_0 - S_{10}} = \frac{1{,}92 - 0.12}{100 - 81.91} = \frac{1.8}{18.09} = 0{,}01 \text{ g biomass/ g of sugar}$$

4.3.7 Overall yield of substrate conversion into product (total organic acids)

$$Y_{P/S} = \frac{dP}{dS} = \frac{P_{10} - P_0}{S_0 - S_{10}} = \frac{2.14 - 0}{100 - 81.91} = 0.12 \text{ g of organic acids/ g of sugar}$$

4.3.8 Overall yield of substrate conversion into lactic acid

$$Y_{P/S} = \frac{dP}{dS} = \frac{P_{10} - P_0}{S_0 - S_{10}} = \frac{1.79 - 0}{100 - 81.91} = 0.10 \text{ g of organic acids/ g of sugar}$$

4.3.9 Proportion lactic acid and total acids at the final product

$$\text{Proportion} = \frac{0.10}{0.12} = 0.83333, \text{ that is, } 83\%.$$

This result confirms that it is a homolactic fermentation.

4.4 Bioactive compounds during fermentation

4.4.1 Caffeine content

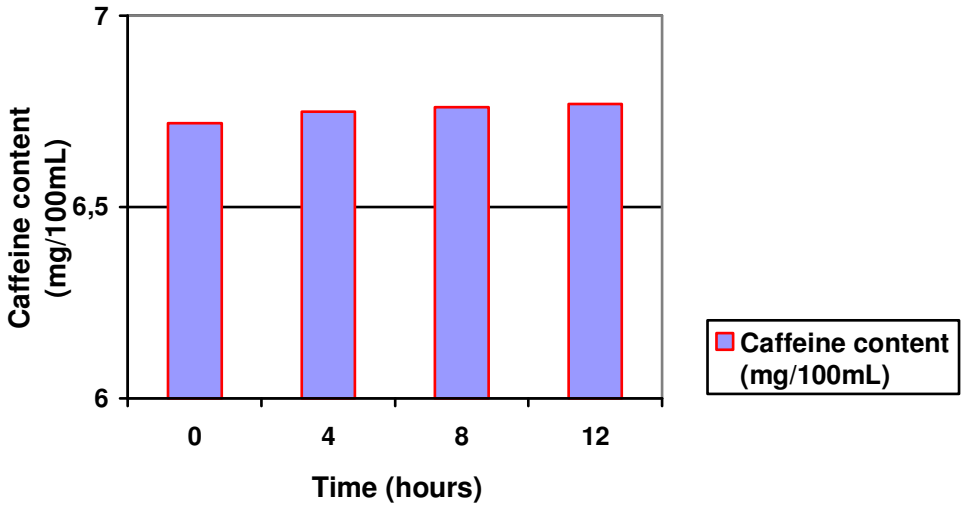


Figure 4.4.1 Caffeine Content (µg/mL) of the Probiotic Beverage during fermentation

4.4.2 Antioxidant content

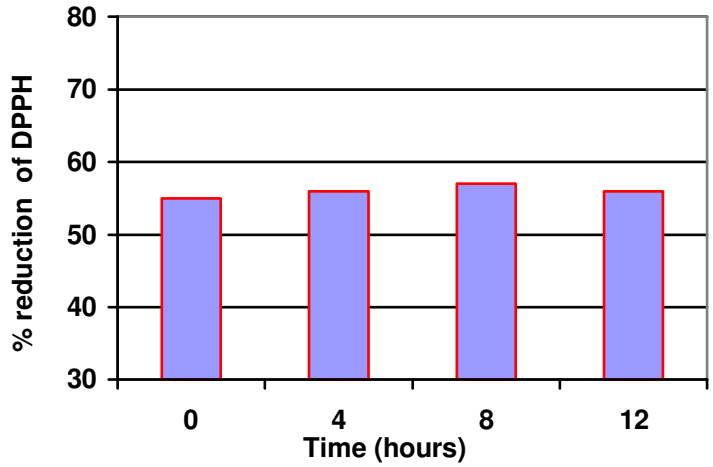


Figure 4.4.2 Antioxidant Activity of the Probiotic Beverage during fermentation (% R of DPPH)

4.5 Sensorial evaluation of the final product

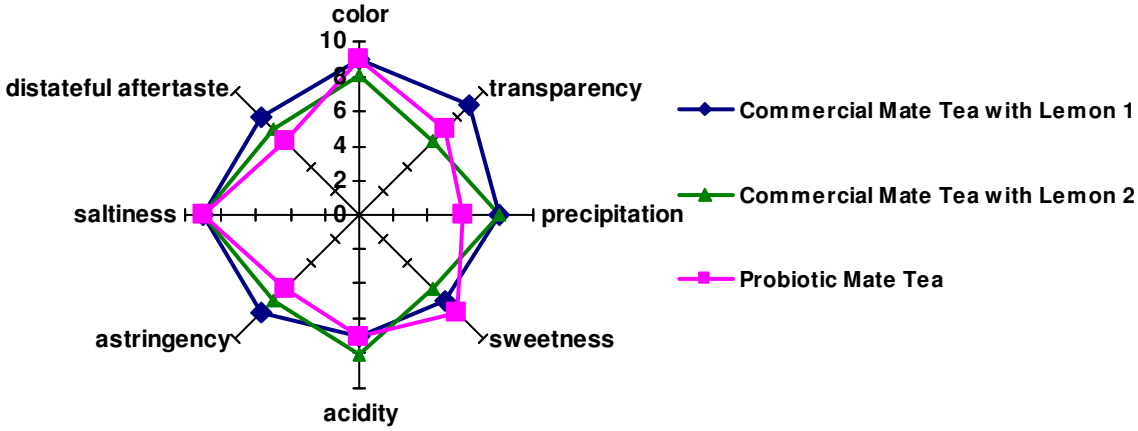


Figure 4.5: Sensorial profile after final fermentation

4.6 Characteristics of the final product

Considering the results of the kinetics of this fermentation, the probiotic beverage fermentation was stopped at 10 hours, with the parameters defined below:

Mate Tea Extract: 15 g ± 1 g of Toasted Mate Leaves in 300 mL of boiling water

Concentration of Commercial honey: 14%

Time of fermentation: 10 hours

Temperature of incubation: 35°C

Condition: Microaerophylic

Final pH: 4.2

Final Acidity: 0.6 mL NaOH 0.1 N

Total Bacterial content :10^{8} CFU/mL

Total Biomass : 2 mg/mL

Total Sugars: 81.91 g/L

Total Organic Acids: 2.14 g/L

Caffeine content: 6.77 mg/ 100 mL

Antioxidant activity: 56% of inhibition of DPPH

4.7 Shelf-life evaluation

4.7.1 Bacterial content and Biomass during shelf-life

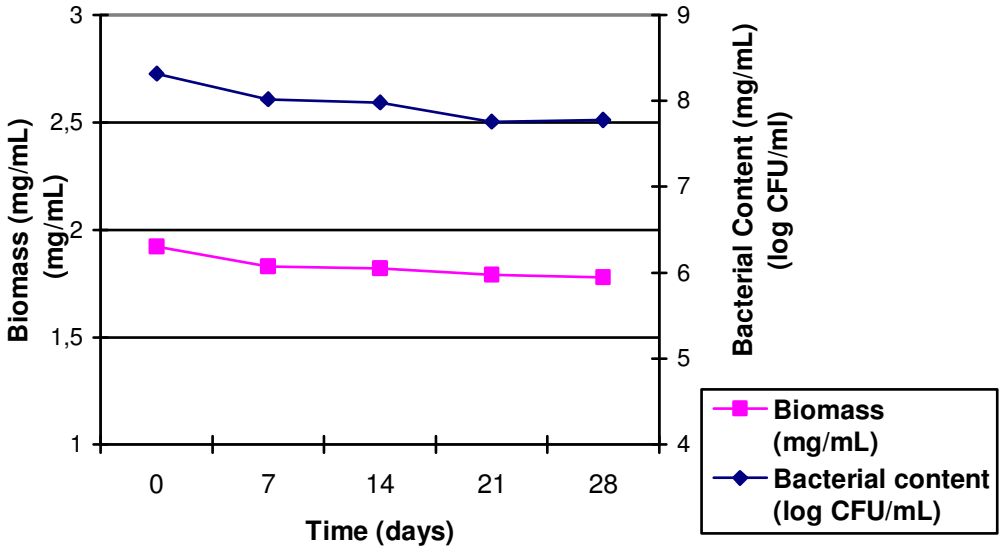


Figure 4.7.1- Bacterial content and Biomass during shelf-life

As showed by these graphics, the stability of L. acidophilus is acceptable, maintaining the minimum until the end of shelf-life.

4.7.2 pH and Acidity during shelf-life

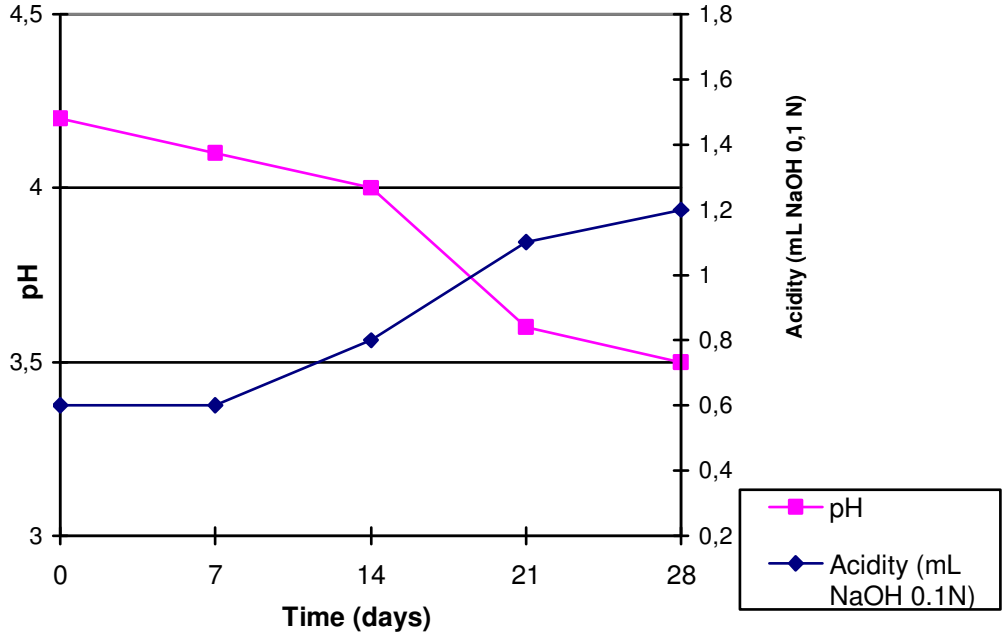


Figure 4.7.2 pH and Acidity profile during shelf-life (temperature 4°C ± 1.0°C)

As shown in the graphic above, during the storage period, the profile of pH and acidity had low variation. The pH remained almost the same till the 14th day, just having a dropping down to 3.5 at the end of shelf-life. The same happened to acidity profile: it had an increasing of level at 14th day, and at the final, just 1.2 mL NaOH 0.1 mL, that is acceptable.

The low variation of pH and acidity are probably due to the kind of strain used. When it is used as inoculum, strains of *L. delbrueckii ssp., L. bulcaricus* and *S. salivarius ssp. thermophilus,* it could be found "sineresis" and D(-) lactic acid accumulation in the final product, that affects pH and acidity. Strains of *L. casei, L. acidophilus* and *Bifidobacterium sp.* give lower post-acidification during storage period (SPREER, 1991).

4.7.3 Sugars content during shelf-life

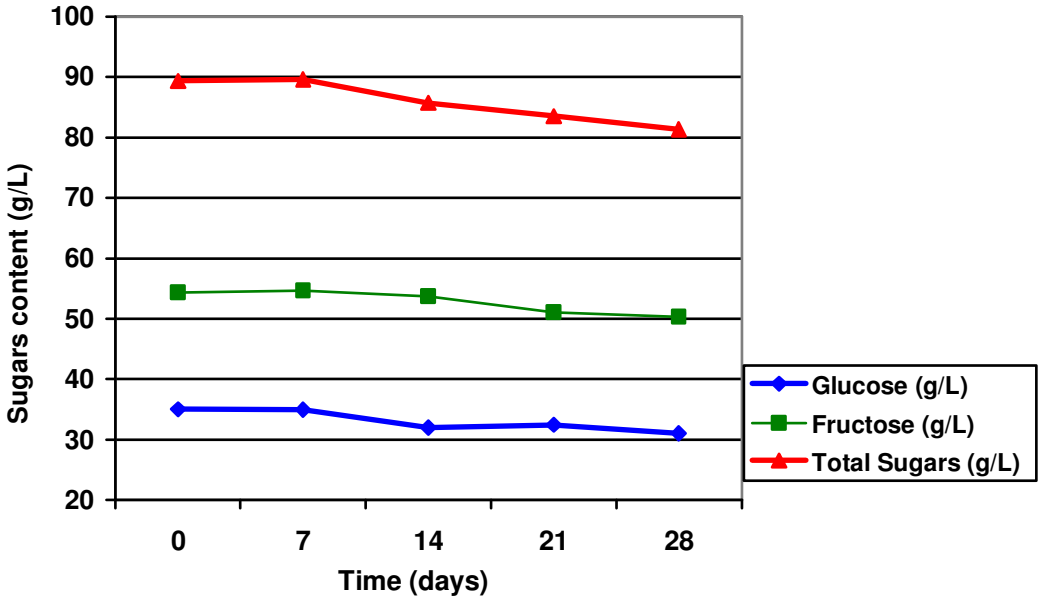


Figure 4.7.3 Sugar content during shelf-life (temperature 4°C ± 1,0°C)

The figure shown above demonstrates that no significant variation of sugars content happened. It's very good, as the main goal of this practical work is to develop an acceptable probiotic beverage, and, in this case, a light sweet taste in the final product is preferable.

4.7.4 Organic acids content during shelf-life

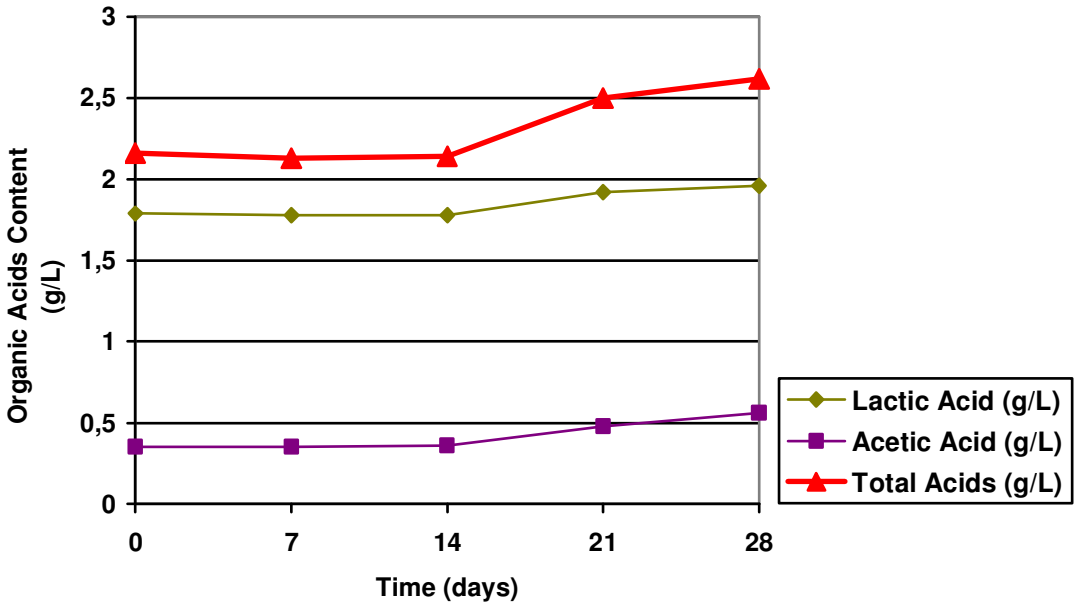


Figure 4.7.4: Organic Acids Content during shelf-life (temperature 4°C ± 1.0°C)

4.7.5 Caffeine content during shelf-life

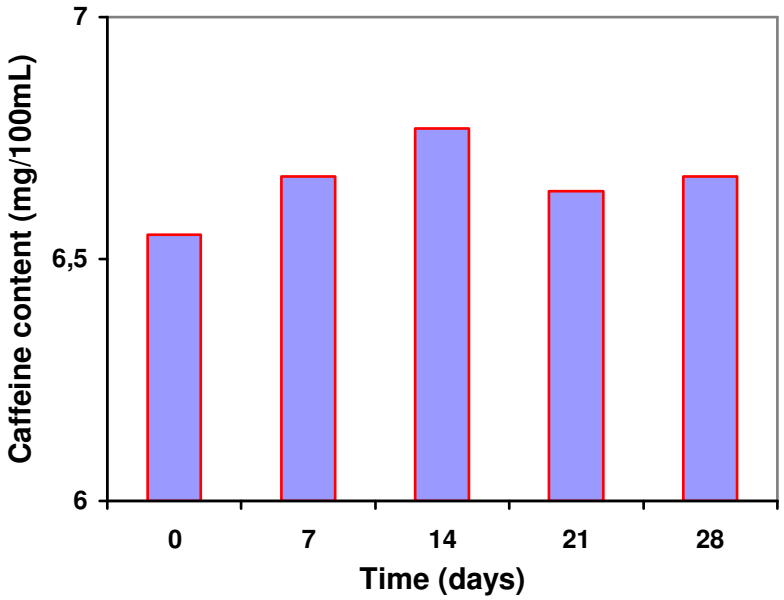


Figure 4.7.5: Caffeine Content during shelf-life (temperature 4°C ± 1,0°C)

4.7.6 Antioxidant activity during shelf-life

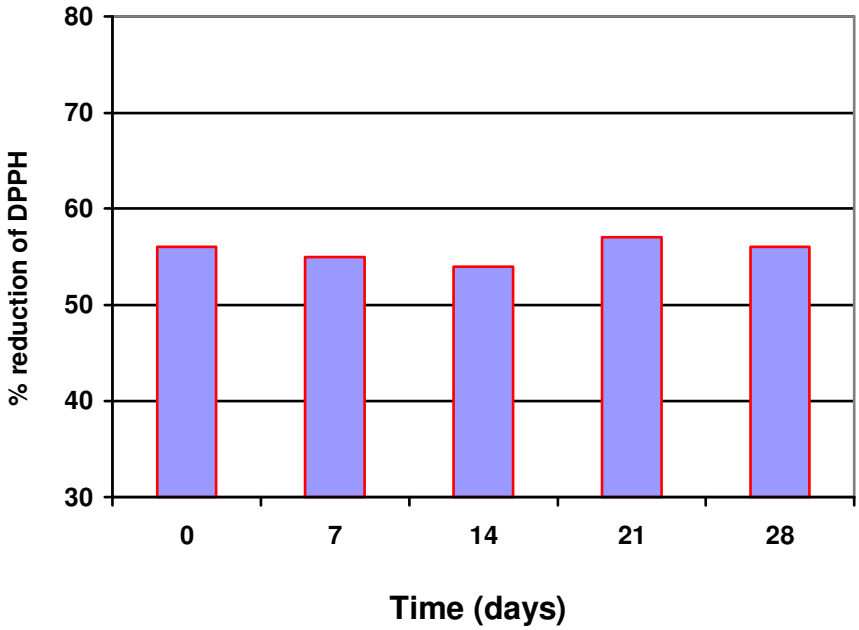


Figure 4.7.6: % reduction (% R) of DPPH during shelf-life **(**temperature 4°C ± 1.0°C)

4.7.7 Sensorial profile during shelf-life

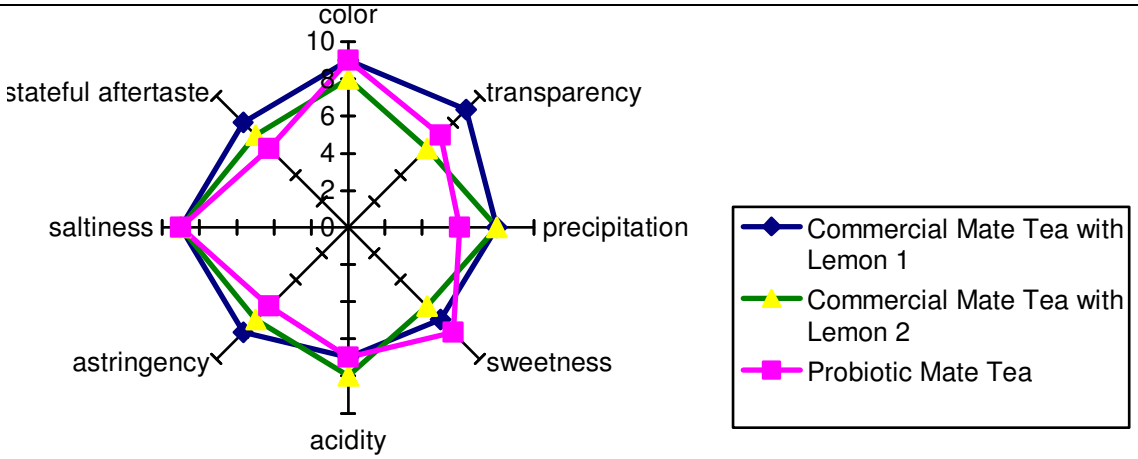


Figure 4.7.7: Sensorial profile after 7 days of shelf-life **(**temperature 4°C ± 1,0°C)

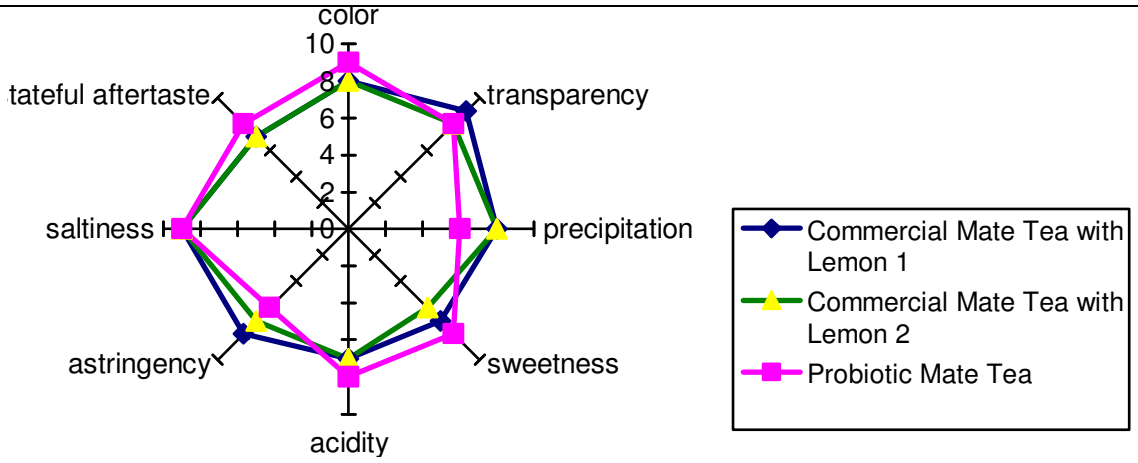


Figure 4.7.8: Sensorial profile after 14 days of shelf-life (temperature 4°C ± 1,0°C)

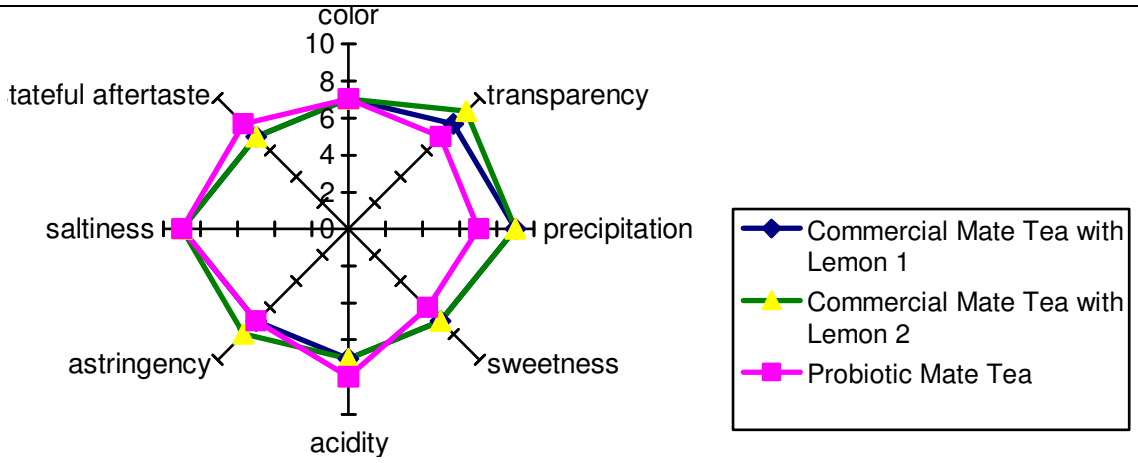


Figure 4.7.9: Sensorial profile after 21 days of shelf-life (temperature 4°C ± 1,0°C)

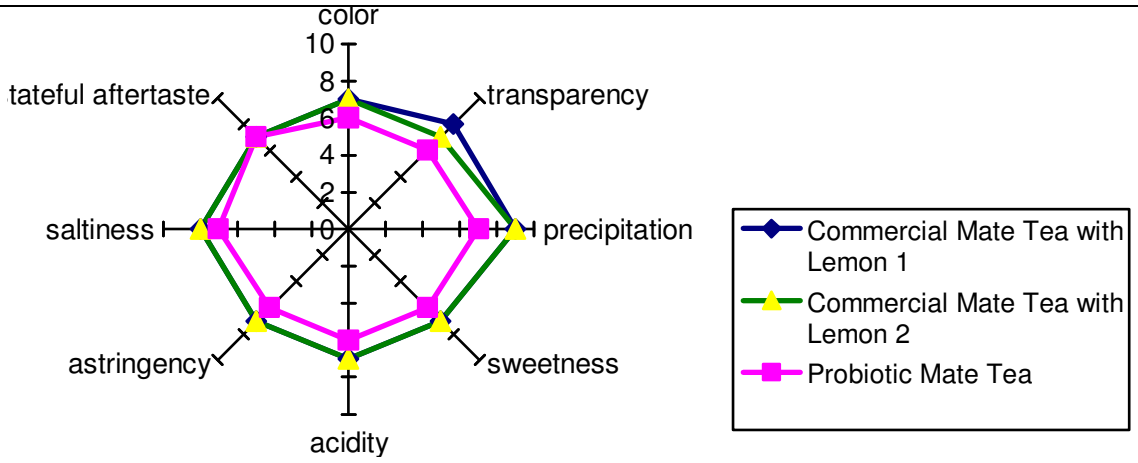


Figure 4.7.10: Sensorial profile after 28 days of shelf-life (temperature 4°C ± 1,0°C)

4.8 Comparison of Antioxidant activity of Ascorbic Acid and the beverage

Oxidation is a very complex process that involves several steps, and all methods used to measure antioxidant activity present specific limitations. The method used in this study compares the in vitro antioxidant activity of mate infusions with a synthetic antioxidant and proves the efficacy mate tea infusion as antioxidants.

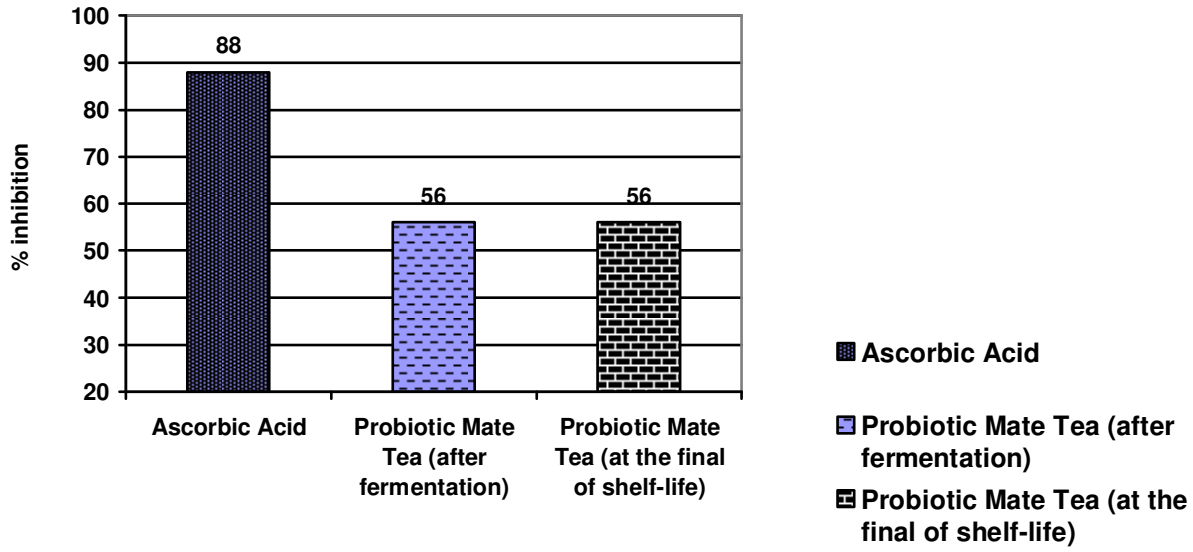


Figure 4.8.1: Antioxidant activity expressed in % of reduction of free radical of DPPH of Ascorbic Acid and the Probiotic Mate Tea Beverage developed.

Although, it results that the antioxidant activity of this Probiotic Mate Tea is lower than Vitamin C, comparing these results with Green Tea and Wines, as showed by BIXBY (2005), Mate Tea has important antioxidant content, as seen below:

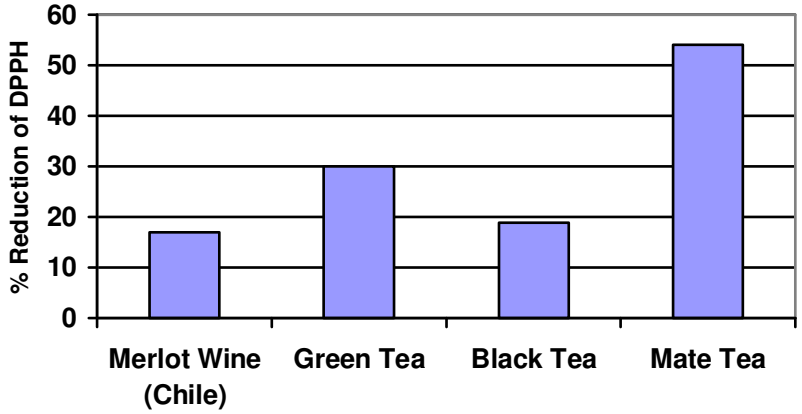


Figure 4.8.2: Comparison of % reduction of DPPH of a red wine and teas (Source: Bixby, 2005)

Also, from a work of LUNCEFORD (2005), it's demonstrated that Mate Tea has higher antioxidant content than green tea by using the AGE fluorescence spectra. SCHINELLA (2000) found radical scavenging capacity of *Ilex paraguariensis* extract was dose-dependent and similar than that of BHT (a food antioxidant) used as referent compound.

5. Conclusions

This study shows the possibility of development and production of a probiotic soft drink with an important content of bioactive compounds, and, also, with positive sensorial characteristics.

The consumption of probiotics in order to prevent intestinal illnesses is known for a long time. However, during acute crises of diarrhea, it is not allowed the lactose intake, and at the same time it is necessary to recover the intestinal microflora. But, unfortunately, nowadays, in the food market, there are only probiotics foods having the lactose as the main ingredient.

One of the advantages of this probiotic beverage is that there's no lactose in it, allowing its consume during diarrhea periods and recovering the intestinal flora.

At the same time, the beverage developed has significant content of antioxidant activity, important to prevent inflammatory points in the intestinal tissue, responsible to the cancer development.

So, the possibility that the beverage (or soft drink), developed in this work, is huge to prevent intestine inflammation and cancer, since, it has, simultaneously, probiotic

characteristics and antioxidant activity, a synergic effect, important to prevent these kind of disease.

Further “in vivo” studies are necessary to evaluate and demonstrate the possibility of use the beverage developed in intestinal diseases, as a prevent therapy.

6. References

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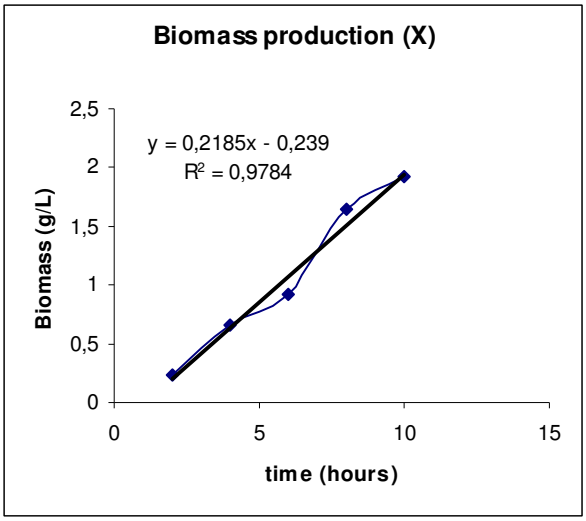
8. Appendices

8.1 Curve of Biomass Production:



9.3 Calibration Curve of Vitamin C