Universidade Estadual Paulista
“Júlio de Mesquita Filho”

Faculdade de Ciências Farmacêuticas

FERNANDA CAMPOS FREIRE

Avaliação em simulador do ecossistema microbiano humano de uma bebida fermentada à base de leite de cabra e subproduto da uva

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Avaliação em simulador do ecossistema microbiano humano de uma bebida fermentada à base de leite de cabra e subproduto da uva

Dissertação apresentada ao Programa de Pós-graduação em Alimentos e Nutrição para obtenção do título de Mestre em Alimentos e Nutrição.

Área de Concentração: Ciências Nutricionais

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RESUMO

Objetivo: Identificar os principais compostos bioativos presentes em uma bebida fermentada probiótica sabor uva, à base de leite de cabra, adicionada ou não de extrato de bagaço de uva e avaliar seu efeito funcional sobre a microbiota intestinal em Simulador do Ecossistema Microbiano Humano (SEMH). **Métodos:** Foram realizadas análises de caracterização química (composição centesimal e fibra alimentar, perfil de ácidos graxos, teor de compostos fenólicos totais e atividade antioxidante) e microbiológicas (viabilidade de *L. rhamnosus* e *S. thermophilus*) das bebidas. O efeito funcional das bebidas foi avaliado através da composição da microbiota intestinal (enumeração de *Lactobacillus* spp., *Bifidobacterium* spp., Anaerobios totais, *Clostridium* spp. e Coliformes totais), produção de ácidos graxos de cadeia curta e íons amônio, bem como sob a capacidade antioxidante. **Resultados:** Os resultados obtidos neste estudo demonstraram que as bebidas formuladas, enriquecidas ou não com extrato de bagaço de uva apresentaram alto ($p \leq 0.05$) teor de fibras dietéticas, ácido oleico, compostos fenólicos e atividade antioxidante. Ainda, mantiveram ($p \leq 0.05$) a viabilidade do *L. rhamnosus* e *S. thermophilus* durante a passagem pelo trato gastrointestinal e, exerceram efeitos positivos sobre a microbiota intestinal, uma vez que alteraram ($p \leq 0.05$) a composição da comunidade microbiana, aumentaram ($p \leq 0.05$) a produção de ácidos graxos de cadeia curta e capacidade antioxidante e diminuíram ($p \leq 0.05$) a concentração de íons amônio. **Conclusão:** Ambas as formulações, adicionada ou não de extrato, exerceram um efeito positivo sobre a microbiota intestinal, uma vez que proporcionaram modificações metabólicas positivas nas diferentes regiões que simulavam os cólons.

Palavras-chave: Alimentos funcionais. Probióticos. Prebióticos. Bagaço de uva. Compostos fenólicos. SEMH.

ABSTRACT

Objective: The objective of this study was to identify the main bioactive compounds present in a grape-flavored fermented probiotic drink made of goat milk, with or without added grape pomace extract and evaluate its effects on gut microbiota in the Simulator of Human Intestinal Microbial Ecosystem (SHIME®). **Methods:** Chemical (centesimal composition and dietary fibre, fatty acid profile, total content of phenolic compounds and antioxidant activity) and microbiological analyses (viability of *L. rhamnosus* and *S. thermophilus*) of the drinks were carried out. The functional effect of the drinks was evaluated through the composition of the intestinal microbiota (enumeration of *Lactobacillus* spp., *Bifidobacterium* spp., *Clostridium* spp., anaerobios total and coliforms total), production of short-chain fatty acids and ammonium, as well as under the antioxidant capacity. **Results:** The results obtained in this study have shown that the drinks, enriched or not with grape pomace extract, showed high ($p \leq 0.05$) content of dietary fibre, oleic acid, phenolic compounds and antioxidant activity. Still, they kept ($p \leq 0.05$) the viability of *L. rhamnosus* and *S. thermophilus* during the passage through the gastrointestinal tract and exerted a positive effects on the intestinal microbiota, since changed ($p \leq 0.05$) the composition of the microbial community, increased ($p \leq 0.05$) the production of short chain fatty acids and antioxidant capacity and decreased ($p \leq 0.05$) the concentration of ammonium. **Conclusion:** Both formulations added or not of grape pomace extract, exerted a positive effect on the intestinal microbiota, since it provided positive metabolic changes in different regions which simulated the human colon.

Key words: Functional foods. Probiotic. Prebiotic. Grape pomace. Phenolic compounds. SHIME®.

LISTA DE ABREVIATURAS

AGGC: ácidos graxos de cadeia curta

SEMH: Simulador do Ecossistema Microbiano Humano

SCFA: short-chain fatty acids

SHIME®: Simulator of Human Intestinal Microbial Ecosystem

ABTS: 2, 2 Azino Bis (3-ethylbenzo thiazoline 6 sulfonic acid) diammoninum salt

ABTS^{+}: ABTS radical cation

AC: ascending colon

TC: transverse colon

DC: descending colon

BCFA: branched-chain fatty acids

PCR: polymerase chain reaction

DGGE: denaturing gradient gel electrophoresis

NH_4^+: ammonium

PCA: principal component analysis

GAE: gallic acid equivalent

TE: trolox equivalent

Rr: richness

PL: Pareto-Lorenz

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1. INTRODUÇÃO

1.1. Subproduto da indústria vinícola – bagaço de uva

A viticultura é considerada uma atividade economicamente importante no mundo globalizado, uma vez que a uva é uma das frutas mais consumidas, tanto na forma *in natura* quanto na forma de vinhos, sucos e derivados (1). No âmbito do mercado mundial são produzidas aproximadamente 67 milhões de toneladas de uvas por ano, sendo cerca de 80% desta produção destinadas a indústria vinícola (2). Os maiores produtores mundiais de uvas são a Itália, França, Estados Unidos, Espanha e China. O Brasil produz cerca de 1,3 mil toneladas de uvas/ano é o considerado 14º maior produtor no ranking mundial (3).

Durante o processamento da uva, seja ele industrial ou artesanal, é gerada uma quantidade expressiva de resíduos. Estima-se que a indústria vinícola produz cerca de 5 a 9 milhões de toneladas de bagaço por ano após o processo de fermentação das uvas (4). O bagaço, composto basicamente por sementes e cascas, é considerado um importante subproduto, uma vez que é rico em fibras e apresenta alto teor de compostos fenólicos (5).

Atualmente, grande parte desse resíduo é descartado no meio ambiente ou tratado como um produto de baixo valor econômico, sendo utilizado como ração para animais ou adubo (6). No entanto, o bagaço é considerado um potencial poluente ambiental, uma vez que possui baixo pH e elevados teores de compostos fenólicos com ação antibacteriana e fitotóxica (7).

Dessa forma, alternativas que viabilizam sua inserção na alimentação humana estão sendo investigadas, uma vez que efeitos positivos sobre a saúde poderiam advir do seu consumo regular (8). Os efeitos benéficos das uvas são atribuídos principalmente aos compostos fenólicos presentes no bagaço (antocianinas, flavonóides, ácidos fenólicos e resveratrol), os quais apresentam atividades antioxidantes, anti-inflamatórias e cardioprotetoras (9,10). Estudos observaram que a administração destes compostos está relacionada com o controle/prevenção de fatores de riscos relacionados à síndrome metabólica e a diversas doenças crônicas, tais como câncer e doenças cardiovasculares (11,12).

Evidências recentes sugerem que a microbiota intestinal desempenha um papel fundamental nesses processos (13,14). Quando presentes no cólon, estes compostos podem ser metabolizados pela microbiota residente originando metabólitos biologicamente ativos (15). Esses metabólitos podem influenciar a composição e a atividade das populações bacterianas intestinais, estimulando ou inibindo grupos específicos e, desta forma, exercendo um efeito de modulação sobre microbiota intestinal (16).

1.2. Microbiota intestinal

A microbiota intestinal é composta por uma diversidade de espécies de microrganismos que variam ao longo do trato gastrointestinal e entre os indivíduos (17). Estima-se que o trato gastrointestinal seja habitado por 10^{11} células por mL de conteúdo luminal, compreendendo cerca de 400 a 500 espécies bacterianas (18). A complexa relação entre o indivíduo e as bactérias do cólon inicia-se logo após o nascimento e se diversifica em função da idade, alimentação e estilo de vida (19). A microbiota é responsável por proteger a mucosa intestinal contra microrganismos patógenos (20), sintetizar as vitaminas K, B1, B2 e B12 (21), degradar os componentes não digeríveis da dieta e produzir metabólitos, tais como os ácidos graxos de cadeia curta (AGCC) (22). Os AGCC são as principais fontes de energia para os colonócitos, em particular o ácido butírico, o qual estimula a proliferação celular do epitélio, o fluxo sanguíneo visceral e intensifica a absorção de sódio e água (23).

Evidências sugerem que a microbiota intestinal desempenha um papel fundamental no estado de saúde e de doença dos indivíduos, uma vez que exerce efeitos importantes sobre o sistema imunológico (24,25). Para assegurar benefícios à saúde do hospedeiro, a microbiota deve permanecer em homeostase, ou seja, em equilíbrio entre as bactérias comensais e patogênicas (26). O desequilíbrio da microbiota está frequentemente associado com a patogênese de doenças agudas e crônicas, tais como a diarreia (20), doenças inflamatórias do intestino (27), obesidade (28), diabetes (29), câncer de cólon (30) e, mais recentemente, em doenças neurológicas como o autismo (31).

O metabolismo e a composição da microbiota são influenciados pelo uso de antibióticos, fatores genéticos e imunológicos, bem como pela dieta (18,32). A ingestão de microrganismos probióticos, ingredientes prebióticos ou combinações simbióticas está fortemente relacionada com a modulação da microbiota intestinal (18,33).

Probióticos são microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro (34). Os probióticos podem estimular o sistema imune e promover resistência gastrintestinal à colonização por patógenos, através da produção de compostos antimicrobianos (35).

Prebióticos, por sua vez, são ingredientes alimentares não digeríveis que possuem a propriedade de serem fermentados de maneira seletiva no cólon, estimulando principalmente o crescimento de bactérias benéficas e alterando a microbiota intestinal a favor de uma composição mais saudável (33).

Ainda, estudos sugerem que alimentos ricos em compostos fenólicos podem ter um impacto significativo sobre a microbiota intestinal (36), uma vez que grande parte dos polifenóis da dieta não são absorvidos no intestino delgado (16). As bactérias presentes no cólon atuam na bioconversão destes compostos, dando origem a metabólitos que contribuem para a modulação da microbiota residente, através da promoção de fatores de crescimento, proliferação e de sobrevivência (37).

Pozuelo et al. (2012) observaram que o consumo de compostos fenólicos e fibras presentes no bagaço de uva, variedade *Cencibel*, estimulou o crescimento de bactérias dos gêneros *Lactobacillus* spp. e *Bifidobacterium* spp. no ceco de ratos (38). Já Touriño et al. (2011) observaram que os metabólitos produzidos após a ingestão de fibras dietéticas antioxidantes presentes nas uvas permaneceram em contato com a mucosa intestinal por mais de 24 horas, podendo estar associados a efeitos positivos a saúde (39).

Saura-Calixto et al. (2010) observaram em um modelo *in vitro* a produção de metabólitos no cólon após a fermentação das protocianidinas, um polifenol presente nas fibras das uvas. Posteriormente, os mesmos metabólitos foram observados no plasma de indivíduos saudáveis após a ingestão deste polifenol. Os autores sugerem que o processo de fermentação das protocianidinas libera metabólitos absorvíveis pela mucosa intestinal com potenciais efeitos benéficos a saúde do indivíduo (40).

Estudos sobre a microbiota intestinal podem ser realizados utilizando modelos *in vivo* e em *in vitro.* Em modelos *in vivo,* as abordagens são mais representativas, uma vez que os parâmetros e as interações fisiológicas com o organismo hospedeiro são levados em conta. Contudo, a composição da população microbiana nas diferentes regiões do cólon não pode ser observada, visto que somente a microbiota fecal é analisada (41,42).

Já os modelos *in vitro* são capazes de fornecer informações sobre as etapas do processo de fermentação nas diferentes regiões do cólon humano. São úteis para investigar a microbiota intestinal, bem como seus metabólitos, além de proporcionar resultados com elevada reprodutibilidade (41,43,44). Dessa forma, o Simulador do Ecossistema Microbiano Humano (SEMH) (figura 1) consiste em um modelo dinâmico do trato gastrointestinal humano, composto por cinco reatores conectados que representam o estômago, o duodeno e os cólons ascendente, transverso e descendente, com seus respectivos valores de pH, tempo de residência, temperatura e capacidade volumétrica (45,46). Atualmente este modelo tem sido bastante utilizado em estudos de nutrição, uma vez que permite analisar a composição e a atividade da população microbiana do intestino (46–50).

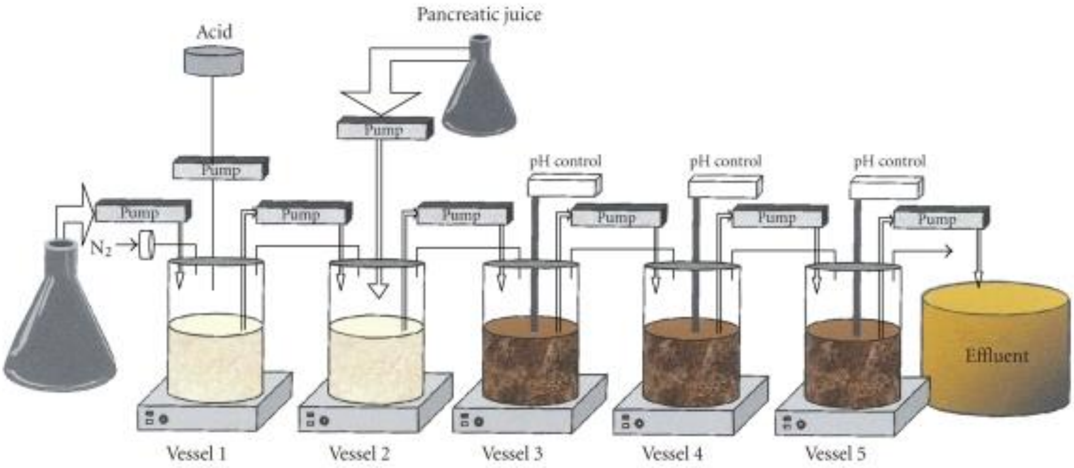

Figura 1. Simulador do Ecossistema Microbiano Humano (SEMH).

Fonte: Sivieri et al, 2014 (50).

1.3. Desenvolvimento de produtos probióticos e prebióticos

A procura por alimentos nutritivos e funcionais tem aumentado nos últimos anos, impulsionando dessa forma, o surgimento de novos produtos no mercado que atendam a essa nova exigência da população. Atualmente, os produtos lácteos probióticos constituem os principais alimentos funcionais comercialmente disponíveis, particularmente os iogurtes, as bebidas lácteas e os leites fermentados (51).

A incorporação de microrganismos probióticos em alimentos é uma alternativa promissora para a modulação das funções fisiológicas dos indivíduos, uma vez que estes promovem resistência gastrintestinal à colonização por patógenos (20), estimulam o sistema imune (26), sintetizam vitaminas (21), aumentam a absorção de minerais e previnem o risco de desenvolvimento de câncer de cólon (30).

No entanto, para exercerem tais efeitos, os probióticos devem ser resistentes às condições gastrointestinais e manter sua viabilidade durante todo o período de armazenamento do produto. Também devem apresentar boas propriedades tecnológicas para sua aplicação em alimentos, não devendo interferir nas características sensoriais desses produtos (52).

As principais cepas probióticas empregadas em alimentos funcionais são as pertencentes aos gêneros *Lactobacillus* spp*.* e *Bifidobacterium* spp*.* (37). Neste contexto, alimentos que utilizaram a cepa probiótica de *Lactobacillus rhamnosus* no processo de fermentação de produtos lácteos são praticamente inexistentes no mercado. No entanto, alguns estudos sugerem que cepas dessa espécie possuem capacidade de sobreviver às condições estomacais e colonizar o trato gastrointestinal de crianças e adultos (53–55), bem como são capazes de restaurar a microbiota urogenital e reduzir o risco de infecções do trato urinário (55-57).

Além dos microrganismos probióticos, a adição de fibras e compostos bioativos presentes em subprodutos agroindustriais é considerada uma inovação em produtos deste segmento, uma vez que efeitos benéficos resultantes de seu consumo já são associados à saúde do consumidor (58,59). O bagaço da uva, o qual é rico em fibras e compostos fenólicos, apresenta atividades antioxidante, anti-inflamatória e cardioprotetora (11,60). Ainda, pode modular a microbiota intestinal, através do aumento da população de bactérias comensais e produção de AGCC, prevenir o câncer de cólon, diminuir o tempo do trânsito intestinal e aumentar da frequência de evacuação (61).

Uma boa alternativa para o desenvolvimento de alimentos funcionais é a utilização de matérias-primas pouco exploradas, como o leite de cabra, principalmente para os individuos que não podem consumir produtos lácteos a base de leite de vaca, devido alergia às proteínas do leite (58,62,63).

Neste contexto, o Brasil é considerado o maior produtor de leite de cabra do continente americano, sendo a Região Nordeste responsável pela concentração de 91,40% do rebanho caprino do país (64). Os produtos lácteos caprinos, especialmente os queijos e iogurtes, são bastantes populares na Península do Mediterrâneo, Médio Oriente, Sul da Rússia e no subcontinente Indiano (65). Entretanto, este mercado ainda é pouco explorado no Brasil, embora o leite de cabra seja reconhecido por seus benefícios nutricionais à saúde. Tais benefícios estão associados à sua elevada digestibilidade, baixo potencial alergênico, maior proporção de ácidos graxos de cadeia curta e média e perfil de oligossacarídeos semelhante ao leite humano (66–68).

Dessa forma, o desenvolvimento de uma bebida funcional, à base de leite de cabra, fermentada com cultura probiótica de *Lactobacillus rhamnosus* e acrescida de fibras e compostos fenólicos obtidos do bagaço da uva, torna-se uma alternativa bastante interessante, tanto para a indústria de alimentos, quanto para os consumidores em sua busca por alimentos mais saudáveis e nutritivos.

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CAPÍTULO 1.

Impact of multi-functional fermented goat milk drink on gut microbiota in a dynamic colon model

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1. Introduction

Gut microbiota is composed of a diversity of microorganism species that vary along the digestive tract and between individuals (Roberfroid et al., 2010). It is estimated that the intestinal tract is inhabited by 10^{11} cells per mL of luminal content, including 400 to 500 bacterial species (Scott, Gratz, Sheridan, Flint, & Duncan, 2013). The microbiota is responsible for protecting the intestinal mucous membrane against pathogenic microorganisms (Lourens-Hattingh & Viljoen, 2001), synthesizing vitamins K, B1, B2 and B12 (Saad, 2006), breaking down the non-digestible components of food and producing metabolites such as short-chain fatty acids (SCFAs) (Ríos-Covián et al., 2016).

Microbiota metabolism and composition are mainly influenced by the use of antibiotics, genetic and immunologic factors and by diet (Scott et al., 2013; Wu et al., 2012). It is known that the ingestion of probiotic microorganisms, prebiotic ingredients or symbiotic combinations is strongly associated with the regulation of gut microbiota (Gibson & Roberfroid, 1995; Scott et al., 2013). Probiotics are living microorganisms which, when administered in adequate quantities, provide the host with health benefits (Hill et al., 2014). Probiotics can stimulate the immune system and promote digestive resistance to colonization by pathogens through the production of antimicrobial compounds (Sanders, 2003). The main probiotic strains used in functional foods belong to genus *Lactobacillus* spp. and *Bifidobacterium* spp. (Hervert-Hernández et al., 2009). However, studies on probiotic *Lactobacillus rhamnosus* strains in the process of fermentation of lactic products are scarce (Jia, Chen, Chen, & Ding, 2016). Some studies suggest that *L. rhamnosus* strains have the ability to survive stomach conditions and colonize the digestive tract of children and adults (Goldin et al., 1992; Millar, Bacon, Smith, Walker, & Hall, 1993; Saxelin, Ahokas, & Salminen, 1993). They have also been found to be able to restore the urinary and genital tract's microbiota and to reduce the risk of urinary tract infections (Reid & Bruce, 2003; Reid, Beuerman, Heinemann, & Bruce, 2001).

Prebiotics are non-digestible ingredients that are able to be fermented in the colon, stimulating the growth of beneficial bacteria and altering gut microbiota in favor of a healthier composition (Gibson & Roberfroid, 1995). Furthermore, studies indicate that foods rich in phenolic compounds also could have a significant impact on gut microbiota (Kemperman et al., 2013). When present in the colon, these compounds can be metabolized by resident microbiota, leading to the production of biologically active metabolites (Duynhoven, Vaughan, Jacobs, Kemperman, & Van Velzen, 2011). These metabolites could influence the composition and activity of intestinal bacteria populations by stimulating or inhibiting specific groups and, as a consequence, regulating the microbiota (Kemperman, Bolca, Roger, & Vaughan, 2010).

Probiotic microorganisms are usually vehicle by dairy products, such as fermented milk and yogurts (Hekmat, Soltani, & Reid, 2009). However, the use of an alternative food matrices, such as goat milk, could be a good option for the production of functional foods, particularly, for individuals that can not ingest bovine dairy products due to the milk protein allergies (dos Santos et al., 2016; Silanikove, Leitner, Merin, & Prosser, 2010). Goat milk is more easily digested by humans than cow milk due to the dimensions of the casein micelles and fat globules (Park, 2007). It also presents an oligosaccharide profile similar to human milk, a larger proportion of short- and medium-chain fatty acids, and a low allergenic potential (Lara-Villoslada et al., 2006; Minervini, Bilancia, Siragusa, Gobbetti, & Caponio, 2009; Raynal-Ljutovac, Lagriffoul, Paccard, Guillet, & Chilliard, 2008).

One interesting option in the development of functional foods is the use of fibers and bioactive compounds from agricultural industry waste.Conveniently, the wine industry processes approximately 50 million tons of grapes every year, and, in doing so, produces 5 to 9 million tons of grape pomace after fermentation (Djilas, Čanadanović-Brunet & Ćetković, 2009). Grape pomace is largely composed of seeds and skins. It is an important byproduct, rich in fiber and with a relevant concentration of phenolic compounds (Cataneo, Caliari, Gonzaga, Kuskoski, & Fett, 2008). The fibers present in grape pomace can have a prebiotic effect on the intestinal mucous membrane, as has been demonstrated by Pozuelo et al. (2012).

Currently, much of the agro-industrial waste is disposed of in the natural environment or is treated as a product of low economic value, being often used as animal feed or fertilizer (de Campos, Leimann, Pedrosa, & Ferreira, 2008). However, many benefits to human health can be attributed to the phenolic compounds present in grape pomace, such as anthocyanins, flavonoids, phenolic acids, and resveratrol, due to antioxidant, anti-inflammatory, and cardio protective effects (Cataneo et al., 2008; Manach, Mazur, & Scalbert, 2005; Puupponen-Pimiä et al., 2001). As a result, options for the use of grape pomace and its bioactive compounds in human food products are being investigated. In this context, foods with various components with bioactive properties can be considered "multifunctional" foods and can promote human health (dos Santos et al., 2016).

The objective of this study was to identify the main bioactive compounds present in a grape-flavored probiotic fermented beverage made of goat milk, with and without grape pomace extract and to evaluate their functional effects on the gut microbiota in a Simulator of Human Intestinal Microbial Ecosystem (SHIME®). It was evaluated the survival of *Lactobacillus rhamnosus* after the passage through the simulated conditions of the stomach and the duodenum. SHIME® is an *in vitro* model that dynamically simulates the human digestive tract which has frequently been used in nutrition studies due to its usefulness in the analysis of intestinal microbial population activity and composition (Barroso et al., 2014; Bianchi et al., 2014; Van de Wiele, Boon, Possemiers, Jacobs, & Verstraete, 2004; Molly, Woestyne, Smet, & Verstraete, 1994; Possemiers, Marzorati, Verstraete, & Van de Wiele, 2010; Sivieri et al., 2014).

2. Material and Methods

2.1. Preparation of the grape pomace extract

Merlot grape pomace was obtained from the Casa Valduga vineyards in the state of Rio Grande do Sul, Brazil. The extraction was performed in a jacketed tank at 50ºC, under mechanical agitation at 48 rpm for 180 minutes. A hydroalcoholic extract solution containing 30% ethanol (v/v) was used at a 7:1 ratio (solvent: substrate). The solid fraction was separated using a basket centrifuge with a 150 µm nylon mesh as a filter. The obtained extract was bottled in previously sanitized plastic canisters and stored at -16±1 ºC.

The extract was then dried in a spray-dryer (Model B190, Buchi®) using maltodextrin at a 10% concentration (MDE 5, Corn Products 1805) as an encapsulating agent. The extract was maintained under constant agitation at room temperature and was sent to the spray dryer's main chamber with a peristaltic pump at a rate of 0.9 L/h. The entry and exit temperatures used in the spray dryer were 180 °C and 70 °C, respectively. It was employed a flow rate of 2.4 bar. The dehydrated extract was kept at -16±1 ºC.

2.2. Fermented beverages preparation

The fermented beverages were produced at the Brazilian Agricultural Research Corporation (EMBRAPA) in Rio de Janeiro, Brazil. Two formulations were produced: fermented milk + grape juice (formulation 1) and fermented milk + grape pomace extract + grape juice (formulation 2). The ingredients used in the beverages, and respective proportions, are presented in Table 1.

Table 1. Ingredients used in the formulation of the fermented beverages studied.

Ingredient (%)	Formulation 1	Formulation 2
Goat milk (Rancho Grande, Brazil)	73.00	67.00
Sugar (União, Brazil)	7.00	7.00
Grape pomace extract (Embrapa Grape and Wine, Brazil)	0.00	6.00
Grape juice (Embrapa Grape and Wine, Brazil)	20.00	20.00

With the exception of the grape juice, the ingredients were blended until they became completely smooth. The mixture was pasteurized at 95±1 ºC for 10 minutes in a water-bath (Sppencer®, Dubnoff). The temperature of the mixture was then reduced to 43±1 ºC for the inclusion of the probiotic culture

(0.3% of *L. rhamnosus*, Sacco®, Brazil) and the starter culture (0.04% of *S. thermophilus*, DuPont®, USA). The mixture was fermented in a BOD incubation chamber (MA1415/780, Marconi®) at 43±1 ºC until a pH of approximately 5.0 was reached. The beverages were cooled to 5±1 °C for 24 hours. Then, the grape juice was added (EMBRAPA Grape and Wine, Brazil) in both formulations. The final products were packed in high-density polyethylene bottles, and stored at 5±1 °C for 28 days.

2.3. Physical and chemical characteristics of the fermented beverages

2.3.1. Determining pH and titratable acidity

The pH and titratable acidity of the fermented beverages were evaluated during the 28 days of refrigerated storage at 5±1 °C using the methods proposed by the Association of Analytical Communities (AOAC, 2012). The analyses were run in triplicate.

2.3.2. Centesimal composition and total dietary fiber

Moisture, ash, protein, fat, carbohydrates content, and total energy values, were determined using the methods proposed by the AOAC (2005). The total dietary fiber content and its fractions (soluble, insoluble, and lignin) were determined according to the methodology proposed by Mañas, Bravo and Saura-Calixto (1994). The analyses were run in triplicate.

2.3.3. Fatty acid profile analysis

Milk fat was extracted according to the method proposed by AOAC (2012). The methyl esters were prepared using the method by Christie (1982). The fatty acid profiles were analyzed in an Agilent 1890 gas chromatographer equipped with a cast silicon capillary column (CP-Sil 88, 100 m x 0.25 mm x 0.20 μm) and a flame ionization detector using the methodology proposed by Cruz-Hernandez et al. (2007). The analyses were run in triplicate.

2.3.4. Extraction of phenolic compounds

Polyphenols were extracted from the fermented beverages using acidified methanol (concentrated HCl, 0.1 g 100 mL^{-1}), as proposed by Karaaslan, Ozden, Vardin, and Turkoglu (2011), with slight modifications. Six mL of acidified methanol was added to each 1.5 g of the fermented bevarages, and the samples were kept overnight at 4±1 ºC. The samples were later centrifuged at 1300 g at 4±1 ºC for 15 minutes. Next, 6 mL of acidified methanol was added to the precipitate, and the samples were centrifuged under the same conditions. The procedure was repeated two more times until a final volume of 24 mL was obtained. The obtained extracts were kept under -16±1 ºC and were used to determine the total phenolic compounds in the fermented beverages.

2.3.5. Determining total phenolic compounds and antioxidant activity

Total phenolic compounds were determined using the Folin-Ciocalteu reagent (Sigma®, USA), according to Georgé, Brat, Alter, & Amiot (2005). The results were expressed as mg gallic acid equivalent (mg GAE 100 g^{-1}). The antioxidant activity was determined using the $ABTS^+$ method proposed by Serpen et al. (2007), and the results were expressed as mmol Trolox equivalent (mmol TE g^{-1}).

2.4. The dynamic colon fermentation model - Simulator of Human Intestinal Microbial Ecosystem (SHIME®)

SHIME® is a dynamic model of the human intestinal tract. It's composed of five connected reactors that represent the stomach (S), the duodenum (D), the ascending colon (AC), the transverse colon (TC), and the descending colon (DC), with their respectives pH values, residence times, temperatures, and volume capabilities (Molly et al., 1994; Possemiers, Verthé, Uyttendaele, & Verstraete, 2004).

At the beginning of the experiments, the AC, TC and DC simulation reactors were inoculated with non-gas producing (methane < 3 ppm) stool samples from three adult volunteers who had not taken antibiotics in the two years prior. The stool inoculum was prepared using the method by Possemiers et al. (2010). The inoculum was stabilized for two weeks in a carbohydrate-based medium, as previously described by Possemiers et al. (2004), so that the microbial community could adapt to the specific conditions within each reactor simulating the different regions of the colon.

The experimental set up of the SHIME® assay included a two-week stabilization period, a two-week control period, and a two-week treatment period, as previously described by Chaikham & Apichartsrangkoon (2014). During the treatment periods, 200 mL of the formulations 1 or 2 were added to the system once a day (containing 7 log CFU mL^{-1} of *L. rhamnosus and S. thermophilus*) and 200 mL of the carbohydrate-based medium was added twice a day. During the control periods, only the carbohydrate-based medium was added to the system (200 mL three times a day). Fig. 1 shows the experimental protocol used in the SHIME®.

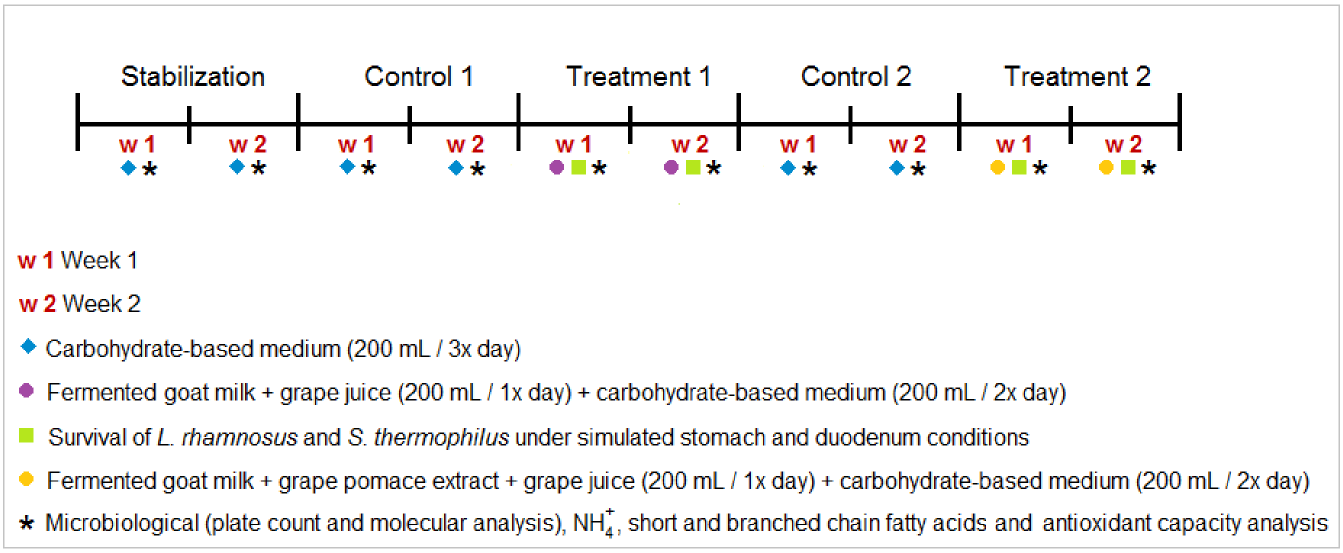

Fig. 1. Experimental protocol used in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®).

All reactors were kept at 37 °C under magnetic stirring. The system was kept under anaerobiose through daily 30-minute injections of nitrogen. The pH of the reactors that simulate the stomach and AC, TC, and DC was controlled by automatically adding sodium hydroxide 1N or hydrochloridric acid 1N (Molly et al., 1994; Possemiers et al., 2004). In addition, 60 mL of artificial pancreatic juice (12.5 g/L of $NaHCO_3$, 6 g/L of Oxgall and 1.9 g/L of pancreatin) was added to the reactor simulating the duodenum at a rate of 4 mL per minute for 15 minutes (Molly et al., 1994; Possemiers et al., 2004).

2.4.1. Survival of *L. rhamnosus* and *S. thermophilus* under the simulated conditions of the stomach and duodenum in the SHIME®

During the treatment period with the fermented beverages, samples were collected from the reactors corresponding to the stomach and duodenum in order to verify the survival of *L. rhamnosus* and *S. thermophilus*. One mL of samples from each reactor were suspended in 9 mL of sterile peptone water and serial dilutions were carried. *L. rhamnosus* was plated in MRS agar (Merck®, Germany) acidified to pH 5.4 and incubated under anaerobiose (Probac, Brazil) at 37±1 ºC for 72h (Oliveira, Sodini, Remeuf, & Corrieu, 2001). *S. thermophilus* was plated in M17 agar (Oxoid®, United Kingdom) containing lactose (5.0 g/L), and incubated under anaerobiose (Probac, Brazil) at 37±1 °C for 48h (Cardarelli, Buriti, Castro, & Saad, 2008).

2.4.2. Microbiological analysis of the SHIME® samples

Changes in the gut microbiota during the experimental protocol was determined based on the enumeration of the populations of *Lactobacillus* spp., *Bifidobacterium* spp., *Clostridium* spp., total anaerobes and total coliforms. The sampling were made after 7 days of each experimental period (control and treatment periods). One mL of samples from each reactor were suspended in 9 mL of sterile peptone water. Serial dilutions were prepared and inoculated in selective culture mediums (Table 2).

Table 2. Culture media and conditions used in the microbiological analysis in the SHIME®.

Genus	Culture Medium	Brand	Time/ Temperature	Oxygen condition	Reference
Lactobacillus spp.	Agar MRS	Himedia (India)	37ºC/ 48h	anaerobiose	Yoshioka et al. (1983)
Bifidobacterium spp.	Agar BIM-25	Difco (France)	37ºC/ 72h	anaerobiose	Munoa & Pares (1988)
Clostridium spp.	Agar RCA	Difco (France)	37ºC/ 48h	anaerobiose	Marzotto et al. (2006)
Total anaerobes	Agar Standart Methods	Acumedia (USA)	37ºC/ 48h	anaerobiose	Yoshioka et al. (1983)
Total coliforms	Petrifilm^{TM} EC plaques	3M	37ºC/ 48h	aerobiose	

The behaviour and diversity of total bacteria throughout during the experimental period was analysed by PCR-DGGE. The DNA from the simulated colon reactor samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Germany), according to the producer's protocols, with slight modifications. The DNA was quantified using a NanoVue^{TM} Plus (GE Healthcare, USA) spectrophotometer. The primers used to replicate the DNA were 968FGC (5’ -CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3’) and 1401R (5-CGG TGT GTA CAA GAC CC-3’) (Engelen et al., 1996). DNA polymerization was performed using a GoTaq$^{®}$ Green Master Mix (Promega, USA). The samples were then amplified in a thermal cycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 95 ºC/min, 35 denaturation cycles at 94 ºC/45 s, annealing at 56 ºC/45s, extension at 72 ºC/min and final extension at 72 ºC/10 min, followed by cooling at 4 ºC.

Electrophoresis was performed in an 8% polyacrylamide gel with a denaturation gradient between 45% and 65% for 16 hours at 75V in a TAE 1 X buffer at a constant temperature of 60 ºC (dos Reis, Carosia, Sakamoto, Varesche, & Silva, 2015). The gels were dyed with ethidium bromide

(Sanguinetti, Dias Neto, & Simpson, 1994), digitized (400 dpi) and later analyzed using the BioNumerics software, version 6.0 (Applied Maths, Belgium).

The ecological analysis (richness and functional organization) was performed based on the study by Marzorati, Wittebolle, Boon, Daffonchio, and Verstraete (2008). Richness (Rr) is correlated with the distribution patterns of the DGGE bands, and with the percentage of denaturing gradient gel required to represent the total diversity of the sample. This concept can be mathematically expressed using the index $Rr = (N^2 \times Dg)$, where N represents the total number of bands in the DGGE line and where Dg represents the denaturing gradient present between the first and last bands.

Pareto-Lorenz distribution curves were created in order to represent the structure and functionality of bacterial communities based on the DGGE profiles (Mertens, Boon, & Verstraete, 2005; Wittebolle, Vervaeren, Verstraete, & Boon, 2008). The respective bands are classified from high to low for each DGGE line according to their intensity. The cumulative value of the Y axis (in this case, the intensity proportion of the bands) corresponds to 20% of the cumulative proportion of the evaluated species (X axis). The more deviation is shown by the Pareto-Lorenz curve from the theoretical perfect line (that is, 45º), the less uniform the structure of the microbial community studied is. Low uniformity means that few different species are present in dominant quantities (Dejonghe, Boon, Seghers, Top, & Verstraete, 2001; Mertens et al., 2005; Wittebolle et al., 2008).

2.4.3. Ammonium (NH_4^+) and short-chain fatty acid (SCFA) analysis

Samples from the colon simulating reactors were collected in each experimental period (control 1 and 2, and treatment 1 and 2), and stored at -20 °C**.** The production of NH_4^+ was determined using anion measurer attached to a ion-selective electrode (Model No. 95-12, Orion) according to Bianchi et al. (2014). The device was calibrated with standard solutions of ammonium chloride at 0.1M in concentrations of 10, 100 and 1000 mg/L of ammonium. A total of 0.2 mL of ISA (Orion) solution was added to each 10 mL of sample material.

For the SCFA analysis, 2 mL of samples were centrifuged at 13.000 g for 5 minutes. Then, 100 µL of the supernatant was diluted in 1900 mL of ultrapure water, 1 g of sodium chloride, 100 mL of chrotonic acid, 70 mL of isobutanol, and 200 µL of sulphuric acid (2M). The SCFAs were analyzed in a Model-2010 gas chromatographer (Shimadzu, Japan) equipped with a split/splitless injector and a flame ionization detector. The SCFAs were separated using an HP-Innowax (30 m x 0.25 mm x 0.25 µm) column (Agilent Technologies, USA). The transporting gas used was hydrogen, and the flow rate was 1.45 mL/min. The temperature of both the injector and the detector was 240 ºC (Adorno, Hirasawa, & Varesche, 2014).

2.4.4. Antioxidant capacity of the SHIME® samples

The antioxidant capacity of the reactor samples simulating the AC, TC, and DC were determined throughout the experimental period according to the method proposed by Re et al. (1999). An aliquot of 30 µL of each sample was mixed with 3 mL of $ABTS^+$, and the absorbance was measured at 734 nm in a SP-220 spectrophotometer (Biospectro, Brazil).

2.5. Statistical analysis

Data had a homoscedastic normal distribution and the results obtained were expressed as mean ± standard deviation. Analysis of variance (ANOVA) and Tukey's test were performed to evaluate the survival of *L. rhamnosus* and *S. thermophilus* under simulated stomach and duodenum conditions in the SHIME®. Student's t-test ($p \leq 0.05$) was applied to comparation between control and treatment periods in each reactor that simulates the colon (AC, TC and DC).

A principal component analysis (PCA) was performed to better understand the results found in the SHIME® during the treatment periods. The variables studied were organized in columns (variables) and the experimental periods were organized into lines (cases). Before the analysis, the data was normalized and the PCA was performed with a correlation matrix and without a rotation factor. All analysis were performed in Statistica 10.0 software (StatSoft® Inc., USA).

3. Results and Discussion

3.1. Physical and chemical characteristics of the fermented beverages

3.1.1. pH and titratable acidity

The pH values of the formulated beverages did not vary during the storage period. Formulations 1 and 2 presented mean pH levels of 4.30 and 4.31, respectively, over the 28 days of storage at 5±1°C. The titratable acidity of formulation 2 (0.61±0.01 g 100 g^{-1}) was higher ($p \leq 0.05$) than that of formulation 1 (0.57±0.01 g 100 g^{-1}), a finding which was likely due to the addition of grape pomace extract, which presented a pH of 3.80. Dos Santos et al. (2016) developed a probiotic fermented goat milk product, using grape pomace extract, and observed pH and acidity levels higher (4.39 and 0.68 g 100 g^{-1}, respectively) than those described in this study. According to the Brazilian Technical Regulations for the Identity and Quality of Fermented Milks, which establishes acidity values at 0.6 to 2.0 g 100 g^{-1} for these products, all of the fermented beverages produced herein adhered to Brazilian legislation.

3.1.2. Centesimal composition and fatty acids profile

Table 3 shows the centesimal composition of the fermented beverages. Statistical differences between the two formulations were detected for all parameters, with the exception of the fat content. Dos Santos et al. (2016) and Salva et al. (2011) developed fermented beverages made of goat milk with

Lactobacillus rhamnosus, and both studies found centesimal composition values similar to those found in this study.

In the current study, the added grape pomace extract contributed to a higher dietary fiber content in formulation 2. However, both formulations adhere to current Brazilian laws and are classified as foods with high dietary fiber content in a daily portion of fermented milk (formulation 1: 5.08 g 200 g^{-1} and formulation 2: 7.84 g 200 g^{-1}). The consumption of dietary fiber is associated with several health benefits, including the regulation of gut microbiota through the increase in the population of commensal bacteria and the production of SCFAs, as well as colon cancer prevention, decreases in the duration of gut transit, and increases in the frequency of defecation (Anderson et al., 2009).

The fat in goat milk is synthesized in the alveoli of the mammary glands and involve the fatty acids present in the blood stream, which are a product of the fermentation that occurs in the rumen. They are composed of triglycerides and a large proportion of medium- and short-chain fatty acids (C4:0 – C16:0) (Jenness, 1980). The fatty acids content in goat milk are different from those found in bovine milk, showing a higher proportion of capric acid (C 10:0), myristic acid (C 14:0), palmitic acid (C 16:0), stearic acid (C 18:0), and oleic acid (C 18:1), as well as branched-chain fatty acids (Ceballos et al., 2009; Park, 2007).

Some components of the lipid fraction may contributes to the prevention of certain diseases, in particular, cardiovascular diseases (Haenlein, 2004). In the current study, formulation 2 was found to have higher concentrations than formulation 1 ($p \leq 0.05$) of oleic acid (C18:1), stearic acid (C18:0), and pentadecanoic acid (iso-C15:0) (Table 3). Several studies suggest that dietary oleic acid (C18:1), commonly known as ω-9, reduces the risk of patients developing atherosclerosis due to its ability to decrease plasmatic cholesterol (Besler & Grimble, 1995; Miles & Calder, 1998; Yaqoob, 1998), induces anti-inflammatory effects in auto-immune diseases (Kremer et al., 1990; Linos et al., 1991), decreases blood pressure (Ferrara et al., 2000), and offer protective effects against breast cancer (Lipworth, Martínez, Angell, Hsieh, & Trichopoulos, 1997; Martin-moreno et al., 1994; Simonsen et al., 1998). Oleic acid intake recommendations are based on the recommendations for intakes of total fat (around 30% of the total energy) and should be therefore in the range of 10–15% (Lopez-Huertas, 2010). All formulations estudied showed concentrations of oleic acid acoording to the intake recommendations.

Stearic fatty acids (C 18:0) have no effect on plasmatic cholesterol; however, once ingested, they are metabolized into oleic acid (Griinari et al., 2000; Grummer, 1991; Matheson et al., 1996). Pentadecanoic acid, on the other hand, is a branched-chain fatty acid (BCFAs), and recent research suggests that its consumption is associated with certain health benefits, including gut microbiota control and an increase in anti-inflammatory cytokine expression (Ran-Ressler, Bae, Lawrence, Wang, & Brenna, 2014).

Higher concentrations ($p \leq 0.05$) of capric acid (C 10:0), myristic acid (C 14:0) and palmitic acid (C 16:0) were observed in formulation 1. Capric acid is one of acids responsible for the peculiar smell of goat milk, which is traditionally described as "goaty" (Haenlein, 2004; Raynal-Ljutovac et al., 2008). From a nutrition point of view, myristic acid (C 14:0) and palmitic acid (C 16:0) are undesirable, because they are associated with an increase in plasmatic cholesterol (Grummer, 1991).

3.1.3. Total phenolic compounds and antioxidant activity

As Table 3 shows, adding grape pomace extract contributed to increase the level of phenolic compounds and antioxidant activity in formulation 2 ($p \leq 0.05$). In this study, the total level of phenolic compounds in both formulations was higher than data reported in literature on fermented milks with added grape pomace (Chouchouli et al., 2013; dos Santos et al., 2016; Frumento et al., 2013; Karaaslan, Ozden, Vardin, & Turkoglu, 2011). Either the addition of grape juice (which was found to have 343.83±4.35 mg GAE 100 g^{-1} of polyphenols) or the type of grape used may have contributed to the higher value of phenolic compounds found in the beverages. Manach et al. (2009) suggests that daily consumption of polyphenols is 100-150 mg for the western population. In this context, both formulations meets of the daily consumption of polyphenols.

Other studies have also described an increase in antioxidant activity in yogurts with adition of grape pomace extract (Chouchouli et al., 2013; Karaaslan et al., 2011). Phenolic compounds have been attracting growing research due to their antioxidant, anti-inflammatory and anti-mutagenic properties. The antioxidant activity of these compounds involves the property of phenols to capture the more reactive varieties of oxygen and to inhibit the self-oxidant potential of cells (Antolovich, Prenzler, Robards, & Ryan, 2000). Increases in oxidant stress may play a fundamental role in the development of chronic diseases, such as heart disease and cancer (Galleano et al., 2012; Prasain, Carlson, & Wyss, 2010).

Table 3. Centesimal composition, fatty acid profiles, and total phenolic compounds in the formulated fermented beverages (formulations 1 and 2).

Parameter	**Formulation 1**	**Formulation 2**	**t (p)**
pH	4.30±0.01	4.31±0.02	-0.63 (0.56)
Titratable acidity (g 100 g^{-1})	0.57±0.01	0.61±0.01	-3.48 (0.03)
Centesimal composition			
Moisture (g 100 g^{-1})	81.67±0.02	78.36±0.06	-85.94 (<0.01)
Ash (g 100 g^{-1})	0.76±0.01	0.89±0.01	31.84 (<0.01)
Total nitrogen (g 100 g^{-1})	0.43±0.01	0.47±0.01	6.97 (<0.01)
Fat (g 100 g^{-1})	2.39±0.01	2.38±0.02	-0.42 (0.70)
Total dietary fiber (g 100 g^{-1})	2.54±0.01	3.92±0.02	-131.23 (<0.01)
Soluble fiber (g 100 g^{-1})	1.88±0.02	2.18±0.01	28.78 (<0.01)
Insoluble fiber (g 100 g^{-1})	0.20±0.01	0.98±0.01	-95.53 (<0.01)
Lignin (g 100 g^{-1})	0.45±0.01	0.76±0.01	-37.97 (<0.01)
Carbohydrate (g 100 g^{-1})	9.96±0.02	11.45±0.01	-141.67 (<0.01)
Total calories (kcal 100 g^{-1})	70.90±0.02	94.90±0.01	-2277.16 (<0.01)
Fatty Acids (g 100 g^{-1} fatty acid methyl esters)			
Capric acid	3.93±0.02	2.91±0.01	79.01 (<0.01)
Myristic acid	10.43±0.02	9.75±0.02	47.03 (<0.01)
Palmitic acid	26.17±0.01	25.75±0.02	40.16 (<0.01)
Stearic acid	6.10±0.02	6.51±0.01	-32.87 (<0.01)
Oleic acid	31.51±0.02	33.07±0.02	-124.54 (<0.01)
Branched-Chain Fatty Acids (g 100 g^{-1} fatty acid methyl esters)			
Iso tetradecanoic acid	0.15±0.03	0.14±0.01	-0.43 (0.69)
Pentadecanoic acid	1.60±0.03	1.52±0.02	-4.13 (0.01)
Iso pentadecanoic acid	0.34±0.02	0.36±0.02	-1.22 (0.29)
Anti-iso pentadecanoic acid	0.63±0.01	0.61±0.02	1.55 (0.20)
Iso hexadecanoic acid	0.37±0.01	0.37±0.01	0.00 (1.00)
Total phenolic compounds (mg GAE 100 g^{-1})	53.16±3.14	73.52±3.13	-7.96 (<0.01)
Antioxidant activity (mmol TE g^{-1})	418.02±16.14	743.78±23.88	-15.98 (<0.01)

Averages ± standard deviation (n=3); Student's t-test ($p \leq 0.05$).

3.2. The effect of grape-flavored probiotic fermented beverages made of goat milk with or without added grape pomace on gut microbiota in a Simulator of Human Intestinal Microbial Ecosystem (SHIME®)

3.2.1. Survival of *L. rhamnosus* and *S. thermophilus* under simulated stomach and duodenum conditions in the SHIME®

Fig. 2 shows the survival of *L. rhamnosus* and *S. thermophilus* in formulations 1 and 2 under the simulated conditions of the stomach and duodenum in the SHIME®.

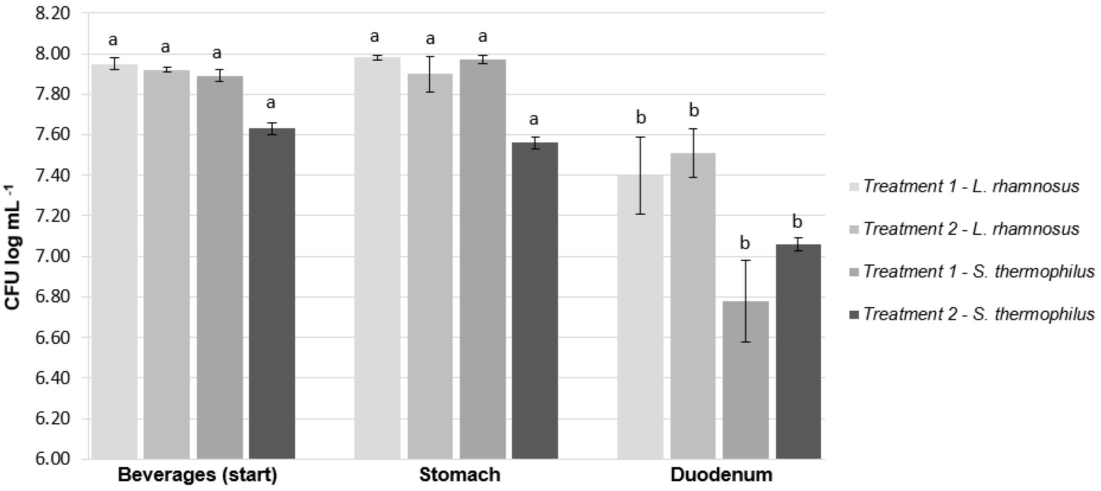

Fig. 2. Survival of L. rhamnosus and S. thermophilus before and after the incubation in the reactors simulating the stomach and duodenum. Quantified by plate counts and expressed as CFU log mL^{-1};Control 1 and 2: carbohydrate-based medium; Treatment 1: carbohydrate-based medium + fermented milk + grape juice; Treatment 2: carbohydrate-based medium + fermented milk + grape pomace extract + grape juice. Different letters presented different results in the Tukey test ($p \leq 0.05$) between the formulated beverages before and after the passage through the reactors simulating the stomach and the duodenum.

The passage of formulations 1 and 2 through the stomach-simulating reactor was not found to affect the survival ($p > 0.05$) of *L. rhamnosus* and *S. thermophilus*. According to Heller (2001), the presence of foods and food ingredients such as dietary fibers (Sendra, Sayas-Barberá, Fernández-López, & Pérez-Alvarez, 2016) may have a protective effect on the viability of the microorganisms during their passage through the stomach. Besides, goat milk can be considered a great option for the incorporation of probiotic strains (dos Santos et al., 2016).

During the passage through the duodenum-simulating reactor, a slight ($p \leq 0.05$) reduction in *L. rhamnosus* survival in both formulations was observed (1: 7.98 to 7.40 log CFU mL^{-1}; 2: 7.90 to 7.51 log CFU mL^{-1}). This behavior is typical of probiotic strains, which are able to resist to the acidic pH of the stomach and the stress conditions of the duodenum (Blanquet-Diot et al., 2012). According to Hill et al. (2014), probiotic products should ideally contain 10^9 CFU of the probiotic strain per portion of food in order to obtain a reasonable expectation of benefits to the host’s wellbeing. Tuo et al. (2013) described that strains of *L. rhamnosus* were capable of surviving gastrointestinal conditions and adhering to Caco-2 cells. Other studies have reported that the strain *L. rhamnosus* GR-1 survived to stomach conditions and to the presence of bile salts, and was capable of colonizing the human intestine for several weeks; the strain was also capable of reducing urinary tract infections and restoring urogenital microbiota (Cadieux et al., 2002; Reid & Bruce, 2003; Reid et al., 2001).

S. thermophilus is a strain that is commonly used as a starter culture in the production of fermented milks, such as yogurts and cheeses. This microorganism is responsible for accelerating of the fermentation process through the production of lactic acid and secondary metabolites, which contribute to the sensory properties of fermented products (Uriot et al., 2016).

A significant decrease in *S. thermophilus* populations was expected after passage through the simulated duodenum conditions. However, a small ($p \leq 0.05$) decrease in the survival of this microorganism was observed for both beverages (1: 7.89 to 6.78 log CFU mL^{-1}; 2: 7.63 to 7.07 log CFU mL^{-1}). Uriot et al. (2016) observed an intense decrease in the population of four *S. thermophilus* strains tested in isolation after their passages through the simulated duodenum conditions in a dynamic *in vitro* model (TIM). This decrease was likely due to the presence of bile salts, which affect phospholipids and proteins of the cellular membrane (Fang, Lai & Chou, 2013). The results obtained in this study showed that the tested food matrices protected both microorganisms present, which were a probiotic and a starter culture.

3.2.2. Microbiological analysis of the SHIME® samples

Table 4 presents the impact of treatments 1 and 2 on the populations of *Lactobacillus* spp., *Bifidobacterium* spp., *Clostridium* spp., total anaerobes and total coliform in the reactors simulating the three portions of the colon (AC, DC, and TC).

Table 4. Microbial counts (CFU log mL^{-1}) of bacteria from different genera in the reactors simulating the ascending colon (AC), transversal colon (TC), and descending colon (DC) during the experimental period.

Genus	Experimental Period	AC	t (p)	TC	t (p)	DC	t (p)
Lactobacillus spp.	Control 1	8.54±0.03	4.76 (0.04)	7.84±0.02	-1.15 (0.37)	7.30±0.15	-6.79 (0.02)
	Treatment 1	7.77±0.26		7.92±0.10		7.81±0.02	
	Control 2	8.23±0.28	-6.81 (0.02)	7.57±0.05	-14.26 (<0.01)	7.08±0.03	-15.16 (<0.01)
	Treatment 2	8.45±0.22		8.02±0.10		7.78±0.05	
Bifidobacterium spp.	Control 1	8.10±0.03	15.59 (<0.01)	7.63±0.16	0.22 (0.85)	7.16±0.05	-14.24 (<0.01)
	Treatment 1	7.92±0.01		7.59±0.12		7.75±0.03	
	Control 2	8.18±0.13	-2.81 (0.10)	7.39±0.21	-3.10 (0.09)	6.96±0.12	-8.73 (0.01)
	Treatment 2	8.31±0.21		7.90±0.07		7.71±0.03	
Clostridium spp.	Control 1	8.69±0.09	20.04 (<0.01)	8.23±0.01	60.84 (<0.01)	8.24±0.29	2.29 (0.15)
	Treatment 1	8.15±0.05		7.89±0.02		7.80±0.03	
	Control 2	8.37±0.05	-5.59 (0.03)	7.94±0.33	-0.25 (0.83)	7.49±0.41	-0.21 (0.85)
	Treatment 2	8.50±0.09		8.00±0.12		7.54±0.02	
Total anaerobes	Control 1	8.94±0.03	13.82 (0.01)	8.47±0.06	13.25 (0.01)	8.34±0.20	5.11 (0.04)
	Treatment 1	7.98±0.15		7.83±0.03		7.80±0.02	
	Control 2	8.94±0.36	0.74 (0.53)	8.08±0.43	0.02 (0.99)	7.72±0.25	-0.40 (0.73)
	Treatment 2	8.70±0.21		8.07±0.15		7.78±0.01	
Total coliforms	Control 1	8.08±0.07	5.57 (0.03)	7.65±0.01	4.00 (0.06)	7.52±0.12	2.84 (0.11)
	Treatment 1	6.22±0.50		6.06±0.70		6.13±0.73	
	Control 2	7.86±0.91	1.49 (0.27)	7.14±0.92	1.15 (0.37)	6.82±0.66	1.30 (0.32)
	Treatment 2	6.79±0.34		6.25±0.42		6.02±0.41	

Averages ± standard deviation (n=6); Student's t test ($p \leq 0.05$).
Control 1 and 2: carbohydrate-based medium; Treatment 1: carbohydrate-based medium + fermented milk + grape juice; Treatment 2: carbohydrate-based medium + fermented milk + grape pomace extract + grape juice.

In the reactor simulating the AC, all bacterial populations analyzed reduced ($p \leq 0.05$) during treatment 1 (fermented milk + grape juice). Meanwhile, in the reactor that simulating the TC, there were reductions ($p \leq 0.05$) in the *Clostridium* spp. and total anaerobes population. In the reactor simulating the DC, there was an increase ($p \leq 0.05$) in *Lactobacillus* spp. and *Bifidobacterium* spp. populations and a decrease ($p \leq 0.05$) in total anaerobes microrganisms.

In treatment 2 (fermented milk + grape pomace extract + grape juice), an increase ($p \leq 0.05$) in *Lactobacillus* spp. and *Clostridium* spp. were found in the reactor simulating the AC. An increase ($p \leq 0.05$) in the *Lactobacillus* spp. population was observed only in the reactor simulating the TC. Increases ($p \leq 0.05$) in *Lactobacillus* spp. and *Bifidobacterium* spp. populations were observed in the reactor simulating the DC.

Recent studies suggest that foods rich in phenolic compounds may influence the gut microbiota composition and activity by stimulating or inhibiting specific bacterial groups (Boto-Ordóñez et al., 2014; Espley et al., 2014; Faria, Fernandes, Norberto, Mateus, & Calhau, 2014; Tabasco et al., 2011). In addition, when present in the colon, these compounds may be metabolized by the resident microbiota producing biologically active metabolites (Duynhoven et al., 2011). Sánchez-Patán et al. (2015) observed decreases in *Lactobacillus* spp., *Bifidobacterium* spp., and *Clostridium leptum* populations after 48 hours of incubation with grape seed extract in the SHIME® model. Barroso et al. (2014) also reported decreases ($p \leq 0.05$) in *Lactobacillus* spp. and *Bifidobacterium* spp. populations after one week of treatment with red wine polyphenols in a SHIME® model. Cueva et al. (2013) observed a decrease in the *Clostridium histolyticum* population after *in vitro* fermentation of flavonoids from grape seeds in a fermentation model using fecal batch-cultures.

In this study, only treatment 2 had a positive influence on the population of beneficial bacteria present in the colon (*Lactobacillus* spp. and *Bifidobacterium* spp). This effect may be attributed to the higher amounts of fibers, stearic acid, oleic acid, pentadecanoic acid, and phenolic compounds, as well as the antioxidant activity present in this formulation.

DGGE analysis on total bacteria was used to evaluate the qualitative changes that potentially occurred within the microbial community during the treatment periods (Fig. 3). The data is presented as a cluster analysis conducted on a composite dataset of the one gel using the unweighted pair group with mathematical averages (UPGMA) and distance matrices of each DGGE gel based on the Pearson correlation similarity coefficients.

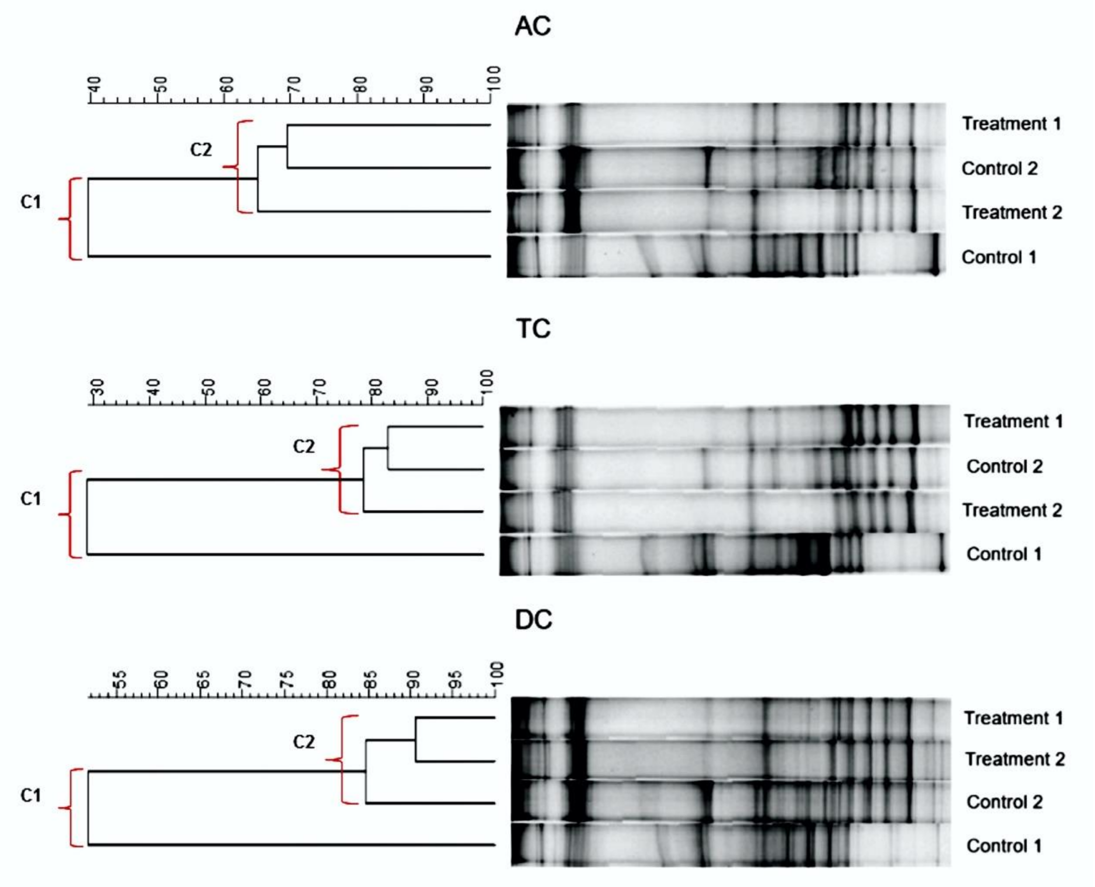

Fig. 3. Denaturing gradient gel electrophoresis (DGGE) of the total bacteria profiles in the reactors simulating the ascending colon (AC), transversal colon (TC), and descending colon (DC) during the experimental period. Control 1 and 2: carbohydrate-based medium; Treatment 1: carbohydrate-based medium + fermented milk + grape juice; Treatment 2: carbohydrate-based medium + fermented milk + grape pomace extract + grape juice.

The cluster analysis in all reactors simulating the different regions of the colon resulted in two distinct clusters (cluster 1: control 1; cluster 2: treatment 1, control 2 and treatment 2). The treatment 1 altered the total bacteria population during control period 1. However, treatment 1 and control 2 showed high similarity in all reactors simulating the different regions of the colon (AC, DC, and TC). The long-term administrations of the treatments induced a change toward the development of a new community structure.

The effect of the treatments on the structure of the total microbial community in the SHIME® system was investigated based on the interpretation of general bacterial DGGE fingerprints, according to a Marzorati et al., (2008). Fig. 4 shows the level of richness (Rr) of the total bacteria population in the reactors simulating the AC, TC, and DC during the experimental period. All the colon reactors presented Rr values over 30. According to Marzorati et al. (2008), richness values below 10 represent environments of restricted colonization. However, values between 10 and 30 correspond to a microbial community of medium richness. Values above 30 represent a microbial community of great diversity typical of a very inhabitable environment. According to this classification, it is possible to state that all of the colon simulation reactors used herein presented an environment of high richness (Rr > 30). Ecological interpretation of general bacterial DGGE fingerprints (Marzorati et al., 2008) showed a reduction in Rr during the treatment periods 1 and 2 in relation to respective control periods, except between the control period 2 and treatment 2 in the reactor simulating the TC. Kemperman et al. (2013) suggest that the polyphenols in red wine extract may have an impact on the composition of total bacteria. These authors showed that the polyphenols in red wine stimulated the growth of some bacterial genera, such as *Klebsiella*, *Alistipes*, *Cloacibacillus*, *Victivallis*, and *Akkermansia.* However, populations of other genera decreased, including *Bifidobacteria*, *B. coccoides*, *Anaeroglobus*, *Subdoligranulum* and *Bacteroides*. Moreover, these authors also reported that some specific groups of bacteria may be vulnerable to the presence of these compounds, while other groups are not affected or even stimulated, thus rendering them more frequent in the microbial community after the consumption of red wine polyphenols.

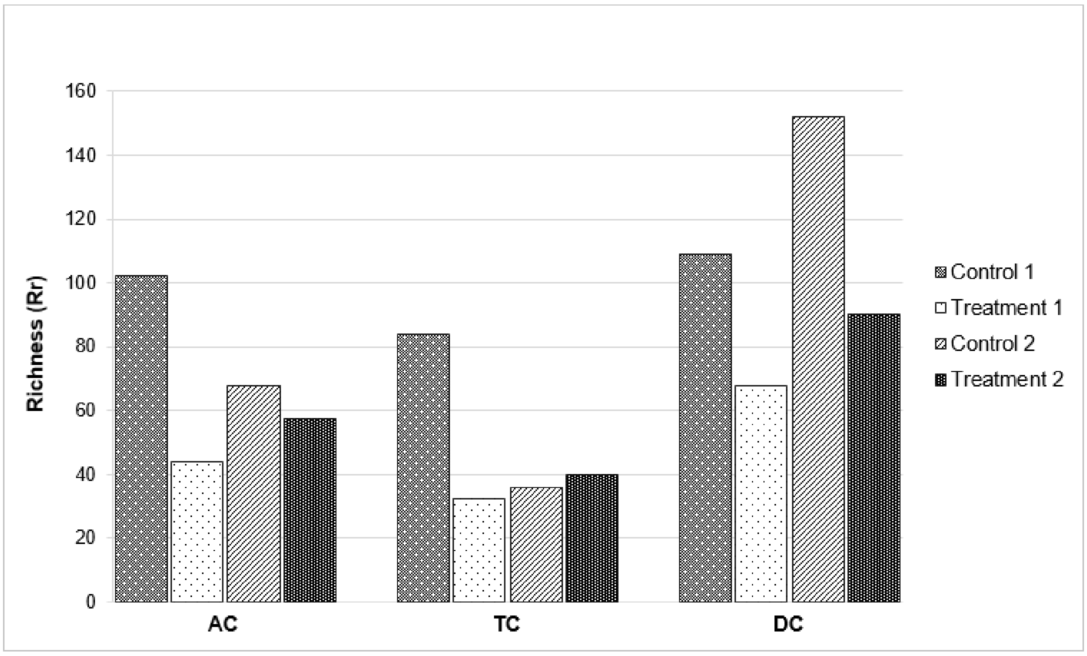

Fig. 4. Richness level (Rr) of total bacteria populations in the reactors simulating the ascending colon (AC), transversal colon (TC), and descending colon (DC) during the experimental period. Control 1 and 2: carbohydrate-based medium; Treatment 1: carbohydrate-based medium + fermented milk + grape juice; Treatment 2: carbohydrate-based medium + fermented milk + grape pomace extract + grape juice.

In terms of the structure and functionality of the bacterial communities, all of the reactors simulating the colon (AC, TC, and DC) presented values close to 45% on the Pareto-Lorenz curve (Fig. 5). According to Marzorati et al.

(2008), points close to a Pareto-Lorenz curve of 45% reflect low uniformity of the microbial community, with most species being dominant. This configuration is commonly described as a well-balanced population of bacteria, with great potential to handle environmental alterations while remaining functional. Similar results were obtained by Sivieri et al. (2014) and Possemiers et al. (2010).

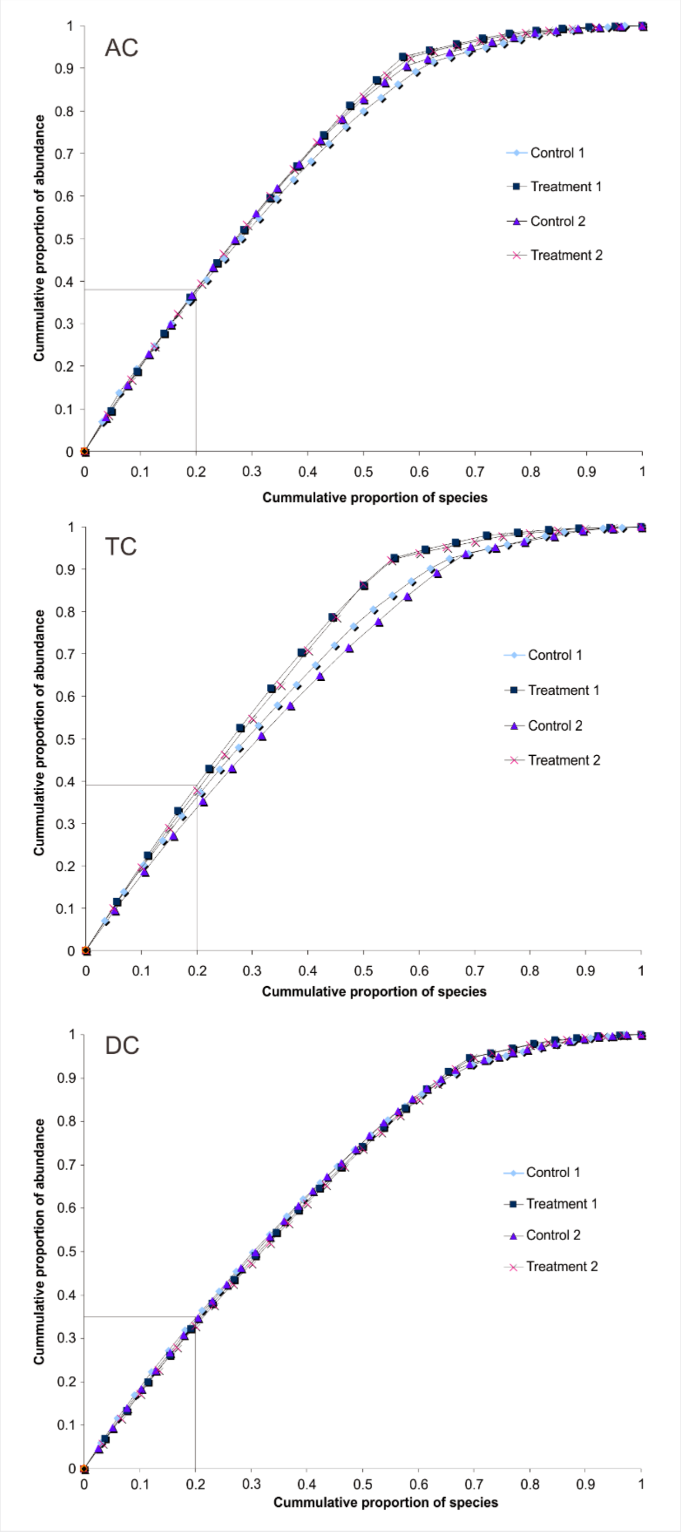

Fig. 5. Pareto-Lorenz curve of the reactors simulating the ascending colon (AC), transversal colon (TC), and descending colon (DC) during the experimental period. Control 1 and 2: carbohydrate-based medium; Treatment 1: carbohydrate-based medium + fermented milk + grape juice; Treatment 2: carbohydrate-based medium + fermented milk + grape pomace extract + grape juice.

3.2.3. Ammonium (NH_4^+) and short- and branched-chain fatty acids (SCFAs) analysis

NH_4^+ corresponds to one of the metabolites resulting from protein fermentation by intestinal bacteria. The fermented beverages formulated herein presented approximately 2.6% protein content. Nonetheless, a reduction ($p \leq 0.05$) in the production of NH_4^+ was observed in all the reactors simulating the regions of the colon (AC, TC, and DC) during treatment periods 1 and 2 (Table 5). Most NH_4^+ are absorbed in the colon, metabolized into urea in the liver, and excreted in urine. NH_4^+ can alter the morphology of intestinal cells and promote carcinogenesis in the colon (Scott et al., 2013).

Table 5. Concentration of NH4+ (mmol/L) in the reactors simulating the ascending colon (AC), transversal colon (TC), and descending colon (DC) during the experimental period.

Experimental Period	AC	t (p)	TC	t (p)	DC	t (p)
Control 1	20.71±0.23		21.23±0.06		17.07±0.06	
Treatment 1	12.12±0.12	97.23 (<0.01)	9.41±0.47	39.60 (<0.01)	9.33±0.31	36.35 (<0.01)
Control 2	12.88±0.03		16.11±0.09		20.32±0.09	
Treatment 2	3.70±0.04	322.21 (<0.01)	3.59±0.05	233.45 (<0.01)	8.87±0.06	617.30 (<0.01)

Averages ± standard deviation (n=3); Student's t test (p≤0.05).
Control 1 and 2: carbohydrate-based medium; Treatment 1: carbohydrate-based medium + fermented milk + grape juice; Treatment 2: carbohydrate-based medium + fermented milk + grape pomace extract + grape juice.

SCFAs are produced by the microbiota as a result of the fermentation of compounds that are not digestible by the gastrointestinal tract. These acids possess less than 6 atoms of carbon and can have linear or branched chains. They are produced by the fermentation of carbohydrates ingested from food. However, the breaking-down of proteins can also result in branched chain fatty acids (BCFAs), such as isobutyrate, isovalerate, and 2-methyl butyrate, thus contributing to 5% of total SCFA production (Ríos-Covián et al., 2016).

In this study, increases ($p \leq 0.05$) in acetic acid and butyric acid were observed in all reactors simulating the different regions of the colon during treatment periods 1 and 2 (Table 6). However, propionic acid decreased ($p \leq 0.05$) during treatment period 1, and a significant increase was observed during treatment period 2 in all colon reactors.

A substantial increase in SCFAs was observed during the treatment period 2. This increase may be attributed to the fibers present in grape pomace extract, since they are a source of carbon and can be metabolized by intestinal bacteria of the genera *Lactobacillus* spp. and *Bifidobacterium* spp. (Fernández et al., 2016). In this context, the treatment 2 showed an increase ($p \leq 0.05$) of *Lactobacillus* spp. and *Bifidobacterium* spp. (Table 4). Similar results were observed by Sivieri et al. (2014) in their study of the prebiotic effect of fruit oligosaccharides on gut microbiota using a SHIME® model.

Some health benefits are attributed to SCFAs. They include decreases in luminal pH (which can inhibit pathogenic microorganisms and increase nutrient absorption), increases in mucin production (which modifies the adhesion of bacteria to intestinal cells) and a stimulation of cellular proliferation in the intestinal epithelium (Ríos-Covián et al., 2016). Acetic acid is the most abundant SCFA in the colon, being responsible for provide energy to cells and induce cholesterol synthesis (Hijova, 2007). Butyric acid is the most important metabolite produced in the colon; it is the main source of energy for the colonocytes, stimulates visceral blood flow, contributes to sodium and water absorption, besides presenting anti-inflammatory and anti-carcinogenic properties (Montalto, D’onofrio, Gallo, Cazzato, & Gasbarrini, 2009; Mortensen

& Clausen, 1996). Propionic acid is responsible for reducing lipogenesis and inhibiting the serum cholesterol synthesis (Hijova, 2007; Hosseini, Grootaert, Verstraete, & Van de Wiele, 2011).

The production of SCFAs observed in this study may have also been influenced by the degradation of phenolic compounds present in the grape pomace extract and grape juice. According to Tuohy, Conterno, Gasperotti, and Viola (2012), gut microbiota can hydrolyze complex phenolic compounds in smaller compounds, thus producing SCFAs. Schneider et al. (1999) and Schoefer et al. (2003) observed that some colon bacteria isolated from human feces used flavonoids as an energy source, thus releasing butyric acid. Bravo (1993) observed that tannic acid and catechins were degraded by the gut microbiota during the fermentation process, producing butyric acid in an in vitro fermentation model inoculated with rat feces.

There was no difference ($p>0.05$) in BCFAs levels between the control periods and respective treatment periods. Studies suggest that BCFAs may have a regulatory effect and may increase anti-inflammatory cytokine expression (Ran-Ressler et al., 2014). Other studies have reported that BCFAs induce apoptosis in breast cancer cells and inhibit the growth of tumors in animal models (Wongtangtintharn, Oku, Iwasaki, & Toda, 2004; Yang et al., 2000).

Table 6. Concentration (mmol/ L) of short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) in the reactors that simulate the ascending colon (AC), transversal colon (TC), and descending colon (DC) during the experimental period.

	Experimental Period	**AC**	**t (p)**	**TC**	**t (p)**	**DC**	**t (p)**
Acetic acid	Control 1	69.27±5.82	-4.50 (0.05)	77.80±6.03	-7.19 (0.02)	73.73±5.72	-11.39 (0.01)
	Treatment 1	112.75±10.94		158.17±13.33		165.75±8.33	
	Control 2	40.16±0.96	-27.51 (<0.01)	48.51±2.66	-13.99 (0.01)	42.55±3.34	-53.63 (<0.01)
	Treatment 2	101.62±2.92		157.02±10.78		168.73±0.74	
Propionic acid	Control 1	15.83±4.16	0.54 (0.64)	20.82±2.62	3.50 (0.07)	17.25±0.45	25.44 (<0.01)
	Treatment 1	13.88±2.10		12.22±1.64		10.20±0.04	
	Control 2	7.54±0.86	-4.40 (0.05)	6.29±0.07	-9.32 (0.01)	7.11±1.05	-5.45 (0.03)
	Treatment 2	16.12±2.53		15.36±1.62		10.54±0.04	
Butyric acid	Control 1	12.85±4.91	-7.82 (0.02)	7.63±1.50	-5.00 (0.04)	2.84±0.03	-33.81 (<0.01)
	Treatment 1	24.38±2.35		33.79±7.57		30.36±1.38	
	Control 2	10.72±1.51	-5.50 (0.03)	13.72±0.66	-23.29 (<0.01)	9.85±0.91	-7.11 (0.02)
	Treatment 2	45.30±9.39		36.85±2.38		28.95±5.56	
Total BCFA	Control 1	2.57±0.20	0.48 (0.67)	2.39±0.10	0.13 (0.91)	2.66±0.41	1.15 (0.33)
	Treatment 1	2.49±0.24		2.38±0.30		2.40±0.19	
	Control 2	2.31±0.14	-0.55 (0.62)	2.30±0.15	-0.68 (0.55)	2.71±0.54	2.00 (0.14)
	Treatment 2	2.39±0.17		2.39±0.23		2.17±0.15	

Averages ± standard deviation (n=3); Student's t test ($p \leq 0.05$).

Control 1 and 2: carbohydrate-based medium; Treatment 1: carbohydrate-based medium + fermented milk + grape juice; Treatment 2: carbohydrate-based medium + fermented milk + grape pomace extract + grape juice.

3.2.4. Antioxidant capacity of SHIME® samples

Fig. 6 presents the antioxidant capacity of the samples collected from the reactors simulating the three regions of the colon (AC, TC, and DC) during both treatment periods. All of the reactors exhibited higher ($p \leq 0.05$) antioxidant capacity in treatment periods 1 and 2 when compared to the respective control periods. Duque et al. (2016) analyzed the effect of orange juice consumption on gut microbiota using a SHIME® model. The authors observed the same behavior in the antioxidant capacity during the treatment and control periods.

Studies suggest that long term consumption of fruits and vegetables plays a central role in the prevention at many chronic diseases (Pandey & Rizvi, 2009; Sun, Chu, Wu, & Liu, 2002). In the gastrointestinal tract, these health-protective effects are partially attributed to their antioxidant properties (Halliwell, Zhao, & Whiteman, 2000), which have been associated with their high phytochemical (phenolic compounds) and antioxidant dietary fibre contents. The result obtained in this study suggests that most of the phenolic compounds present both in grape pomace extract and in grape juice were available in the reactors simulating the colon for fermentation by intestinal microbiota.

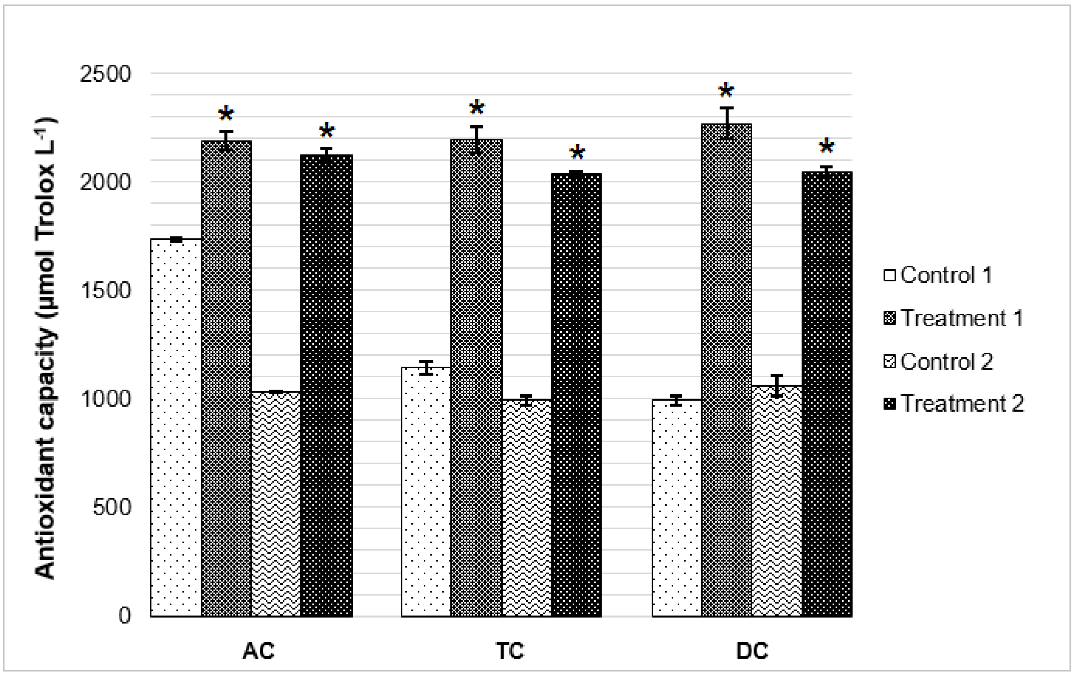

Fig. 6. Antioxidant capacity of the reactors simulating the ascending colon (AC), transversal colon (TC), and descending colon (DC) during the experimental period. Control 1 and 2: carbohydrate-based medium; Treatment 1: carbohydrate-based medium + fermented milk + grape juice; Treatment 2: carbohydrate-based medium + fermented milk + grape pomace extract + grape juice. Averages with * have significantly different values on Student's t-test (p≤0.05) in control period 1 versus treatment period 1 and in control period 2 versus treatment 2 in the different reactors.

3.3. Principal Component Analysis (PCA)

A PCA was performed throughout the experimental period in the SHIME®, and the components explained 83.78% of the total variation of results (Fig. 7A). Principal component 1 explained 50.05% of data analyzed and was described by the production of acetic acid and butyric acid, antioxidant capacity and *Lactobacillus* spp. and *Bifdobacterium* spp. populations.

Principal component 2 explained 33.73% of the total variation of results and was described by *Clostridium* populations, total coliforms, propionic acid, and $NH4^+$ production.

Fig. 7B shows that the control and treatment periods were grouped in two distinct clusters. The treatment periods were described by increases in acetic acid and butyric acid production, antioxidant activity, and *Lactobacillus* spp. and *Bifdobacterium* spp. populations. Meanwhile, the control periods were described by the increase in *Clostridium* spp. populations and in the total coliforms, as well as by the production of propionic acid and $NH4^+$ ions.

These results suggest that both treatment periods had a positive effect on gut microbiota, since they allowed for positive metabolic changes in the different regions simulating the colon (AC, TC, and DC).

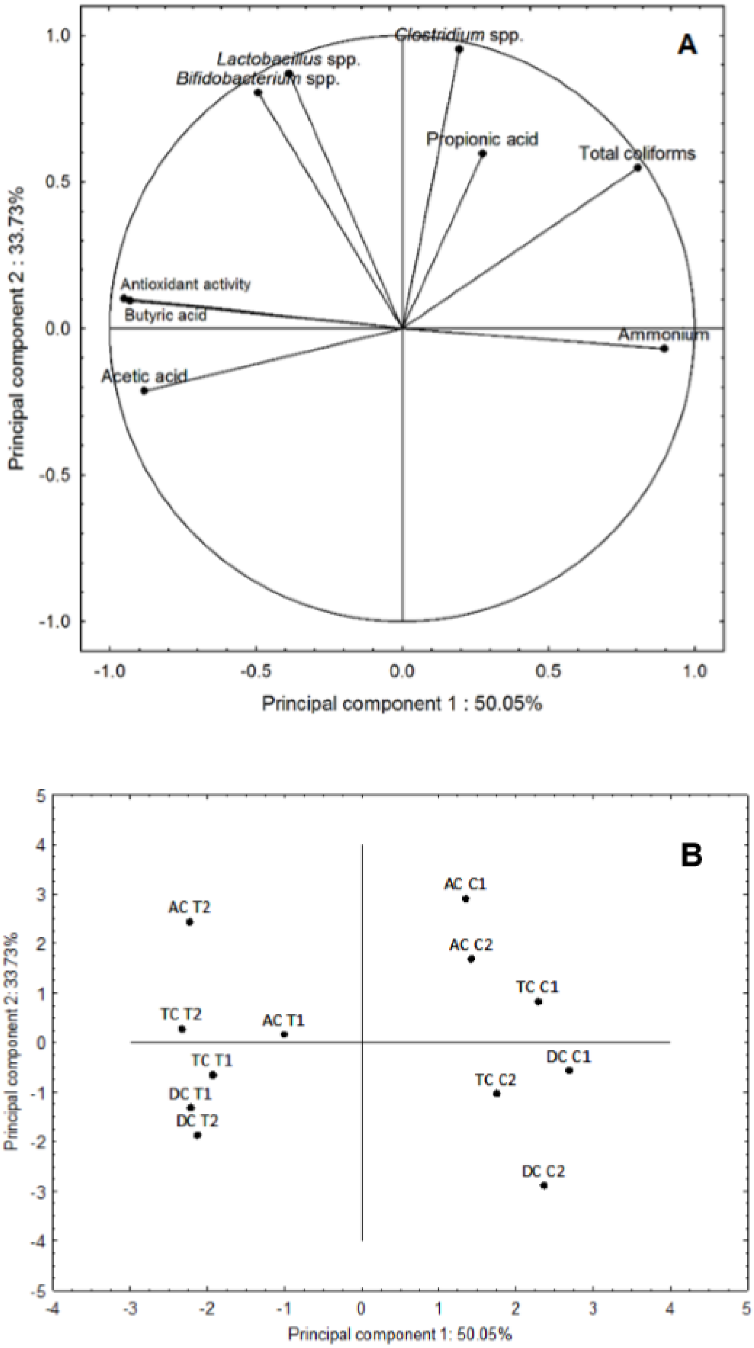

Fig. 7. Principal component analysis. (A: variable projection; B: sample projection) of the in vitro SHIME® model. AC: ascending colon; TC: transversal colon; DC: descending colon; C1: control 1 (carbohydrate-based medium); C2: control 2 (carbohydrate-based medium); T1: treatment 1 (carbohydrate-based medium +fermented milk + grape juice); T2: treatment 2 (carbohydrate-based medium +fermented milk + grape pomace extract + grape juice).

4. Conclusion

Foods comprised of different types of bioactive compounds may be considered "multifunctional foods." The results observed in this study demonstrated that probiotic fermented beverages made of goat milk and grape juice, with or without grape pomace extract, had high amounts of dietary fiber, oleic acid and phenolic compounds content, which are considered biologically active. The beverages had a protective effect on *L. rhamnosus* and *S. thermophilus* microorganisms during their passage through the gastrointestinal tract, and were also found to have a positive effect on gut microbiota. They altered the composition of the microbial structure and improved bacterial metabolism in the different regions simulating the colon. In summary, the beverages formulated in this study can be considered a multifunctional food and offer a new perspective for the production of foods with potential positive effects on human health.

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3. CONSIDERAÇÕES FINAIS

A partir dos resultados apresentados, conclui-se que:

1. As formulações desenvolvidas, enriquecidas ou não com extrato de bagaço de uva, apresentaram alto teor de fibras dietéticas, ácido oleico, compostos fenólicos e atividade antioxidante.

2. Em ambas as formulações, a viabilidade *do L. rhamnosus* e do *S. thermophilus* foi preservada durante a passagem pelo trato gastrointestinal simulado e exerceram efeitos positivos sobre a microbiota intestinal, uma vez que alteraram a composição da comunidade microbiana, aumentaram a produção de ácidos graxos de cadeia curta e capacidade antioxidante e diminuíram a produção de íons de amônio.

3. A Análise de Componentes Principais apresentou dois agrupamentos distintos, um com os períodos de tratamento e outro com os períodos controle, demostrando que ambas as formulações exerceram um efeito sobre a microbiota intestinal, em termos de composição e metabolismo.

4. Estudos *in vivo*, especialmente estudos clínicos, são necessários para uma comprovação dos resultados encontrados *in vitro*.