CLAUDIA CRISTINA AULER DO AMARAL SANTOS

FERMENTAÇÃO DE EXTRATO HIDROSSOLÚVEL À BASE DE AMENDOIM E SOJA POR BACTÉRIAS E LEVEDURAS NO DESENVOLVIMENTO DE BEBIDA POTENCIALMENTE PROBIÓTICA

LAVRAS - MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para obtenção do título de Doutor.

Orientador
Profª. Dra. Rosane Freitas Schwan

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Dra. Anne Christine Gschaedler Mathis	CIATEJ
Dr. Javier Placido Arrizon Gaviño	CIATEJ
Dr. Anderson de Souza Sant'Ana	UNICAMP/FEA
Dr. Disney Ribeiro Dias	UFLA.
Dr. Whasley Ferreira Duarte	UFLA

Dra. Rosane Freitas Schwan
Orientador

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DEDICO

RESUMO

O amendoim e a soja são considerados alimentos funcionais por possuírem componentes benéficos para o corpo capazes de reduzir o risco de certas doenças. O consumo de produtos derivados dessas oleaginosas proporciona benefícios para a saúde devido à riqueza em proteínas, minerais e ácidos graxos essenciais. O extrato hidrossolúvel produzido a partir dessas matérias-primas pode servir como alternativa ao leite de vaca para vegetarianos e indivíduos intolerantes à lactose. Visto que essas leguminosas podem ser utilizadas como ricas fontes de nutrientes seu consumo deve ser aumentado, especialmente nos países em desenvolvimento. A fermentação do extrato de amendoim e de soja pode originar um novo produto com excelente qualidade físico-química e nutricional, sendo que a adição de micro-organismos probióticos e substâncias prebióticas conferirão a esse produto característica de bebida simbiótica. Além disso, a associação do amendoim com a soja pode promover maior aceitação do produto em relação ao sabor e, consequentemente, elevar o consumo desses alimentos. Os objetivos deste trabalho foram descrever o preparo do extrato hidrossolúvel de amendoim e soja; realizar fermentações em culturas simples e mistas; avaliar parâmetros físico-químicos do substrato não fermentado e fermentado, além de selecionar micro-organismos para serem utilizados na fermentação do produto, juntamente com bactérias probióticas (comerciais) a fim de se obter um novo produto funcional sensorialmente aceitável.

Palavras-chave: Bebida funcional. Simbiótico. Bactérias probióticas. Leveduras. Cocultura. Fermentação lática.

ABSTRACT

The peanut and soybean are considered functional foods due to their ability to reduce the risk of certain diseases. The consumption of derived products from these oilseeds provides health benefits due to the protein, minerals and essential fatty acids richness. The milk produced from these raw materials can serve as an alternative to cow's milk for vegetarians and individuals with lactose intolerance. Since these oilseeds have a role to play in combating malnutrition, their consumption should be increased, especially in developing countries. The peanut and soy milks fermentation can give a new product with excellent chemical and nutritional quality, with the addition of probiotic microorganisms and prebiotic substances that confer a symbiotic beverage characteristics. Moreover, the use of mixed peanut and soy milks can promote wider acceptance of the product related to flavor, hence increase consumption of these valuable crops. The purpose of this study were: 1) the development and characterization of peanut-soy milk; 2) the use of peanut-soy milk as substrate for single and co-culture fermentations; 3) evaluate physico-chemical parameters of unfermented and fermented substrate; and 4) selection of lactic acid bacteria, including probiotic ones (commercial) and yeasts in order to obtain a new functional product sensorially acceptable by the consumers.

Keywords: Functional beverage. Symbiotic. Probiotic bacteria. Yeast. Co-culture. Lactic fermentation.

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LISTA DE ABREVIATURAS E SIGLAS

ABENUTRI	Associação Brasileira das Empresas de Produtos Nutricionais
ABIA	Associação Brasileira das Indústrias da Alimentação
ABRAN	Associação Brasileira de Nutrologia
BAL	Bactérias do Ácido Lático
CFU	*Colony Forming Unit*
FAO	*Food and Agriculture Organization*
FOS	Fruto-oligossacarídeos
GRAS	*Generally recognized as safe*
LAB	*Lactic acid Bacteria*
LGG	*Lactobacillus "Gorbach and Goldin"*
MRS	*The Man Rogosa Sharp Medium*
PM	Peanut milk
PSM	Peanut-soy milk
VC	*Viable count*
YPD	*Yeast Peptone Dextrose Medium*
WHO	*World Health Organization*

SUMÁRIO

1 INTRODUÇÃO

Os alimentos são essenciais e indispensáveis à vida humana e fornecem todos os elementos necessários para o homem desenvolver não só o seu físico, mas também suas atividades intelectuais. Numerosos estudos mostram claramente que a qualidade de vida está intimamente associada ao tipo de alimentação diária, bem como ao estilo de vida de cada indivíduo (BELLO, 1995; NORDSTROM; BISTROM, 2002; URALA; LÄHTEENMÄKI, 2007; GRANATO et al., 2010).

Pesquisas específicas em biotecnologia estão recebendo atenção redobrada e, mais especificamente, a investigação sobre alimentos funcionais está ganhando importância. A elaboração desses alimentos é atualmente uma das áreas mais visadas para o desenvolvimento de produtos no mundo inteiro, visto o interesse em nutrição e em alternativas para melhorar a saúde na sociedade em geral (NIVA; MÄKELÄ, 2007). A pressão do mercado por esse tipo de alimentos é crescente, devido à conscientização do consumidor em relação ao consumo de alimentos benéficos (NORDSTROM; BISTROM, 2002). Formulados para serem fontes significativas de determinados nutrientes ou outros componentes de importância nutricional, os alimentos funcionais têm o potencial de causar grande impacto, tanto sobre o abastecimento de alimentos quanto na saúde pública (MOGELONSKY, 1999).

O leite e outros produtos lácteos recebem grande destaque na alimentação diária, principalmente por causa de seu alto valor nutritivo. Em muitos países, no entanto, e de modo especial naqueles em desenvolvimento, há grave insuficiência no fornecimento de nutrientes, incluindo o leite e seus derivados. A escassez na oferta de leite e de seus nutrientes essenciais tem levado a esforços para o desenvolvimento de alternativas a esse produto (NELSON; STEINBERG; WEI, 1976; ASIEDU, 1994; AIDOO et al., 2010). O

extrato hidrossolúvel, que é produzido a partir de matérias-primas de origem vegetal, pode servir como ótima alternativa para tais países onde o fornecimento de leite é inadequado.

O amendoim (*Arachis hypogaea* L.) e a soja (*Glycine max* L.) são importantes fontes de óleo comestível e de proteína e, portanto, considerados altamente valiosos na nutrição humana e animal. Nos últimos anos, no Brasil, ocorreu grande expansão da área cultivada, produção e principalmente aumento consistente em produtividade dessas culturas. Em termos produtivos, o amendoim brasileiro é proveniente, em maior escala, da região Sudeste. Com relação à soja, atualmente o Brasil é o maior produtor mundial, ultrapassando os Estados Unidos na safra 2012/2013 (CONAB, 2013).

Apesar do grande potencial do extrato hidrossolúvel de soja, este obteve no passado baixa aceitação no Brasil, basicamente devido ao sabor e aroma desagradáveis ao paladar dos consumidores brasileiros. Porém, os benefícios da soja à saúde humana são claramente um ponto importante para a promoção deste alimento junto ao consumidor, desde que eficientemente informados, apontando os benefícios de sua ingestão sobre a saúde e, desta forma, desenvolvendo ou reforçando uma atitude positiva do indivíduo em relação ao produto (BEHRENS; ROIG; SILVA, 2001). O extrato hidrossolúvel de amendoim e produtos derivados desta cultura tem benefícios nutricionais para jovens e idosos, devido à extrema riqueza em proteínas, minerais e ácidos graxos essenciais, como os ácidos linoléico e oléico, considerados altamente valiosos na nutrição humana (ISANGA; ZHANG, 2009).

Visto que essas leguminosas têm um papel a desempenhar na luta contra a desnutrição, seu consumo deve ser aumentado especialmente nos países em desenvolvimento. A fermentação dos extratos de amendoim e de soja pode originar um novo produto com excelente qualidade físico-química, nutricional e ainda com ação prebiótica e probiótica. Além disso, a associação do amendoim

com a soja pode promover maior aceitação do produto em relação ao sabor e, consequentemente, elevar o consumo dessas valiosas culturas (YADAV et al., 2003; YADAV et al., 2010). São, portanto, necessários mais estudos para responder adequadamente a investigação sobre a possibilidade de processamento dessas matérias-primas em produtos úteis e comestíveis.

2 REFERENCIAL TEÓRICO

2.1 Alimentos funcionais

Os alimentos que oferecem, além das funções nutricionais básicas, benefícios adicionais para a saúde, apareceram pela primeira vez no mercado em 1960. Naquela época, a mídia começou a destacar e reforçar a importância de uma boa alimentação e que os alimentos processados ou não, devem manter os atributos naturais de seus ingredientes. Os consumidores começaram a tomar consciência de que havia uma ligação entre uma série de enfermidades (tais como a constipação, doenças cardiovasculares, obesidade e hipertensão) e a ingestão de quantidade excessiva de determinados alimentos. Além disso, as consequências para a saúde pelo fato de se comer mal, ter hábitos sedentários e uma dieta pobre foram agravadas pelo estresse inerente à vida moderna (BELLO, 1995).

Dentro desse contexto, uma nova categoria de alimentos chamados alimentos funcionais foi lançada no mercado em meados de 1980. O termo “alimentos funcionais” foi proposto, em 1984, pelos cientistas japoneses que estudavam as relações entre nutrição, satisfação sensorial, fortificação e modulação de sistemas fisiológicos (MENRAD, 2003). As práticas alimentares da população do Extremo-Oriente, principalmente do Japão e China, contribuíram decisivamente para o conceito dos alimentos funcionais, ou seja, alimentos industrializados que contenham ingredientes que ajudem nas funções específicas do corpo, além de serem nutritivos (HASLER, 1998; BUTTRISS, 2000).

Não há dúvida de que o interesse japonês por alimentos funcionais também trouxe a conscientização para a necessidade de tais produtos em locais como Europa, Estados Unidos e no Brasil, mais recentemente. Especialistas,

nesses países, perceberam que além de serem capazes de reduzir os custos com cuidados de saúde, devido ao envelhecimento da população, os alimentos funcionais também representam um crescente potencial comercial para a indústria de alimentos (SIRÓ et al., 2008).

O mercado global de alimentos funcionais deve faturar em 2014 US$ 29,8 bilhões, aumento de 22,8% em relação a 2010. O Japão, maior mercado consumidor e produtor de alimentos funcionais, deve faturar US$ 11,3 bilhões em 2014; nos Estados Unidos, as vendas devem crescer 20,7% no mesmo período, chegando a US$ 9,1 bilhões (ABRAS, 2012). Segundo dados da Abenutri (2010) - Associação Brasileira das Empresas de Produtos Nutricionais, atualmente o setor movimenta cerca de R$ 500 milhões por ano no país, representando aproximadamente 1% do total das vendas da indústria brasileira de processamento de alimentos. De acordo com a Associação Brasileira das Indústrias da Alimentação (ABIA), organização que representa os interesses da indústria brasileira de alimentos lácteos funcionais, os produtos nutracêuticos e produtos à base de soja ocupam um promissor nicho de mercado (ALIMENTO SEGURO, 2005).

Ainda, segundo a Abenutri (2010), o mercado de suplementos nutricionais no Brasil tem um potencial incrível, mas está "engatinhando" e é praticamente irrelevante quando comparado aos mercados americano, europeu, asiático e de vários outros países. Esse atraso na área de suplementos alimentares se deve à atual regulamentação desses alimentos, com medidas antiquadas e proibitivas, contrariando a tendência mundial dos alimentos funcionais e suplementos para melhorar a qualidade de vida, contribuindo negativamente para o crescimento financeiro, econômico e industrial do país.

Produtos com propriedades funcionais foram lançados principalmente na indústria de laticínios, produtos de confeitaria, de panificação, refrigerantes e no mercado de alimentos para bebês (KOTILAINEN et al., 2006; MENRAD,

2003). Os alimentos funcionais apresentam as seguintes características (ROBERFROID, 2002):

a) devem ser alimentos convencionais e serem consumidos na dieta normal/usual;
b) devem ser compostos por componentes naturais, algumas vezes em elevada concentração ou presentes em alimentos que normalmente não os supririam;
c) devem ter efeitos positivos, além do valor básico nutritivo, que podem aumentar o bem-estar e a saúde e/ou reduzir o risco de ocorrência de doenças, promovendo benefícios à saúde e aumento da qualidade de vida, incluindo os desempenhos físico, psicológico e comportamental;
d) alegação da propriedade funcional embasada cientificamente;
e) podem ser alimentos naturais ou alimentos nos quais um ou mais componentes tenham sido removidos;
f) podem ser alimentos onde a natureza de um ou mais componentes tenha sido modificada;
g) podem ser alimentos no quais a bioatividade de um ou mais componentes tenha sido modificada.

Os alimentos e ingredientes funcionais podem ser classificados de dois modos: quanto à fonte de origem, vegetal ou animal, ou quanto aos benefícios que oferecem, atuando em seis áreas do organismo: no sistema gastrointestinal; no sistema cardiovascular; no metabolismo de substratos; no crescimento, no desenvolvimento e diferenciação celular; no comportamento das funções fisiológicas e como antioxidantes (SOUZA; SOUZA NETO; MAIA, 2003). Alguns autores relatam que esses alimentos podem auxiliar na proteção contra

doenças como hipertensão, diabetes, câncer, osteoporose, coronariopatias e doenças crônico-degenerativas (NEUMANN et al., 2002; TAIPINA; FONTS; COHEN, 2002; SOUZA; SOUZA NETO; MAIA, 2003).

2.1.1 Probióticos

O uso dos probióticos não é novo, e, de fato, eles vêm sendo consumidos pelos seres humanos na forma de alimentos fermentados há milhares de anos (KOPP-HOOLIHAN, 2001). Seus benefícios para a saúde foram muito analisados, como, por exemplo, por Hipócrates e outros cientistas, sendo relatado que o leite fermentado pode curar alguns distúrbios do sistema digestivo. Escrituras bíblicas mencionam o uso de probióticos no tratamento de doenças do corpo (LOURENS-HATTINGH; VILJOEN, 2001).

Em 1907, o cientista russo Elie Metchnikoff propôs pela primeira vez o conceito dos probióticos como é conhecido hoje. Ele observou que o consumo de grandes quantidades de produtos lácteos fermentados com lactobacilos prolongava a vida. Formulou a primeira explicação científica para os efeitos benéficos do ácido lático e das bactérias do ácido lático presentes no leite fermentado (RASIC, 2003). Hoje é aceita que a ingestão diária desses probióticos contribui para melhorar e manter equilibrada a microbiota intestinal e evitar distúrbios gastrointestinais (LAVERMICOCCA, 2006).

De acordo com a definição da *Food and Agriculture Organization*/*World Health Organization* (FAO/WHO, 2001), os probióticos são "micro-organismos vivos que quando administrados em quantidades adequadas conferem um benefício à saúde do hospedeiro". Pela legislação brasileira, probióticos são definidos como: "micro-organismos vivos capazes de melhorar o equilíbrio microbiano intestinal produzindo efeitos benéficos à saúde do indivíduo" (ANVISA, 2002). São também conhecidos como

bioterapêuticos, bioprotetores ou bioprofiláticos e utilizados na prevenção de infecções entéricas e gastrointestinais (REIG; ANESTO, 2002).

Aplicações de alimentos como probióticos são encontradas principalmente em produtos lácteos, como iogurtes, kefir e bebidas inoculadas com tais micro-organismos. O desenvolvimento científico desses produtos tem mostrado a sua elevada aceitação sensorial (ALMEIDA et al., 2008; ZOELLNER et al., 2009). Aplicações alimentares emergentes incluem queijo probiótico e sorvetes (CRUZ et al., 2009a; 2009b), barras de cereais, fórmulas infantis e muitos outros (GRANATO et al., 2010). Alguns exemplos comerciais são citados no Quadro 1.

Quadro 1 Exemplos comerciais de produtos probióticos

Produto	**Descrição**	**Produtor**
Actimel	Leite fermentado com *Lactobacillus casei Imunitass*	Danone, França
Activia	Iogurte cremoso contendo *Bifidus regularis*	Danone, França
Gefilus	Uma vasta gama de produtos com LGG*	Valio, Finlândia
Hellus	Produto lácteo contendo *Lactobacillus fermentum ME-3*	Tallinna Piimato¨o¨ stuse AS, Estônia
Jovita Probiotisch	Mistura de cereais, frutas e iogurte probiótico	H&J Bruggen, Alemanha
Pohadka	Iogurte com culturas probióticas	Valasské Mezirící Dairy, República Tcheca
Pro Viva	Bebida de frutas refrescante e iogurte natural em diversos sabores, contendo *Lactobacillus plantarum*	Skane mejerier, Suécia
Rela	Iogurtes, leites e sucos cultivados com *Lactobacillus reuteri*	Ingman Foods, Finlândia
Revital Active	Iogurte e bebida láctea com probióticos	Olma, República Tcheca
Snack Fibra	Lanches e barras com fibras naturais, minerais e vitaminas	Celigueta, Espanha
SOYosa	Gama de produtos à base de soja e aveia. Inclui uma bebida refrescante e um iogurte probiótico de aveia-soja	Bioferme, Finlândia
Soytreat	Produto tipo kefir com seis probióticos	Lifeway, EUA

"continua"

Quadro 1 “conclusão”

Produto	Descrição	Produtor
Yakult	Leite fermentado contendo *Lactobacillus casei* Shirota	Yakult, Japão
Yosa	Iogurte de aveia aromatizado com frutas naturais e *berries*, contendo bactérias probióticas (*Lactobacillus acidophilus*, *Bifidobacterium lactis*)	Bioferme, Finlândia
Vitality	Iogurte com pré e probióticos e ômega-3	Muller, Alemanha
Vifit	Leites fermentados com LGG, vitaminas e minerais	Campina, Países Baixos

Fonte: Siró et al., 2008. [*]*Lactobacillus “Gorbach and Goldin”* – presentes naturalmente na microbiota intestinal de um indivíduo saudável.

O mercado global de ingredientes, suplementos e alimentos probióticos movimentou cerca de 14,9 bilhões de dólares em 2007 e atingiu 16 bilhões de dólares em 2008. Os probióticos do gênero *Lactobacillus* representaram a maior parte, computando 61,9% das vendas totais em 2007 e os iogurtes foram os principais produtos com utilização desses micro-organismos, totalizando 36,6% (FOOD PROCESSING, 2009).

Uma nova análise de mercado do *Dairy Reporter* informou que o mercado global de probióticos aumentará em 6,8% ao ano nos próximos cinco anos, direcionado pela região da Ásia-Pacífico, “que deverá ser o mercado mais proeminente no futuro”. Em termos de vendas, o relatório citou vendas de US$ 27,9 bilhões em 2011, devendo alcançar US$ 44,9 bilhões em 2018. Entretanto, questões de preços, cultura e falta de padronização das especificações dos produtos deverão ter um efeito inibidor no crescimento do mercado. O relatório destacou que as áreas de interesse para probióticos são lácteos, cereais, produtos fermentados de carne, alimentos desidratados, entre outros. A Ásia-Pacífico, China e Japão, dominam a receita do mercado para probióticos, com Índia e outras regiões também mostrando um crescimento significante. A Europa, Alemanha e Reino Unido estão entre os mercados mais atrativos. Os mercados da América do Norte e dos países emergentes como Brasil também estão

mostrando um grande potencial para crescimento na demanda (DAIRY REPORTER, 2013).

Os benefícios à saúde do hospedeiro, atribuídos à ingestão de culturas ou alimentos probióticos ou ambos, são: controle da microbiota intestinal, estabilização da microbiota intestinal após o uso de antibióticos, promoção da resistência gastrointestinal à colonização por patógenos, aumento da concentração dos ácidos acético e lático, de bacteriocinas e outros compostos antimicrobianos, promoção da digestão da lactose em indivíduos intolerantes a esse carboidrato, estimulação do sistema imune, alívio da constipação e aumento da absorção de minerais e vitaminas (SAAD, 2006).

Uma população correspondente a 10^9 células viáveis ou 10^7 células viáveis/100mL é a dose mínima diária recomendada para se obter os efeitos benéficos através de um alimento probiótico (FAO/WHO, 2001). Considerando que bactérias probióticas colonizam o trato intestinal transitoriamente, grandes populações precisam ser ingeridas diariamente para que os efeitos positivos sejam mantidos (REID et al., 2003).

Em um intestino adulto saudável, a microbiota predominante se compõe de micro-organismos promotores da saúde, em sua maioria pertencentes aos gêneros *Lactobacillus* e *Bifidobacterium*. Os *Lactobacillus,* geralmente citados como probióticos são: *L. casei*, *L. acidophilus, L. brevis, L. cellibiosus, L.lactis*, *L. fermentum, L. plantarum* e *L. reuteri*. As espécies de *Bifidobacterium* com atividade probiótica são: *B. bifidum, B. longum, B. infantis, B. adolescentis, B. thermophilum* e *B. animalis* (KALANTZOPOULOS, 1997). Segundo Holzapfel e Schillinger (2002), outras bactérias com propriedades probióticas são: *Enterobacter faecalis, Enterococcus faecium* e *Sporolactobacillus inulinus*, enquanto os micro-organismos *Bacillus cereus, Bacillus subtilis, Escherichia coli* Nissle, *Propionibacterium freudenreichii, Saccharomyces boulardii* e *Saccharomyces cerevisiae* têm sido citados como micro-organimos não láticos

associados a atividades probióticas, principalmente para uso farmacêutico ou em animais.

Diferentes isolados de *Lactobacillus*: *L. acidophilus, L. casei, L. crispatus, L. fermentum, L. gasseri, L. johnsonii, L. paracasei, L. plantarum, L. reuteri, L. rhamnous, L. helveticus, L. Lactis*; de *Bifidobacterium*: *B. bifidum, B. brevis, B. infantis, B. longum, B. lactis, B. adolescentis, B. essensis, B. laterosporus* e outras espécies como *E. coli nissle, Saccharomyces boulardii, Enterococcus francio, Propionibacterium, Pediococcus* e *Leuconostoc* podem ser considerados como as principais espécies e gêneros microbianos que têm sido usados como probióticos (SENOK; ISMAEEL; BOTTA, 2005; SHAH, 2007).

Esses probióticos utilizados em alimentos são principalmente adicionados como parte do processo de fermentação. No entanto, cada vez mais eles vêm sendo adicionados como suplementos. Há também tendência crescente na utilização de probióticos como nutracêuticos, estando disponíveis em várias formas, como em cápsulas. Entretanto, essa tendência na utilização de probióticos pode levar à redução na eficácia da capacidade funcional devido à exclusão dos potenciais efeitos sinérgicos dos alimentos (RANADHEERA; BAINES; ADAMS, 2010).

Independentemente da diversidade desses micro-organismos, os principais preparados probióticos disponíveis atualmente no mercado pertencem a um grupo de bactérias designadas bactérias do ácido láctico (BAL), por exemplo, *Lactobacillus* e *Streptococcus,* que incluem a actinobactéria *Bifidobacterium.* As bactérias láticas são Gram-positivas, não esporuladas e produzem ácido lático como principal ou único produto da fermentação. As homofermentativas produzem duas moléculas de ácido lático, enquanto que as heterofermentativas produzem ácido lático, etanol e uma molécula de dióxido de carbono a partir de uma molécula de glicose (CHANG; STONE, 1990).

Particularmente, *Streptococcus salivarius* subsp. *thermophilus* e *Lactobacillus delbrueckii* subsp. *bulgaricus* têm sido tradicionalmente usados na produção de iogurte. Esses micro-organismos não são capazes de sobreviver e crescer no trato intestinal do hospedeiro, portanto não são classificados como probióticos pela maioria dos cientistas e são considerados apenas BAL iniciadoras de iogurte (SENOK; ISMAEEL; BOTTA, 2005).

Alimentos fermentados por BAL apresentam melhores valores nutricionais em função da digestão parcial de proteínas, gorduras, carboidratos e aumentam as vitaminas do complexo B, além de produzirem a β-D-galactosidase. Efeitos indiretos dessa fermentação constituem inibição de fatores antinutricionais, produção de toxinas e produção de compostos antimicrobianos (DEMIATE; OETTERER; WOSIACKI, 1994).

2.1.1.1 A importância dos produtos probióticos não lácteos

Além de produtos lácteos, outros alimentos que podem ser parte de uma dieta diária têm sido investigados como carreadores de probióticos. A pesquisa atual está focada no desenvolvimento de novos alimentos probióticos não lácteos para atender às necessidades mais amplas de sabores e abrir novas áreas de mercado (CHAMPAGNE; GARDNER, 2005; FARNWORTH; MAINVILLE; DESJARDINS, 2007; RANADHEERA; BAINES; ADAMS, 2010; RIVERA-ESPINOZA; GALLARDO-NAVARRO, 2010; RATHORE; SALMERÓN; PANDIELLA, 2012; SALMERÓN; THOMAS; PANDIELLA, 2014).

É muito difícil encontrar dados que relatam as vendas com base em produtos não lácteos probióticos, uma vez que essa categoria é relativamente nova no mercado. Além disso, esses produtos não são comumente encontrados em muitos países, como no Brasil, onde existem poucas marcas que

comercializam o iogurte de soja com polpas de frutas diferentes. Seria interessante se mais dados a respeito das vendas de alimentos probióticos estivessem disponíveis, pois a indústria poderia dirigir o desenvolvimento do produto para um segmento específico da população ou para um determinado tipo de produto bem aceitável (GRANATO et al., 2010).

Os produtos probióticos não lácteos têm grande importância devido à crescente tendência do vegetarianismo e à alta prevalência de intolerância à lactose em muitas populações, em todo o mundo. No entanto, não há dúvida de que o setor de laticínios, que está fortemente ligado aos probióticos é o maior mercado de alimentos funcionais, respondendo por quase 33% do mercado em geral (RIVERA-ESPINOZA; GALLARDO-NAVARRO, 2010). Segundo pesquisa Ibope feita em 2010, o Brasil conta com pouco mais de 15 milhões de vegetarianos (pessoas que não se alimentam de carne, mas que podem ou não consumir outros produtos de origem animal), ou 8% da população do país (NATURAL TECH, 2013).

Os dados sobre a incidência de intolerância à lactose não são facilmente encontrados. No Brasil, embora existam poucos estudos, a incidência de intolerância à lactose atinge cerca de 40% da população (AGÊNCIA BRASIL, 2013). A intolerância à lactose é a incapacidade de digerir o principal carboidrato do leite, resultado da deficiência ou ausência da enzima intestinal β-galactosidase, ou, simplesmente, lactase (LONGO, 2006). Essa enzima possibilita a quebra da molécula do carboidrato em monossacarídeos (galactose e glicose), facilitando a sua absorção pelo intestino. As pessoas deficientes em lactase não têm essa capacidade e quando consomem leite a lactose não é desdobrada no intestino delgado, não sendo absorvida. Desta forma, a pressão osmótica do intestino aumenta e uma quantidade considerável de água é retirada dos tecidos vizinhos. Então, a lactose passa para o intestino grosso, onde é fermentada por grupos microbianos produtores de gases e água ou é hidrolisada

por bactérias em ácidos orgânicos de cadeia curta. Os gases que não são absorvidos causam inchaço e os ácidos produzidos irritam a parede intestinal e aumentam a motilidade que, combinados com a água secretada no intestino resultam em diarréia (FERREIRA, 1997; BARBOSA; ANDREAZZI, 2009).

Recentemente, uma pesquisa sinalizou que os iogurtes disponíveis no mercado brasileiro são impróprios para consumo por indivíduos intolerantes à lactose, devido à pequena redução do teor de lactose durante o tempo de vida útil (BATISTA; SILVA; DA CRUZ, 2008). Portanto, a literatura especializada tem apontado para um interesse emergente em estudos regionais, assim como para o fato de que indústrias de alimentos estão se adaptando às significativas diferenças regionais em tradições, culturas, costumes e práticas.

2.1.2 Prebióticos

Os prebióticos são oligossacarídeos não digeríveis, porém fermentáveis por micro-organismos, cuja função é mudar a atividade e a composição da microbiota intestinal com a perspectiva de promover a saúde do hospedeiro. As fibras dietéticas e os oligossacarídeos não digeríveis são os principais substratos para o crescimento dos micro-organismos nos intestinos (MORAES; COLLA, 2006).

Nos últimos anos houve um rápido aumento no uso de prebióticos como alimentos funcionais e novos produtos estão sendo desenvolvidos a todo o tempo, sendo adicionados a suplementos, produtos lácteos, pães e bebidas (WILLIAMSOM, 2009). A procura por prebióticos no mundo é estimada em cerca de 167.000 toneladas e gira em torno de 390 milhões de euros por ano. Entre eles, fruto-oligossacarídeos (FOS), inulina, lactulose, isomalto-oligossacarídeos (IMO), polidextrose e amido resistente são considerados os principais componentes prebióticos disponíveis no mercado (SIRÓ et al., 2008).

Oligossacarídeos desempenham um importante papel no controle da obesidade, resultante do aumento da saciedade e redução da fome (BOSSCHER; VAN LOO; FRANK, 2006). Alguns outros efeitos atribuídos aos prebióticos são: a modulação de funções fisiológicas-chave, como a absorção de cálcio, o metabolismo lipídico, a modulação da composição da microbiota intestinal, a qual exerce um papel primordial na fisiologia intestinal e na redução do risco de câncer de cólon (ROBERFROID, 2002).

Um produto em que estão combinados um probiótico e um prebiótico é denominado simbiótico. A interação entre o probiótico e o prebiótico *in vivo* pode ser favorecida por uma adaptação do probiótico ao substrato prebiótico anterior ao consumo. Isto pode, em alguns casos, resultar em uma vantagem competitiva para o probiótico, se ele for consumido juntamente com o prebiótico (SAAD, 2006). Há uma sinergia entre probióticos e prebióticos em produtos simbióticos. Prebióticos são compostos consumidos por probióticos como carbono ou fonte de energia no cólon. Isso resulta em aumento da contagem do probiótico e na redução de micro-organismos patogênicos no intestino (HOMAYOUNI et al., 2008).

Os prebióticos estimulam o crescimento dos grupos endógenos da população microbiana, tais como as bifidobactérias e os lactobacilos, que são ditos como benéficos para a saúde humana (BLAUT, 2002). Dentre as características de um prebiótico temos: resistência às enzimas salivares, pancreáticas e intestinais, bem como ao ácido estomacal; não deve sofrer hidrólise enzimática ou absorção no intestino delgado; quando atingir o cólon deve ser metabolizado seletivamente por número limitado de bactérias benéficas; deve ser capaz de alterar a microbiota colônica para uma microbiota bacteriana saudável e ser capaz de induzir efeito fisiológico que seja importante para a saúde; e ser osmoticamente ativa (RODRÍGUEZ et al., 2003).

A soja contém prebióticos como rafinose e estaquiose que são promotores naturais do crescimento de bifidobactérias (TAMIME; MARSHALL; ROBINSON, 1995). Os produtos da soja vêm sendo avaliados como veículos para probióticos e estudos recentes têm indicado uma série de benefícios para a saúde. Os efeitos sinérgicos entre os probióticos e produtos da soja sugerem que essa combinação pode exercer efeitos vantajosos e aumentar valores terapêuticos nos alimentos (WOO et al., 2009).

Um grupo específico de oligossacarídeos que tem atraído interesse comercial é o dos frutooligossacarídeos (FOS). Os FOS são formados por uma molécula de sacarose com uma, duas ou três unidades de frutose unidas, mediante ligações β-(1-2) à molécula de sacarose (FERREIRA; TESHIMA, 2000). Esses compostos, comercializados como Raftilose e NutraFlora, podem ser obtidos de fontes naturais (por exemplo, a inulina) ou sintetizados enzimaticamente a partir da sacarose por ação de b-frutofuranosidase fúngica (CRITTENDEN, 1999; KAPLAN; HUTKINS, 2000). Os FOS foram considerados pela FDA (*Food and Drug Administration*) como substância GRAS (geralmente reconhecidas como seguro) e foram adicionados a fórmulas para lactentes, iogurtes e suplementos alimentares (SPIEGEL et al., 1994).

Os efeitos benéficos da ingestão de FOS têm sido comprovados por diversos estudos. Esses açúcares não convencionais foram classificados como assistentes dos micro-organismos benéficos presentes no trato intestinal, como *Lactobacillus* e *Bifidobacterium*. Eles melhoram o metabolismo das bifidobactérias e diminuem o pH no intestino grosso, inibindo bactérias putrefativas. A ingestão diária desses carboidratos resulta em aumento da quantidade de bifidobactérias no trato intestinal (HARTEMINK; DOMENECH; ROMBOUTS, 1997; PASSOS; PARK, 2003).

2.2 Extrato hidrossolúvel vegetal e derivados

A produção de extrato vegetal usando leguminosas e sementes oleaginosas é uma tecnologia antiga, relatada desde o século 13. Com o tempo, a tecnologia foi aprimorada para incluir o desenvolvimento de alternativas vegetais ao leite de vaca, especialmente na formulação de alimentos infantis, uma vez que são ricos em proteínas, minerais e vitaminas. As principais matérias-primas que têm sido utilizadas na produção de extratos hidrossolúveis incluem a soja, o fejão-caupi, feijão alado ou de asa, amendoim e sementes de melão (NELSON; STEINBERG; WEI, 1976; AIDOO et al., 2010).

Em muitos países, especialmente naqueles em desenvolvimento, o abismo crescente entre os recursos disponíveis e a população tem resultado em grave insuficiência no fornecimento de nutrientes. Neste déficit, pode ser incluído o leite e seus derivados, principalmente por causa de suas características únicas de fornecer nutrientes essenciais. A escassez na oferta de leite tem levado a esforços para o desenvolvimento de produtos alternativos a esta matéria-prima (NELSON; STEINBERG; WEI, 1976; ASIEDU, 1994).

O extrato hidrossolúvel que é produzido a partir de matérias-primas de origem vegetal, como o de amendoim e de soja pode servir como uma ótima alternativa para os países onde o fornecimento de leite é inadequado. Leguminosas e sementes oleaginosas possuem características que as tornam convenientes para realizar combinações de duas ou mais espécies a fim de se obter um produto aceitável. Um produto composto, utilizando amendoim e soja como fontes de nutrientes, como proteínas, fibras, ácido fólico, outras vitaminas e minerais seria um bom substituto do leite animal (AIDOO et al., 2010).

Atualmente, o interesse em extrato hidrossolúvel vegetal tem sido renovado por causa da crescente conscientização dos consumidores em relação aos benefícios nutricionais de alimentos à base de plantas (DIARRA; NONG,

2005). Este cenário vai ao encontro do grande número de pessoas que está aderindo a uma alimentação e estilo de vida mais voltados ao bem-estar e à ética alimentar. Consequentemente, os extratos de soja e de amendoim, separadamente, têm sido utilizados em vários produtos, incluindo bebida fermentada probiótica (CAMARGO et al., 2000), creme de café (MULANDO; RESURRECCIÓN, 2006), bebida láctea sabor chocolate (DESHPANDE; CHINNAN; PHILLPS, 2008), coalhada (YADAV et al., 2010), combinação de extrato hidrossolúvel de soja, da polpa de manga e leite de búfala (KUMAR; MISHRA, 2004) e iogurte (ISANGA; ZANG, 2009).

2.2.1 Extrato hidrossolúvel de amendoim e derivados

O amendoim (*Arachis hypogaea* L.) é uma leguminosa oleaginosa de origem sul-americana, considerada altamente valiosa na nutrição humana e animal. Rica em óleo, proteínas e vitaminas era uma importante fonte de energia e aminoácidos utilizada intensamente na alimentação dos indígenas, antes da colonização. No século XVIII foi introduzido na Europa. No século XIX difundiu-se do Brasil para a África e do Peru para as Filipinas, China, Japão e Índia. Nos dias atuais o amendoim é um produto conhecido e apreciado em praticamente todos os países do mundo, pelo seu incomparável sabor e versatilidade de uso em pratos salgados, doces e na indústria (ARAÚJO et al., 2000). O produto destina-se principalmente à indústria de confeitaria, venda/consumo *in natura*, exportação e fabricação de óleo.

Há algum tempo é sabido que o extrato de amendoim e produtos derivados de amendoim têm benefícios nutricionais para jovens e idosos devido à extrema riqueza em proteínas, minerais e ácidos graxos essenciais, como os ácidos linoléico e oléico (ISANGA; ZHANG, 2009). São amplamente utilizados na Índia e em outros países em desenvolvimento, por vegetarianos e, mais

recentemente, por crianças alérgicas a proteínas do leite de vaca (KOUANE; ZHANG; CHEN, 2005). A figura 1 mostra alguns dos benefícios à saúde através do consumo de amendoim.

Figura 1 Benefícios que o amendoim pode trazer à saúde. Fonte: Abicab, 2013. Disponível em: http://www.abicab.org.br/amendoim/o-amendoim-e-seus-beneficios/

Nos últimos 50 anos, muitas maneiras de produzir extrato de amendoim foram desenvolvidas por diversos pesquisadores (CHAN; BEUCHAT, 1992; KOUANE; ZHANG; CHEN, 2005), mas todos esses métodos são modificações do processo de *Illinois* para o preparo de extrato de soja (CHAN; BEUCHAT, 1992). A composição do extrato de amendoim depende do modo de preparo, mas em todos os casos esse extrato hidrossolúvel de baixo custo tem elevado conteúdo de proteína e é conhecido por ser rico em energia (KOUANE; ZHANG; CHEN, 2005).

O extrato hidrossolúvel de amendoim pode ser produzido por maceração e trituração dos grãos crus de amendoim com água até se obter uma pasta, sem prejuízo para a filtração (CHAN; BEUCHAT, 1992). Alternativamente, pode também ser produzido pela moagem de amendoins crus e/ou torrados, com teor de gordura integral ou parcialmente desengordurado, em forma de farinha para que a água possa ser adicionada posteriormente, obtendo-se emulsão.

Outra maneira é usar o extrato de amendoim ou proteína isolada de amendoim para suplementar o leite de origem animal (leite tonificado). Em todos os casos a relação de farinha de amendoim ou amendoim e água variam muito de um produtor para outro. Os produtos não lácteos obtidos são em seguida homogeneizados e pasteurizados, em muitos casos, da mesma maneira que o leite fresco. Além de serem suplementados com vitaminas e minerais podem também ser saborizados (CHAN; BEUCHAT, 1992).

O Brasil, até o início dos anos 70 do século passado, foi importante produtor de amendoim, ocupando papel expressivo tanto no suprimento interno de óleo vegetal quanto na exportação de subprodutos. A maior produção ocorreu em 1972, com 970 mil toneladas, sendo que o principal produto era o óleo, muito utilizado na culinária. A partir de 1974, devido, dentre outros fatores, à contaminação por aflatoxina e a maior disponibilidade de óleo de soja,

o preço do produto nos mercados interno e externo caiu desestimulando o plantio. A partir daquele período até a década de 1990 houve forte redução na área plantada e produção em nosso país (ARAÚJO et al., 2000).

Entretanto, nos últimos anos, no Brasil, ocorreu grande expansão da área cultivada, produção e principalmente aumento consistente em produtividade. Da produção de 142 mil toneladas em 1995, chegamos a 326,3 mil toneladas em 2012/13, enquanto a produtividade passou de 1.740 kg/ha em 1994/96 para 3.379 kg/ha em 2012/13 (FREITAS et al., 2005; CONAB, 2013). O uso de cultivares de porte rasteiro e a mecanização das operações de plantio e colheita são tecnologias que refletiram em maior produtividade (ARAÚJO et al., 2000).

Em termos produtivos, o amendoim brasileiro é oriundo, em maior escala, da região Sudeste, seguida pela Centro-Oeste e Nordeste. O estado de São Paulo é o maior produtor, responsável por cerca de 80% da produção nacional (Figura 2). A produção de amendoim é feita em duas épocas: a primeira, conhecida como safra das águas, representa 75% do volume total e corresponde aos plantios realizados em outubro/novembro nas regiões Sudeste e Sul. A segunda, chamada de safra da seca, complementa o montante, sendo os plantios realizados no mês de março nas regiões Sudeste e Nordeste (ARAÚJO et al., 2000).

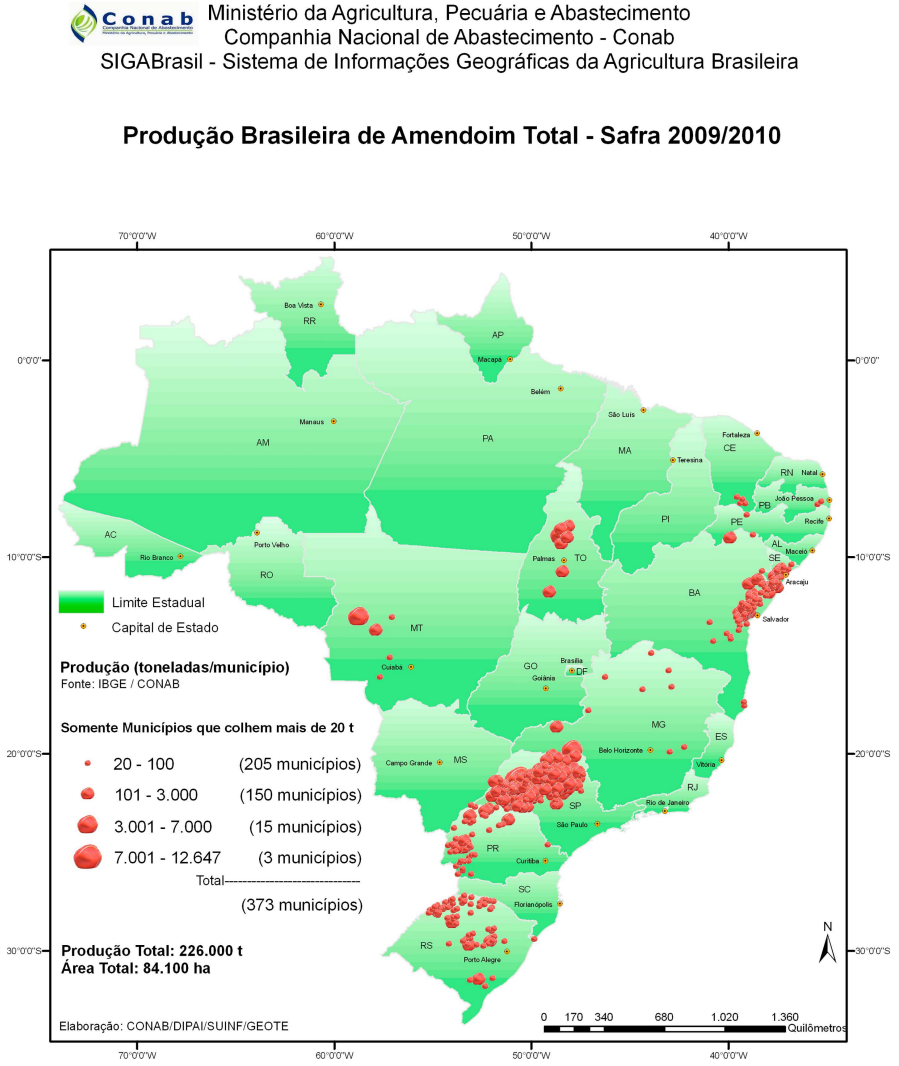

Figura 2 Produção brasileira de amendoim total – safra 2009/2010. Fonte: CONAB, 2010. Disponível em: http://www.conab.gov.br/OlalaCMS/uploads/arquivos/13_08_19_16_50_01_ bramendoimtotal2010.png

2.2.2 Soja e derivados

A soja (*Glicine max* L.) é uma leguminosa produzida no Brasil desde 1920. A partir de 1950, esse produto tem sido utilizado na produção de alimentos ricos em proteína, incluindo farinha, concentrado protéico e isolado protéico de soja. Apesar de conhecida e explorada no Oriente há mais de cinco mil anos (é reconhecida como uma das mais antigas plantas cultivadas do planeta), o Ocidente ignorou o seu cultivo até a segunda década do século XX, quando os Estados Unidos iniciaram sua exploração comercial (primeiro como forrageira e, posteriormente, como grão). Em 1940, no auge do seu cultivo como forrageira, foram cultivados, naquele país, cerca de dois milhões de hectares com tal propósito (MARTINS, 1995).

A produção de soja no mundo em 2011/12 foi de 236,03 milhões de toneladas. Já no Brasil, que era o segundo maior produtor mundial do grão, a produção foi de 65,50 milhões de toneladas. Na safra de 2012/13 o Brasil superou os Estados Unidos produzindo 81,00 milhões de toneladas, representando um aumento de 23,7% na produção. Os Estados Unidos, no mesmo período, produziram 73,27 milhões de toneladas, ocupando o segundo lugar na produção do grão (SCOT CONSULTORIA, 2013).

A Agroconsult prevê que a área de plantio com soja no Brasil na safra 2013/14, em fase inicial de preparo, deve crescer 5,8%, alcançando 29,3 milhões de hectares em comparação com 27,7 milhões de hectares no período anterior. Com o aumento da área, a produção brasileira de soja pode alcançar 88,4 milhões de toneladas na safra 2013/14 (AGROCONSULT, 2013).

Uma grande proporção de proteínas vegetais é utilizada na agroindústria. O isolado protéico de soja possui alto valor nutricional e vem sendo utilizado na formulação de suplementos visando à substituição parcial ou total da proteína do leite (GIESE, 1994). As proteínas da soja possuem

excelentes propriedades nutricionais e funcionais, sendo amplamente utilizadas em diversos produtos alimentares. O consumo da proteína de soja, que é rica em isoflavonas, pode ter um efeito benéfico sendo observados resultados no combate aos desconfortos causados pelo climatério, já que tem ações estrogênicas e antiestrogênicas, predominantemente sobre receptores de estrogênios. Tem também um efeito antioxidante, diminuindo radicais livres e inibindo os danos causados pelos raios ultravioletas que causam problemas na pele, tornando-a fina, ressecada e sem elasticidade (CLAPAUCH et al., 2002).

Um exemplo de linha de bebidas à base dessa leguminosa é a Sollys, da Nestlé, que oferece a versão Original (conhecida popularmente como leite de soja) e a versão Frutas (alimento com soja e suco de frutas em cinco sabores: abacaxi, laranja, maçã, pêssego e uva). Segundo a empresa, trata-se da única marca do segmento de bebidas à base de soja a apresentar o selo de aprovação da ABRAN – Associação Brasileira de Nutrologia. A linha é vendida em embalagens de 1 litro e de 200 mL e os produtos contêm 0% Lactose e 0% Colesterol, além de serem fontes de cálcio e de vitamina C – contribuindo para a saúde dos ossos e dentes e auxiliando na proteção do organismo. Além disso, os produtos da linha Sollys são acrescidos de proteínas de soja ACTISOY. A proteína isolada da soja proporciona melhor sabor aos produtos quando comparados a outros que são acrescidos de extrato de soja (BERTI, 2010).

Produtos à base de soja trazem vantagens à saúde por apresentarem propriedades hipolipêmicas, anticolesterol e baixa alergenicidade, embora o seu consumo direto seja limitado devido ao sabor e indução de flatulências (KARLESKIND et al., 1991). A falta de β-galactosidases, que hidrolisam os carboidratos na soja (sacarose, rafinose e estaquiose), resulta na produção de gases no trato gastrointestinal humano. Os sabores desagradáveis (*off flavor*) dos produtos de soja são atribuídos à ação das enzimas lipoxigenases formando

hidroperóxidos dos ácidos graxos poli-insaturados (RACKIS; SESSA; HONIG, 1979).

2.3 Fermentação de produtos à base de soja e de amendoim

A fermentação é um processo biológico em que as multiplicações bacterianas e fúngicas alteram as características sensoriais e produzem metabólitos que preservam ou enriquecem a composição nutricional (DEMIATE; OETTERER; WOSIACKI, 1994). As bactérias probióticas geralmente não crescem rapidamente em leite de vaca. Assim, na fabricação do iogurte elas não alcançam elevado número como outras BAL (CHAMPAGNE et al., 2009).

Por outro lado, muitos estudos indicam que a soja é um bom substrato para as bactérias probióticas, mas não para a tradicional cultura iniciadora de iogurte *L. delbrueckii* subsp. *bulgaricus* (KARLESKIND et al., 1991). Estes resultados sugerem que algumas bactérias probióticas poderiam competir melhor com culturas iniciadoras de iogurte em um substrato à base de soja. As principais bactérias probióticas já aplicadas em bebidas de soja são *Lactobacillus acidophilus, Lactobacillus fermentum* e *Bifidobacterium* sp.. Entretanto, pouco se sabe sobre *Lactobacillus rhamnosus, Lactobacillus helveticus, Lactobacillus delbrueckii* subsp. *lactis*. Uma característica da fermentação da soja por probióticos é a taxa de acidificação de acordo com a linhagem utilizada. Segundo Chumchuere & Robinson (1999), as culturas selecionadas para a fermentação de extrato hidrossolúvel de soja (12% de sólidos totais) utilizaram efetivamente os respectivos oligossacarídeos e reduziram o pH da bebida.

Com relação ao extrato hidrossolúvel de amendoim, ao longo dos anos foi convertido com êxito em produtos alimentares de baixo custo, com alto

valor nutritivo. Nesta área, os pesquisadores se concentraram em produtos resultantes da fermentação, tais como o iogurte, o leitelho e o queijo curado, entre outros (CHAN; BEUCHAT, 1992). O preparo e a fermentação do extrato de amendoim podem servir como forma de aumentar o consumo dessa valiosa cultura (SUNNY-ROBERTS; OTUNOLA; IWAKUM, 2004).

Sunny-Roberts, Otunola e Iwakum (2004) prepararam um produto semelhente a iogurte a partir de extrato de amendoim e encontraram um aumento nos teores de cinzas totais, cálcio, potássio e fósforo, quando comparado com uma amostra de extrato não fermentado. O teor de proteína aumentou de 2,98% para 5,95% na amostra fermentada. Foi observado também aumento de lisina, metionina e triptofano de 3,90 para 7,28; 1,12 para 3,15; e 1,41 para 2,43 g/16g de nitrogênio, respectivamente. Por outro lado, foram observados decréscimos com relação à fibra bruta e conteúdo de gordura total. Os mesmos autores relataram que os iogurtes de extrato de amendoim com sabor de baunilha e morango tinham quase a mesma aceitabilidade sensorial quando comparados ao produto comercial do leite de origem animal, podendo ser bons substitutos, especialmente quando o custo foi levado em consideração.

Os avanços na fabricação de produtos fermentados revelaram que o hexanal, um dos compostos responsáveis pelo sabor indesejado de nozes no extrato de amendoim, desapareceu completamente como resultado da fermentação (LEE; BEUCHAT, 1991). Neste sentido, frutas como morango, manga, uva, pêssego, abacaxi, laranja, maçã e maracujá têm provado ter a capacidade de mascarar o sabor forte de bebidas baseadas em soja. Essas frutas são populares por fornecerem um perfil de sabor suave e agradável, podendo, desta forma, também serem saborizantes para bebida fermentada à base de amendoim.

Isanga e Zhang (2007) observaram textura inferior do iogurte à base de extrato de amendoim, quando comparado ao do iogurte de leite de vaca, embora

fosse aceitável. Beuchat e Nail (1978) observaram textura semelhante a um creme no extrato fermentado de amendoim com *Lactobacillus bulgaricus* 1909 e *Lactobacillus acidophilus* B-1910 e relataram que o soro excessivo tornou o produto indesejável para os substitutos do iogurte.

Diante do exposto, estudos mais diversificados devem ser realizados a fim de melhorar a estabilidade, superar o sabor de nozes e problemas sensoriais encontrados durante a produção do extrato hidrossolúvel de amendoim e seus derivados.

2.4 Emprego de coculturas

Industrialmente, curtos tempos de fermentação são preferíveis a fim de se aumentar a produção das plantas industriais, bem como reduzir a contaminação por micro-organismos indesejáveis. Além disso, as culturas probióticas por si só podem gerar produtos com *flavour* desagradável. Uma potencial solução para esses dois problemas é o uso de culturas probióticas misturadas a culturas iniciadoras de iogurte. O emprego de coculturas permite a fermentação em tempos aproximadamente duas vezes menores do que aqueles nos quais são utilizadas culturas puras, tornando-se adequado para a indústria (MACEDO; SOCCOL; FREITAS, 1998).

O uso simultâneo de culturas probióticas com culturas iniciadoras de iogurte pode ser muito prejudicial para algumas cepas. Assim, o desenvolvimento de um produto fermentado contendo probióticos requer seleção de linhagens que apresentem boa capacidade de crescer no substrato, bem como habilidade de competir ou até mesmo criar uma sinergia entre as linhagens (MACEDO; SOCCOL; FREITAS, 1998). A combinação mais apropriada de uma cultura iniciadora a uma bactéria probiótica específica deve ser determinada por processo de seleção, que irá avaliar o impacto das

diferentes culturas sobre as propriedades sensoriais e a sobrevivência das bactérias probióticas.

Em função da perda de viabilidade, o emprego de culturas probióticas associadas a culturas de suporte compostas, de preferência de *S. thermophilus* ou de outra cultura de iogurte ou culturas mesofílicas com diferentes combinações de cepas de *Lactococcus,* é aconselhável. Em determinados casos, a combinação de cepas de culturas probióticas com culturas iniciadoras de iogurte resulta na diminuição da contagem da cultura probiótica durante a vida de prateleira do produto. Esse fato demonstra que a seleção de uma cultura de suporte apropriada para cada cultura probiótica é fundamental para a obtenção de produtos frescos fermentados, com boa sobrevivência de culturas probióticas durante sua vida útil (SAXELIN et al., 1999; SAARELA et al., 2000).

Culturas iniciadoras que são capazes de crescer durante a fermentação e são termofílicas devem ser preferencialmente utilizadas, uma vez que a maioria dos probióticos multiplica-se bem a 37ºC, sendo esta a temperatura ideal para o processo fermentativo utilizando tais micro-organismos. Adicionalmente, a taxa de multiplicação da cultura iniciadora deve ser moderada, permitindo a multiplicação da bactéria probiótica. A cultura iniciadora também deve produzir compostos que favoreçam a multiplicação da cultura probiótica ou promover redução da tensão de oxigênio (SAARELA et al., 2000).

Em determinados casos, as cepas probióticas são adicionadas ao leite após a fermentação, o que vem a encarecer o produto. Entretanto, essa adição posterior não propicia a multiplicação do probiótico, podendo, inclusive, resultar em diminuição de sua viabilidade. Assim sendo, é recomendável a adição do probiótico antes ou concomitantemente à adição da cultura iniciadora (SAARELA et al., 2000).

Tradicionalmente e economicamente, as leveduras são os mais importantes micro-organismos explorados pelo homem. As leveduras têm uma

longa história de uso seguro, comprovado na fermentação de alimentos e bebidas. Por outro lado, as leveduras também podem causar deterioração nestes produtos (JAKOBSEN; NARVHUS, 1996). O ecossistema microbiano de bebidas e alimentos fermentados é complexo, composto por uma variedade de micro-oganismos como bactérias, leveduras e, às vezes, fungos filamentosos (BLANDINO et al., 2003). Desta forma, também há a possibilidade da utilização de leveduras em cocultura para a produção de fermentados alcoólicos e não alcoólicos.

Soulides (1955) mostrou que leveduras *Torulopsis* sp. (agora *Candida* sp.) podem estender a viabilidade de bactérias do iogurte durante meses. Um estudo mais recente mostra a bioestabilização de kefir com *Saccharomyces cerevisiae* devido à formação de etanol e à utilização de açúcar (KWAK; PARK; KIM, 1996). Algumas evidências indicaram que as leveduras podem ajudar na estabilização da população de bactérias do ácido láctico no queijo e iogurte (ARFI et al., 2004; FREITAS et al., 2009).

Liu e Tsao (2009) estudaram os efeitos de leveduras sobre a sobrevivência de BAL probióticas e não probióticas em leite fermentado, em condições não refrigeradas (30°C), visando desenvolver um leite fermentado que apresentasse características favoráveis para a sobrevivência das bactérias. Os resultados obtidos forneceram evidência definitiva de que as leveduras possuem efeitos que aumentam a estabilidade de BAL em laboratório e que os efeitos específicos destas sobre a estabilidade variam de acordo com as leveduras e com as BAL utilizadas.

As indústrias de alimentos, cada vez mais visam um mercado de consumo específico e competitivo. O resultado é uma demanda por ingredientes funcionais com diversas aplicações em alimentos formulados, sendo que as proteínas do amendoim e da soja preenchem essas condições com qualidade e baixo custo. Visto que essas matérias-primas podem desempenhar importante

papel na luta contra a desnutrição, o baixo nível atual no seu consumo, especialmente nos países em desenvolvimento, deve ser aumentado.

Diante do exposto, as crescentes evidências científicas a respeito dos efeitos benéficos promovidos pelos alimentos probióticos e prebióticos, associadas à exigência por parte do mercado consumidor por alimentos ditos mais saudáveis, surgem como fatores positivos para o desenvolvimento de um extrato hidrossolúvel vegetal simbiótico. São, portanto, necessários estudos para a adequada investigação sobre a possibilidade de processamento do amendoim e da soja em novos produtos úteis, comestíveis e com boas características nutricionais e sensoriais.

Desta forma, neste trabalho o extrato hidrossolúvel de amendoim foi utilizado como principal matéria-prima para formular uma bebida probiótica, que poderá resultar em benefícios para a saúde, além de explorar o potencial mercado para o extrato hidrossolúvel de amendoim e amendoim em geral. O estudo teve como objetivo descrever o processo de preparação do extrato fermentado de amendoim e soja, utilizando leveduras, bactérias do ácido lático e bactérias probióticas comerciais, além de avaliar parâmetros físico-químicos e metabólitos produzidos durante a fermentação em culturas simples e em cocultura.

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CAPÍTULO 2 Development and characterization of a nutritional peanut-soy milk for a fermented beverage

Claudia Cristina Auler do Amaral Santos[1], Bárbara da Silva Libeck[2], Rosane Freitas Schwan[3]

[1] Department of Biology, Federal University of Lavras (UFLA), CP 3037 - Campus Universitário, CEP 37.200-000 Lavras, MG, Brazil

[2] Department of Food Science, , Federal University of Lavras (UFLA), CP 3037 - Campus Universitário, CEP 37.200-000 Lavras, MG, Brazil

[3] Department of Food Science, Federal University of Lavras (UFLA), CP 3037 - Campus Universitário, CEP 37.200-000 Lavras, MG, Brazil. Corresponding author. [telephone (+55) 35 3829 1614; fax (+55) 35 3829 1100; e-mail: rschwan@dbi.ufla.br

ABSTRACT

Non-dairy formulations using different peanut-soy milk (PSM) blends were investigated to develop a new nutritive substrate for fermentation. The PSM was found to be rich in essential amino acids, notably leucine, isoleucine, lysine, and threonine [2.90, 1.78, 1.75 and 1.71 (g/100g) of PSM, respectively]. The contents obtained of crude protein, fat, dry matter, fiber and carbohydrate were 4.00, 3.17, 9.30, 0.39 and 1.62 (g/100 mL), which can be compared to cow's milk. The PSM feasibility for growth and acidification by lactic acid bacteria and yeasts was studied by inoculation with 1% (v/v) of the strain of interest and fermentation was followed over a 16 h period at 37 ºC. The *Pediococcus acidilactici* (UFLA B FFCX 27.1), *Lactobacillus rhamnosus* (LR 32) and *Lactobacillus acidophilus* (LACA 4) probiotics, and the yeast *Kluyveromyces marxianus* (CCT 3172) reached populations above 8 log CFU/mL after 24 hours of fermentation. Drops in pH values (approximately 4.00 after 12 h) were found for the PSM inoculated with *Bifidobacterium longum* (BL 04), *Streptococcus thermophilus* (TA 40) and with *Lactobacillus fermentum* (CC 305). The *Kluyveromyces marxianus* (CCT 3172), *Saccharomyces cerevisiae* (UFLA Y FFFW 15), *Saccharomyces cerevisiae* (UFLA Y FFCX 101.8), and *Debaryomyces etchelsii* (UFLA Y FFBM 5.56) yeasts consumed about 90% of available carbohydrates. Despite the high use of the sugars these yeasts provided moderate acidification of the substrate, reaching final pH above 5.00.

Keywords: Peanut and soy beverage. Mixed substrates. Lactic acid fermentation. Probiotic culture.

1 INTRODUCTION

In some developing countries, where milk and dairy products are usually priced too high for low income earners and are also invariably produced in insufficient quantities, the use of vegetable source is excellent option to aid the problem of protein deficiency (KPODO et al., 2013). The demand for alternatives to cow milk is growing, mainly due to problems with allergenicity, desire for vegetarian products, and high cholesterol levels (SUNNY-ROBERTS; OTUNOLA; IWAKUN, 2004, FARNWORTH; MAINVILLE; DESJARDINS, 2007, SALMERÓN; THOMAS; PANDIELLA, 2014).

Increasing consumer awareness towards healthy diets and changing eating habits due to urbanization has created a huge market demand for new functional foods with a beneficial effect on health (RATHORE; SALMERÓN; PANDIELLA, 2012). Worldwide, the dairy sector, which is strongly linked to probiotics, is the largest functional food market, accounting for nearly 33% of the broad market, but vegetables might be a healthier option for the development of new non-dairy probiotic foods (LEATHERHEAD FOOD INTERNATIONAL, 2006, PRADO et al., 2008). The vegetables are rich in functional food components such as minerals, vitamins, dietary fibers, antioxidants, and do not contain any dairy allergens that might prevent usage by certain segments of the population (LUCKOW; DELAHUNTY, 2004). Therefore, the application of probiotic cultures in non-dairy products and environments represents a great challenge.

Probiotic bacteria are defined as “live microorganisms that when administered in adequate amounts confer a health benefit on the host” (FAO, 2001). Many studies indicated that soy (*Glycine max*) is a good substrate for functional foods since fermentation by probiotics has the potential to reduce the levels of some carbohydrates (FARNWORTH; MAINVILLE; DESJARDINS,

2007, CHAMPAGNE et al., 2009). These sugars can be responsible for gas production in the intestinal system, increase free isoflavone levels and favour desirable changes in bacterial populations in the gastrointestinal tract. The main probiotic bacteria studied for growing in soy beverages are *Lactobacillus acidophilus, Lactobacillus fermentum, Bifidobacterium* sp., *Lactobacillus rhamnosus* and *Lactobacillus paracasei s*ubsp. *paracasei* (BOUHNIK et al., 2004; WEI; CHEN; CHEN, 2007; CHAMPAGNE et al., 2009).

It has been proven that peanuts (*Arachis hypogaea* L.) have the potential for LAB growth (LEE; BEUCHAT, 1991, SUNNY-ROBERTS; OTUNOLA; IWAKUN, 2004, YADAV et al., 2010), including probiotic ones (BEUCHAT; NAIL ,1978, SCHAFFNER; BEUCHAT, 1986). Peanut is a major source of edible oil and protein meal, and therefore it is considered to be highly valuable in human and animal nutrition (NWOKOLO, 1996). Fermentation of peanut milk may serve as one effort that can increase the consumption of this valuable crop, and hence improve protein availability for consumers (SUNNY-ROBERTS; OTUNOLA; IWAKUN, 2004).

There is no data available characterizing the PSM as a substrate for fermentation by LAB, including probiotic ones and yeasts. Yeasts have a long history of proven safe use in the fermentation of foods and beverages. Several yeasts could assist the starter cultures in cheeses and yogurts by proteolytic activity and possibly participate in the formation of aroma components. These activities are expected to vary among isolates and to be controlled by environmental conditions (JAKOBSEN; NARVHUS, 1996).

In this context, it would be desirable to know if LAB, including probiotic bacteria and yeasts, can be as starter cultures into a PSM formulation for fermentations and development of a new beverage. Therefore, the purpose of this study was to elaborate a novel nutritive substrate based on peanut and soybean milks. The growth ability of several strains, including probiotic

microorganisms (commercial), lactic acid bacteria, and yeasts were evaluated. Fermentation parameters of the strains in this novel vegetable based milk were observed and the efficiency was measured by determination of cell growth, consumption of available carbohydrates, pH and production of lactic acid.

2 MATERIALS AND METHODS

2.1 Vegetable milks preparation

Peanut milk was prepared as previously described (SALUNKHE; KADAM, 1989) with minor modifications. The seeds were sorted to remove discolored grains and any foreign material. Selected peanut seeds were roasted at 130 °C for 20 min in a ventilated oven. The seeds were peeled and weighed before being soaked in 0.5 g/100 mL $NaHCO_3$ for at least 12 h. The shelled seeds were then washed with water and mixed with distilled water in a ratio of 1:5 [peanuts (g):water (mL)] and transferred to a stainless steel blender (Cemaf, São Paulo, Brazil) for 5 min. The resulting paste was filtered through a double layered cheese cloth.

For the soymilk preparation, 250 g of selected beans were initially soaked in 1 L of water for 16 h at 25 °C. Subsequently, 190 g of these moistened beans were soaked in 500 mL water in a stainless steel blender and mixed for 3 minutes. Then, the resulting slurry was filtered through double layered cheese cloth and boiled for 5 min (CHAMPAGNE et al., 2009).

2.2 Proportions of extracts and standardization

Aiming to develop a new substrate in which the peanut was preferably the predominant raw material, the peanut and soybean milks were mixed in three different ratios (3:1, 2:1 and 1:1) and investigated. The heat treatment was carried out by pasteurization at 90 °C/20min. The formulation to be later used for fermentation were chosen after chemical composition analysis of samples.

2.3 Microorganisms growth

The commercial microorganisms were obtained from Danisco. The other strains of lactic acid bacteria and yeasts used belonged to the collection of the Microbial Physiology Laboratory/Department of Biology, Federal University of Lavras (UFLA), Brazil (Table 1).

Table 1 Cultures used in this study

Microorganisms	**Number Code**	**Source**
Probiotic bactéria		
Bifidobacterium longum	BL 04	Flora Fit - Danisco, USA
Lactobacillus rhamnosus	LR 32	Flora Fit - Danisco, USA
Lactobacillus acidophilus	LACA 4	Yo Mix - Danisco, Deutschland
Lactobacillus paracasei subsp. *paracasei*	LBC 81	Yo Mix - Danisco, France
Yogurt starters		
Lactobacillus delbrueckii subsp. *bulgaricus*	LB 340	Yo Mix - Danisco, France
Streptococcus thermophilus	TA 40	Yo Mix - Danisco, France
UFLA bacteria collection		
Lactobacillus fermentum[1]	UFLA B FFEA 6.01	UFLA/DBI collection
Lactobacillus fermentum[2]	CCT 305	André Toselo collection
Lactobacillus plantarum[2]	CCT 203	André Toselo collection
Pediococcus acidilactici[3]	UFLA B FFCX 27.1	UFLA/DBI collection
Lactococcus lactis[2]	CCT 0360	Collection of tropical cultures
UFLA yeasts collection		
Kluyveromyces marxianus[2]	CCT 3172	Collection of tropical cultures
Saccharomyces cerevisiae[4]	UFLA Y FFFW 15	UFLA/DBI collection
Saccharomyces cerevisiae[1]	UFLA Y FFBM 18.03	UFLA/DBI collection
Saccharomyces cerevisiae[3]	UFLA Y FFCX 101.8	UFLA/DBI collection
Debaryomyces etchelsii[1]	UFLA Y FFBM 5.56	UFLA/DBI collection

Isolated from: [1]- Fermentation of cocoa beans (Pereira, et al., 2012); [2]- Cocoa fermentations – André Toselo (Schwan et al., 1997); [3]- indigenous beverage *caxiri* (Santos et al., 2012); [4]- Raspberry wine (Duarte et al., 2010).

The commercial cultures activation was performed according to the manufacturer's instructions. For all strains, stock cultures were prepared by mixing culture medium Man Rogosa Sharp broth (MRS, Merck Whitehouse Station, USA) for bacteria and glycerol (40 g/100 mL) in the proportion 1:1 placing 1 mL in Cryovials and freezing at - 20 º C.

YPD agar [10 g/L yeast extract (Merck, Darmstadt, Germany), 10 g/L peptone (Himedia, Mumbai, India), 20 g/L glucose (Merck, Darmstadt, Germany), 20 g/L agar (Merck, Darmstadt, Germany) pH 3.5] with glycerol (40 g/100 mL), were used to stock the yeasts. The glycerol solution was previously sterilized at 121 °C for 15 minutes (CHAMPAGNE et al., 2009).

To perform the activation of the interest strains, 100 mL of sterile MRS broth were inoculated with 1 mL of a thawed stock culture and incubated at 37 ºC for 16 h. The yeasts were grown in YPD broth, pH 3.5, and incubated at 30 °C for 24 h.

2.4 Fermentations

The PSM (150 mL) was placed in flasks (200 mL) and heat treated. Subsequently, it was rapidly refrigerated at 4ºC. The inoculum was added at 1 mL/100 mL to provide initial counts about of 10^7 CFU/mL. The suspension with inoculated cells was incubated at 37 °C for up to 24 h. Samples were taken at 0, 12 and 24 h of the fermentation process, for analysis of pH, titratable acidity, total sugars, and viable count (VC).

2.5 Viable count of inoculated strains (VC)

One milliliter of sample was taken from each fermentation flask. Serials tenfold dilution was prepared in a solution of NaCl (0.9 g/100 mL) and bacto

peptone [(Difco)(0.1 g/100 mL)]. Viable counts of the LAB were obtained by spreading 0.1 mL sample of an appropriate dilution onto the surface of MRS plates containing 0.1 g/100 mL of cysteine-HCl under microaerophilic conditions during incubation (WANG; YU; CHOU, 2002). Yeasts were grown on YPD agar pH 3.5 at 28 ºC for 5 days. Violet Red Bile Agar with glucose (VRBG) (Oxoid, Hampshire, England) was used for bile-tolerant Gram-negative *Entorobacteriaceae* detection, the plates were incubated at 37°C for 24-48 h. Colony forming units (CFU) were enumerated in plates containing 30 to 300 colonies, and cell concentration was expressed as log CFU/mL of fermented PSM.

2.6 Analytical methods

Moisture, crude protein, fiber, fat, ash, and titratable acidity (TA) were determined in accordance with standard procedure (AOAC, 2000). The measurement of total available carbohydrate (TAC) was carried out by the colorimetric Anthrona method as described by Dishe (1962). Calcium was quantified using an atomic absorption spectrophotometer (PERKIN ELMER CORP, 1980). Potassium was determined by a flame photometric method and phosphorus with a Technical Analyzer (EEL 201) (AOAC, 1985). The pH of the fermenting PSM samples was measured with a pH meter (Tecnal, Tec-3MT, São Paulo, Brazil). All determinations were carried out in triplicate.

The determinations of total amino acids were performed by High Performance Liquid Chromatography (HPLC) as described by White, Hart and Fry (1986). After a 24 h hydrolysis in 6 M HCl at 100 °C, the amino acids reacted with phenyl isothiocyanate, and the derivatives separated using a Luna C-18, 100 Å; 5 µ, 250 mm × 4.6 mm (00G-4252-E0, Torrance, CA, USA) column, at 50 °C. The detection was by ultraviolet absorption measurements

using a fixed wavelength (254 nm) detector. Quantification was carried out by comparison with a standard mixture (Thermo Scientific, Rockford IL, USA) and DL-2-aminobutyric acid was used as an internal standard from Sigma–Aldrich Corp., St. Louis, MO (HAGEN; FROST; AUGUSTIN, 1989).

2.7 Data presentation and statistical analyses

The results are expressed as means ± standard deviation (SD). In all of analyses, the assumption of normality was tested using Shapiro and Wilk test. The assumption of homogeneity of variances was tested using Barlett test. As both assumptions of normality and homogeneity of variances were satisfied, parametric testing was performed. The data discussed are the averages from three independent assays and were subject to analysis of variance (ANOVA), followed by Fisher's Least Significant Difference (LSD) test ($p < 0.05$) (GRANATO; ARAÚJO CALADO; JARVIS, 2014). Different letters were used to label values with statistical differences among them, using a model containing the fixed effects of treatment (strains), time of fermentation, and the interactions between the treatment and the fermentation time. The statistical analyses were performed using the Sisvar 5.3 software (Ferreira, 2010), except the Bartlett test, which was performed using the Assistat software (SILVA; AZEVEDO, 2009).

Principal component analyses were performed with the software XLSTAT 7.5.2 (Addinsoft, New York, N.Y., USA) for group data from VC, TAC, TA expressed as lactic acid and pH during PSM fermentation.

3 RESULTS AND DISCUSSION

3.1 Substrates characteristics

Among oilseeds, the peanut is present in the country's usual diet, and provide mild flavor to its derivates. Diarra, Nong and Jie (2005) reported that peanut milk can be converted into a multitude of high nutritious products. The only limitation is one's imagination and inventiveness. In this area, peanut milk has been successfully converted into low cost edible products with high nutritional value, such as yogurt, buttermilk, ripened cheese analogs, and curd or "tofu" (DIARRA; NONG; JIE, 2005).

In this work we formulate different blends of peanut and soy milks in order to develop a new nutritional substrate. The results of chemical composition of the unfermented PSM are presented in Table 2. In previous studies, there were variations in the yields and chemical composition of the vegetable milks. These differences were probably due to inherent characteristics of the raw materials and the different preparation methods. Diarra, Nong and Jie (2005) pointed out the different ways of producing peanut milk and peanut milk products showing the effects of processing conditions on peanut milk.

The percentage of solids and carbohydrates calculated for soymilk were similar to the results reported (9.2 and 1.7 g/100 mL, respectively) by Champagne et al. (2009). Sunny-Roberts, Otunola and Iwakun (2004) found for groundnut formulation 89.4 g/100 mL of moisture, 2.98 g/100 mL of crude protein, 0.78 g/100 mL of crude fiber, 5.47 g/100 mL of fat content, 0.42 g/100 mL of ash, and 1.32 g/100 mL of carbohydrate. According to Bucker et al. (1979), the composition of peanut milk was 90.6 g/100 mL of moisture, 0.2 g/100 mL of ash, 2.8 g/100 mL of protein, 2.0 g/100 mL of carbohydrate, 0.05 g/100 mL of glucose, 0.63 g/100 mL of sucrose, 0.20 g/100 mL of starch, and

4.4 g/100 mL of fat. Lam and De Lumen (2003) stated that although soybean and peanuts contain low contents of carbohydrate, 32% and 14%, respectively, these carbs generally have good bioavailability.

Variations in PSM concentrations influenced the chemical composition and physicochemical properties of blends to varying levels. The different ratios of peanut:soy milk (1:1; 2:1; 3:1) showed values of moisture content, ash, fiber, proteins, fat, carbohydrates and potassium significantly different ($p < 0.05$) (Table 2).

Increasing soy concentrations caused significant raise in protein content, whereas increasing peanut concentrations increased fat content. Kpodo et al. (2013) found crude protein content of soymilk (4.8 g/100 mL) and peanut milk (3.71 g/100 mL), separately. The amounts of proteins commonly found in peanut and soybean milks are generally higher than cow milk (2.84 g/100 mL). This demonstrates the possibility of using these products in partial or total replacement of animal milk, primarily because these oilseeds are abundant sources of proteins.

Table 2 Chemical and mineral composition of the peanut, soybean, and mixed extracts in different proportions, using different heating treatments

Sample	Peanut extract	Soybean extract	ME 1:1	ME 2:1	ME 3:1	ME 2:1 pasteurized
Moisture (mL/100 mL)	91.55±0.01 [a]	90.49±0.12 [d]	90.59±0.15 [cd]	90.70±0.04 [c]	90.95±0.04 [b]	90.65±0.03 [c]
Dry matter (g/100 mL)	8.45±0.01 [d]	9.51±0.17 [a]	9.41±0.07 [ab]	9.30±0.09 [b]	9.05±0.02 [c]	9.35±0.03 [b]
Ash (g/100 mL)	0.08±0.00 [c]	0.20±0.00 [a]	0.12±0.02 [bc]	0.12±0.01 [bc]	0.14±0.02 [b]	0.12±0.01 [bc]
Fiber (g/100 mL)	0.78±0.03 [a]	0.06±0.01 [d]	0.07±0.01 [d]	0.39±0.01 [c]	0.73±0.01 [b]	0.38±0.02 [c]
Protein (g/100 mL)	3.13±0.04 [e]	5.48±0.04 [a]	4.53±0.04 [b]	4.00±0.03 [c]	3.73±0.04 [d]	4.01±0.03 [c]
Fat (g/100 mL)	3.93±0.03 [a]	1.90±0.00 [e]	2.88±0.02 [d]	3.17±0.03 [c]	3.63±0.06 [b]	3.17±0.10 [c]
Carbohydrate (g/100 mL)	0.53±0.03 [e]	1.87±0.03 [a]	1.81±0.02 [b]	1.62±0.05 [c]	0.82±0.03 [d]	1.67±0.03 [c]
Phosphorus (g/100 mL)	0.01±0.00 [b]	0.02±0.00 [a]	0.01±0.00 [b]	0.02±0.00 [a]	0.01±0.00 [b]	0.02±0.00 [a]
Potassium (g/100 mL)	0.01±0.00 [a]	0.08±0.00 [e]	0.05±0.00 [b]	0.03±0.00 [c]	0.02±0.00 [d]	0.03±0.00 [c]
Calcium (g/100 mL)	0.03±0.00 [c]	0.04±0.00 [b]	0.05±0.00 [a]	0.05±0.00 [a]	0.05±0.00 [a]	0.05±0.00 [a]

ME – Mixed extract (peanut:soybean). Data are the means of three independent assays.

[abc]: Values followed by the same letter in the same column are not significantly different ($p > 0.05$) by Fisher's Least Significant Difference (LSD) test ($p < 0.05$).

The moisture content of formulations increased with the raise of the peanut milk content (Table 2). The solids content of PSM formulations with different proportions of peanut milk and soymilk did not differ ($p < 0.05$), with the exception of the mixture in the ratio 3:1. Generally, cow milk has higher total solids content (13.9 g/100 mL) than other aqueous extracts of oilseeds (SCHAFFNER; BEUCHAT, 1986, CHAMPAGNE et al., 2009, KPODO et al., 2013).

The calcium, phosphorus and potassium contents in this work did not exceed 0.05, 0.02, and 0.08, respectively. This could probably be due to the relatively lower levels in the raw materials and for different production methods. Sunny-Roberts, Otunola and Iwakun (2004) studying groundnut milk reported that there is a decrease in minerals contents in the unfermented milk compared to the seeds/nuts. This loss could occur after the fine paste adhering to the cheesecloth, in the wash water or during the heat treatment. Calcium bioavailability from soybean products is essentially equivalent to that from milk, but the balance of calcium to phosphorus is inadequate, since the ratio is 1:2, whereas the ideal is 2:1. Legume contain very low (0.05 g/Kg) amount of sodium (LAM; DE LUMEN, 2003), which is good for health because of the relationship that low sodium diets has to hypertension in humans (APATA; OLOGHOBO, 1994).

The formulation selected for the development of a novel beverage was the 2:1, peanut milk:soybean milk ratio. This proportion shows comparative contents of fiber, proteins, lipids and carbohydrates to that of cow milk. The largest proportion (67 mL/100 mL) of peanut milk is ideal, since there are few studies of this feedstock fermentation or as a functional beverage. Furthermore, it has been demonstrated that soybean milk has a strong beany flavour, making it unpopular, while peanuts have minimal beany flavour (NADUTEY, 1999). It is already known , the soymilk acceptance is increased when associated with

additives, ingredients or other raw materials, which enhance the flavor and aroma (JAEKEL; RODRIGUES; SILVA, 2010). Then, the combination of both extracts at a 2:1 ratio might reduce the unpleasant flavor of soybean beverage. Besides, the combination of soybean with other vegetables is desirable for proper balancing of essential amino acids (MAIA et al., 2000).

The total amino acid composition of a food protein, particularly of the essential amino acids, has classically been considered a measure of its biological adequacy. The total amino acid composition of PSM is presented in Table 3. These values are compared to the official amino acid profile of an ideal protein as established by Who (2007). The results here appear to suggest that the product could be a good source of essential amino acids.

Table 3 Total amino acid composition of the peanut-soy milk (2:1), compared with the WHO standard (2007)

Amino acid	**Requirements WHO/FAO/ONU (g/100g of protein, dry basis)**			**Peanut-soy milk (2:1) (g/100g of milk, dry basis)**
	Children (age)		**Adults**	
	1-2	3-10		
Histidine (Hys)	-	-	1.5	1.12±0.14
Isoleucine (Ile)	-	-	3.0	1.78±0.08
Leucine (Leu)	-	-	5.9	2.90±0.11
Lysine (Lys)	5.2	4.8	4.5	1.75±0.11
Methionine (Met)	-	-	1.6	0.50±0.10
Cysteine (Cys)	-	-	0.6	0.67±0.02
Phenylalanine (Phe)	-	-	-	1.54±0.34
Tyrosine (Tyr)	-	-	-	1.61±0.20
Threonine (Thr)	2.7	2.5	2.3	1.71±0.14
Tryptophan (Trp)	7.4	6.6	0.6	nd
Valine (Val)	-	-	3.9	2.01
Total essential amino acids	15.3	13.9	23.9	15.59
Arginine (Arg)	-	-	-	4.80±0.43
Alanine (Ala)	-	-	-	1.92±0.12
Aspartic acid (Asp)	-	-	-	5.13±0.26
Glutamic acid (Glu)	-	-	-	9.44±0.48
Glycine (Gly)	-	-	-	1.93±0.08

“continua”

Table 3 “conclusion”

Amino acid	Requirements WHO/FAO/ONU (g/100g of protein, dry basis)			Peanut-soy milk (2:1) (g/100g of milk, dry basis)
	Children (age)		Adults	
	1-2	3-10		
Proline (Pro)	-	-	-	2.40±0.14
Serine (Ser)	-	-	-	2.20±0.08
Total amino acid				43.37%

Nd – Not determined.

3.2 Fermentation

The PSM were inoculated with 1 mL/100 mL culture suspension of different strains aiming to achieve about 7 log CFU/mL, which would be the cell concentration of probiotic required at the time of consumption (based on a 100 mL daily, intake of the fermented product) (RATHORE; SALMERÓN; PANDIELLA, 2012). However, inoculation of 1 mL/100 mL resulted in initial populations ranging from 6.9 to 8.0 log CFU/mL. The difference of almost 1 log CFU/mL has influenced the profile of acidification of PSM.

3.2.1 Acidification profile and sugar metabolism

Data on TAC, TA, pH and VC are shown in Table 4. Yeast strains were more efficient than bacteria in relation to the consumption of the available carbohydrates, showing an average of 0.15 g/100 mL of residual sugar after 24 h of fermentation. These yeasts produced ethanol and organic acids, giving odor of bread and alcohol to the fermented PSM, besides an undesirable appearance with irregular coagulation and excessive syneresis (Fig 1).

Table 4 Effects of inoculation on the concentrations of total sugars, titratable acidity, pH and microbial population in mixed extract (2:1) inoculated with different LAB and yeast strains and at different periods of fermentation

Strain	TAC (g/100 mL)		TA (g/100 mL)		pH		Log CFU/mL	
	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h
Commercial probiotic bacteria								
B. longum BL 04	1.29±0.18 ^{cdefA}	0.91±0.05 ^{cdB}	0.77±0.02 ^{abB}	1.35±0.02 ^{aA}	4.08±0.03 ^{bA}	4.00±0.05 ^{bA}	7.77±0.03 ^{fA}	7.77±0.04 ^{eA}
L. rhamnosus LR 32	1.41±0.19 ^{abc}	1.01±0.09 ^{bc}	0.12±0.02 ^{cdeA}	0.08±0.02 ^{jA}	6.10±0.10 ^{cA}	5.75±0.02 ^{dB}	8.89±0.02 ^{aA}	8.55±0.03 ^{bB}
L. acidophilus LACA 4	1.46±0.26 ^{abcA}	0.40±0.04 ^{fB}	0.10±0.01 ^{deB}	0.30±0.05 ^{fA}	4.94±0.04 ^{fA}	4.50±0.06 ^{hB}	7.89±0.01 ^{eB}	8.09±0.00 ^{cA}
L. paracasei subsp. *paracasei* LBC 81	1.51±0.04 ^{abA}	0.62±0.09 ^{eB}	0.73±0.02 ^{bB}	1.16±0.02 ^{bA}	4.55±0.03 ^{gA}	4.12±0.08 ^{gB}	7.52±0.01 ^{hB}	7.69±0.02 ^{eA}
Commercial yogurt starters bactéria								
L. delbrueckii subsp. *bulgaricus* LB 340	0.96±0.13 ^{fgA}	0.69±0.07 ^{deB}	0.15±0.01 ^{cdB}	0.75±0.02 ^{eA}	6.50±0.02 ^{bA}	5.58±0.11 ^{deB}	8.29±0.02 ^{cA}	7.76±0.03 ^{eB}
S. thermophilus TA 40	1.46±0.23 ^{abcA}	0.36±0.02 ^{fgB}	0.79±0.01 ^{aB}	0.93±0.03 ^{dA}	4.19±0.01 ^{hA}	4.00±0.05 ^{hB}	7.19±0.01 ^{jA}	7.01±0.06 ^{jB}
UFLA bacteria collection								
L. fermentum UFLA B FFEA 6.01	1.59±0.19 ^{aA}	1.01±0.09 ^{bcB}	0.17±0.01 ^{cA}	0.19±0.01 ^{ghA}	6.00±0.01 ^{cdA}	5.50±0.10 ^{eB}	7.87±0.10 ^{eA}	7.30±0.05 ^{gB}
L. fermentum CCT 305	1.18±0.19 ^{defA}	1.13±0.08 ^{bA}	0.82±0.00 ^{aB}	0.98±0.04 ^{cA}	4.05±0.07 ^{hA}	4.00±0.01 ^{hA}	8.42±0.08 ^{bA}	7.36±0.04 ^{gB}
L. plantarum CCT 203	1.35±0.14 ^{bcdA}	0.98±0.14 ^{bcB}	0.17±0.03 ^{cdA}	0.19±0.02 ^{ghA}	6.00±0.05 ^{cdA}	5.50±0.03 ^{eB}	7.64±0.03 ^{gB}	7.87±0.05 ^{dA}
P. acidilactici UFLA B FFCX 27.1	1.30±0.04 ^{cdeA}	0.14±0.03 ^{kB}	0.13±0.01 ^{cdeA}	0.13±0.03 ^{hA}	6.00±0.06 ^{cdA}	6.00±0.04 ^{cA}	8.17±0.05 ^{dB}	8.91±0.06 ^{aA}
Lc. lactis CCT 0360	1.11±0.26 ^{efA}	1.11±0.02 ^{bcA}	0.16±0.02 ^{cA}	0.13±0.02 ^{hA}	6.55±0.07 ^{bA}	6.45±0.09 ^{bA}	7.59±0.02 ^{ghA}	7.29±0.02 ^{ghB}
UFLA yeasts collection								
S. cerevisiae UFLA Y FFFW 15	0.16±0.03 ^{hA}	0.16±0.04 ^{hA}	0.16±0.02 ^{cB}	0.26±0.04 ^{fA}	6.05±0.07 ^{cA}	6.00±0.08 ^{cA}	7.42±0.02 ^{iA}	7.48±0.03 ^{fA}
S. cerevisiae UFLA Y FFBM 18.03	1.53±0.10 ^{abA}	1.49±0.06 ^{aA}	0.11±0.01 ^{cdeA}	0.14±0.04 ^{hiA}	7.04±0.07 ^{aA}	6.95±0.15 ^{aA}	7.57±0.02 ^{ghA}	7.17±0.03 ^{iB}
S. cerevisiae UFLA Y FFCX 101.8	0.19±0.03 ^{hA}	0.16±0.03 ^{hA}	0.14±0.02 ^{cdeA}	0.17±0.07 ^{ghiA}	5.90±0.10 ^{dA}	5.50±0.09 ^{eB}	7.34±0.02 ^{iA}	7.20±0.09 ^{hiB}
D. etchelsii UFLA Y FFBM 5.56	0.17±0.04 ^{hA}	0.15±0.01 ^{hA}	0.09±0.03 ^{eB}	0.20±0.06 ^{gA}	5.50±0.13 ^{eA}	5.50±0.28 ^{eA}	7.63±0.03 ^{gA}	7.54±0.07 ^{fA}
K. marxianus CCT 3172	0.11±0.01 ^{hA}	0.11±0.01 ^{hA}	0.15±0.02 ^{cdA}	0.20±0.05 ^{gA}	5.60±0.10 ^{eA}	4.95±0.06 ^{fB}	8.10±0.04 ^{dA}	8.01±0.11 ^{cB}

TAC- total available carbohydrate (initial TAC -1.62 g/100 mL±0.28); TA- titratable acidity (initial TA - 0.06 g/100 mL). Initial pH 7.00. For each column, mean values with different lowercase letters are significant at $p < 0.05$. For each row, mean values with different capital letters are significant at $p < 0.05$.

The *S. cerevisiae* (UFLA Y FFBM 18.03) yeast was the exception, metabolizing 0.13 g/100 mL of TAC resulting in a fermented beverage with good aspect, without undesirable odor (Fig 1). The use of yeasts to ferment the PSM may give rise to a new class of products where the addition of yeast serve as a source of protein and vitamin B (STEINKRAUS, 1996).The researched literature has not shown any previous work on yeast fermentation of vegetables, like peanut and soybean.

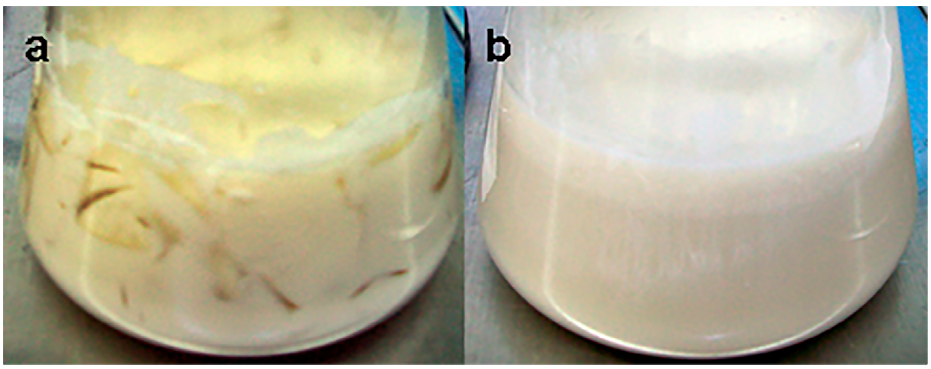

Figure 1 Appearance of the extract fermented by: A) *K. marxianus* (CCT 3172); B) *S. cerevisiae* (UFLA YFFBM 18.03)

The commercial probiotic bacteria *L. acidophilus* (LACA 4), *L. paracasei* subsp. *paracasei* (LBC 81), the commercial yogurt starter cultures (LB 340) and *S. thermophilus* (TA 40) were efficient in the consumption of TAC. These strains were able to metabolize 1.22 g/100 mL, 1.00 g/100 mL, 0.93 g/100 mL and 1.26 g/100 mL of available sugar in PSM, respectively. *P. acidilactici* (UFLA FFCX 27.1) consumed 1.48 g/100 mL of carbohydrate, and was the LAB that consumed more sugars from the substrate ($p < 0.05$).

Different levels of carbohydrate would directly affect theoretical maximum amounts of acid which could be formed by various organisms. By our finds, there are plenty carbohydrates in PSM to carry out a fermentation that would attain the pH of yogurt-like products (pH 4.2–5.0). However, the

acidification pattern will depend on the sugar assimilation profile of the microorganisms (CHAMPAGNE et al., 2009). The *L. fermentum* (CC 305), *S. thermophilus* (TA 40), and *B. longum* (BL 04) lactic acid bacteria were the major producers of lactic acid (0.82 g/100 mL, 0.79 g/100 mL, and 0.77 g/100 mL, respectively) and quickly lowered the pH of PSM, reaching values below 4.5 after 12 hours of fermentation (Table 4). In general, the results of the pH values followed a trend opposite to that observed for titratable acidity measurements, as the acidity increased, the pH decreased. Hou et al. (2000) reported that various strains of *B. longum* were able to grow in soymilk, rapidly reducing the pH and consuming sugars like raffinose and stachyose, available in the substrate. Bucker, Mitchell and Johnson (1979) examined the ability of 19 lactic acid bacteria strains to ferment peanut milk prepared from blanched, full fat Runner Variety. *L. acidophilus, L. plantarum*, *P. acidilactici*, and *S. thermophilus* produced the highest levels of acid, reaching TA of 0.25, 0.20, 0.17 and 0.27 g/100 mL, respectively, expressed as lactic acid, after 48h. These results suggested that the addition of soymilk to peanut milk favors the growth of LAB, increasing the production of lactic acid, quickly lowering the pH of the PSM.

Studies have shown that pH around 3.5 to 4.0 inhibit *Enterobacteriaceae* and Gram-negative bacteria (MUYANJA et al., 2003). Indeed, in our work, the fermentations with pH of 4.5 or below, were kept free of Gram-negative *Entorobacteriaceae*. On the other hand, the assays that pH remained above 5.5, populations until 2 log CFU/ml were found. Champagne et al. (2009) suggested that the pH of cow milk yogurt, typically around pH 4.3, is unnecessary for soy beverage. According to these authors, the very low pH does not result in the highest probiotic population in the fermented soy products by the uncoupling of growth and acid production. Moreover, unwanted appearance, extremely acid odor, and excessive syneresis (data not shown) resulted from pH

lower than 4.5, showing to be undesirable for the preparation of the fermented PSM. The vegetable milk coagulation was advanced enough at pH 5.5 for PSM assays.

It is known that other factors may play a role in the development of increased viscosity. The curd formation is related to the amount and type of protein present, as well as the acidity, with more protein and more acid producing a firmer curd (SCHAFFNER; BEUCHAT, 1986). Approximately 90% of the proteins in soybeans is a mixture of globulins. The remaining proteins are composed of intracellular enzymes (lipoxygenase, urease, amylase), hemagglutinins, protein inhibitors and membrane lipoproteins. The protein precipitated at pH 4.5 traditionally has been called glycinin. However, numerous studies have shown that soy proteins are quite heterogeneous (WOLF, 1977, KINSELLA, 1979). Thus, the isoelectric point of soy and also peanuts proteins can be quite variable.

The extracts fermented by *L. fermentum* (UFLA B FFEA 6.01 and CCT 305) and *L. plantarum* (CCT 203) showed an unpleasant aspect for a novel beverage with granule formation and unsuitable consistency (Figure 2). This was probably due to the production of acetic acid, ethanol and CO_2, since these microorganisms are known heterofermentative strains (CHANG; STONE, 1990).

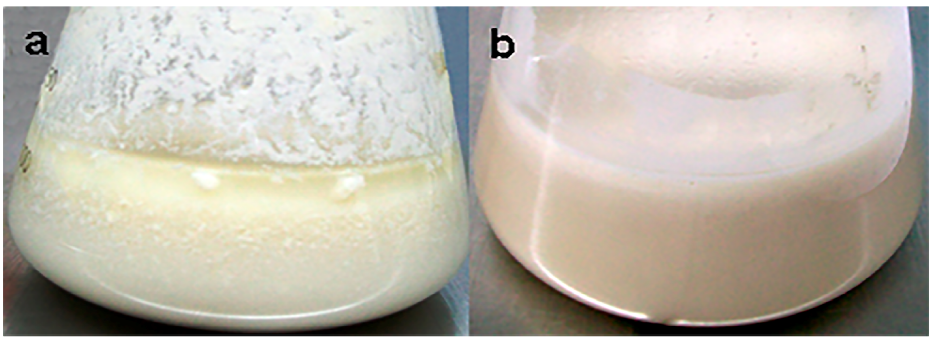

Figure 2 Undesirable appearance of the extract fermented by: A) *L. fermentum* (UFLA B FFEA 6.01), and B) *L. plantarum* (CCT 203)

L. rhamnosus (LR 32) reached the largest population among all strains ($p < 0.05$) at 12 h of fermentation, followed by the *L. fermentum* (CCT 305), *L. delbrueckii* subsp. *bulgaricus* (LB340), and *P. acidilactici* (UFLA B FFCX 27.1). Among the yeasts, only *K. marxianus* (CCT 3172) was able to achieve population above 8 log CFU/mL of PSM). All strains tested were able to reach a minimum population of 7 log CFU/mL (Table 4), which is the recommended intake for probiotic microorganisms. Nevertheless, *L. rhamnosus* (LR 32), *L. delbrueckii* subsp. *bulgaricus* (LB340), *S. thermophilus* (TA 40), *L. fermentum* (UFLA B FFEA 6.01 and CCT 305), *Lactococcus lactis* (CCT 0360), and *S. cerevisiae* (UFLA Y FFBM 18.03 and UFLA Y FFBM 101.8) showed significant decreases ($p < 0.05$) in their populations after 24 h. The inability of some strains to keep the viable population was probably due (1) to the exhaustion of all fermentable carbohydrates, (2) high contents of organic acids, excessively decreasing the pH, affecting the survival of the culture, (3) excessively long fermentation time. On the other hand, *L. acidophilus* (LACA 4), *L. paracasei* subsp. *paracasei* (LBC 81), *L. plantarum* (CCT 203) were able to continue growing in the substrate, reaching significantly higher populations in 24 hours of fermentation.

The results obtained for the VC, TAC, and acidification parameters in PSM fermentation were submitted to PCA to correlate the different microbial groups (Figure 3). The first (PC 1) and second (PC 2) principal components explain 46.16% and 22.57%, respectively, of the total variance. On the positive side of PC 1, the strains *L. fermentum* (CCT 305), *B. longum* (BL 04) and *L. paracasei* subsp. *paracasei* (LBC 81) were grouped as the major producers of lactic acid. The probiotic *L. acidophilus* (LACA 4) also appear on that side, but ungrouped in view of its higher carbohydrate consumption and viable populations (above 8 log CFU/mL), indicating that this microorganism was able to convert part of the consumed sugar in biomass. The yogurt starter culture *S.*

thermophilus appears isolated on the negative side of the PC 2. This LAB consumed 77.8% of available sugars and quickly acidified the substrate. However, their final population was the lowest among all the tested microorganisms. On the negative side of PC 1 and the positive side of PC 2 was the probiotic *L. rhamnosus* (LR 32), the strain was correlated with highest VC with a minor carbohydrate consumption.

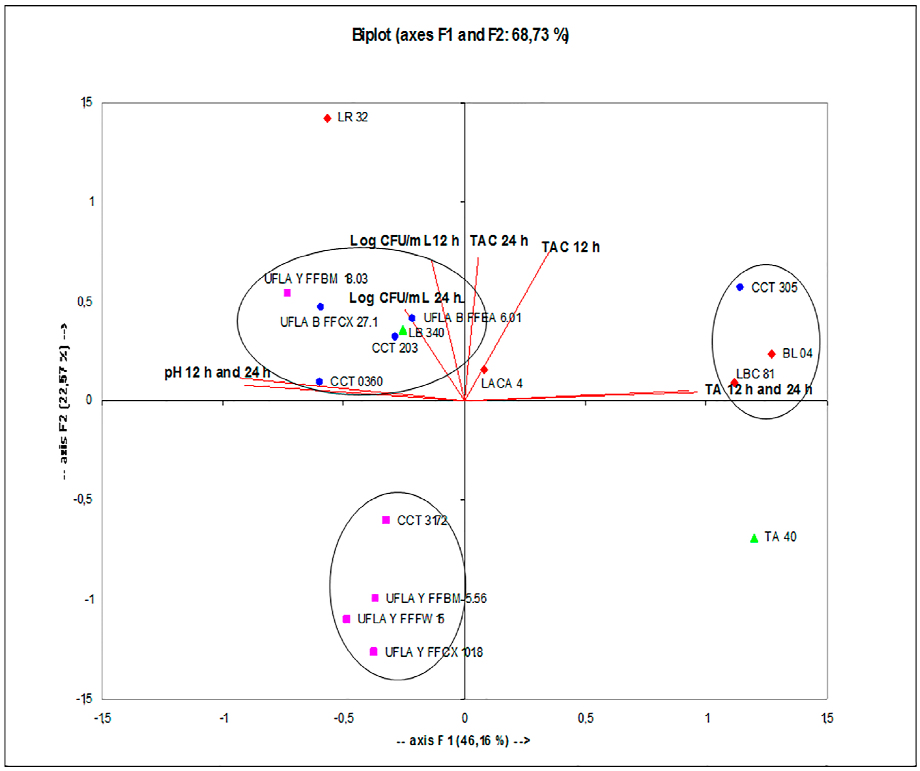

Fig. 3 Principal component analysis (PCA) of the viable count and physicochemical parameters during the fermentation of peanut-soy milk

A large group was formed by the UFLA bacteria collection along with the yogurt starter culture *L. delbrueckii* subsp. *bulgaricus* (LB 340) and *S. cerevisiae* (UFLA Y FFBM 18.03) yeast. This group was primarily correlated with a moderate intake of carbohydrates, low acidity, with consequent minor

reduction in pH value. The remaining yeasts were grouped on the negative side of PC 1 and PC 2, the most efficient strains in the use of available carbohydrates, but with low capacity of acidification.

4 CONCLUSION

One of the aims of this work was to develop a novel substrate with adequate nutritional quality, to be a viable source of protein and essential amino acids for low income people, vegetarian and lactose intolerant consumers. This study is the first report of the potential use of PSM for a new beverage development, which may give rise to a new class of products based on peanuts and soybean milks. PSM proved to be a suitable substrate with high nutritional profile enough to sustain the growth of different LAB, including probiotics and of yeasts. Furthermore, fermented PSM presented low pH being safe for consumption. Thus, further research is required to study the potential of using mixed cultures in order to evaluate the physicochemical changes, metabolites produced, probiotic survival and sensory acceptance of a novel functional beverage.

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CAPÍTULO 3 Co-culture fermentation of peanut-soy milk for the development of a novel functional beverage

Claudia Cristina Auler do Amaral Santos[4], Bárbara da Silva Libeck[5], Rosane Freitas Schwan[6]

[4] Department of Biology, Federal University of Lavras (UFLA), CP 3037 - Campus Universitário, CEP 37.200-000 Lavras, MG, Brazil

[5] Department of Food Science, , Federal University of Lavras (UFLA), CP 3037 - Campus Universitário, CEP 37.200-000 Lavras, MG, Brazil

[6] Department of Food Science, Federal University of Lavras (UFLA), CP 3037 - Campus Universitário, CEP 37.200-000 Lavras, MG, Brazil. Corresponding author. [telephone (+55) 35 3829 1614; fax (+55) 35 3829 1100; e-mail: rschwan@dbi.ufla.br

ABSTRACT

Most of the commercial probiotic products are dairy-based, and the development of non-dairy probiotic products could be an alternative for new functional products. The peanut-soy milk (PSM[7]) was inoculated with six different lactic acid bacteria (LAB), including probiotic strains and yeasts and fermentation was accomplished for 24 h at 37 ºC and afterwards, another 24 h at ± 4 °C. The *Pediococcus acidilactici* (UFLA BFFCX 27.1), *Lactococcus lactis* (CCT 0360), *Lactobacillus rhamnosus* (LR 32) probiotic LAB, and the *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB 340) yogurt starter culture reached cell concentrations of about 8.3 Log CFU/mL during fermentation. However, these strains were not able to acidify the substrate when inoculated as pure culture. The *Lactobacillus acidophilus* (LACA 4) probiotic produced significant amounts of lactic acid (3.35 g/L) and rapidly lowered the pH (4.6). *Saccharomyces cerevisiae* (UFLA YFFBM 18.03) did not completely consume the available sugars in PSM and consequently produced low amounts of ethanol (0.24 g/L). In pure culture, *S. cerevisiae* (UFLA YFFBM 18.03), *L. rhamnosus* (LR 32), *L. acidophilus* (LACA 4), and *P. acidilactici* (UFLA BFFCX 27.1) promoted the increase of total amino acids (48.02%, 47.32%, 46.21% and, 44.07%, respectively). However, when in co-cultured, the strains consumed the free amino acids favoring their growth, and reaching the population of 8 log CFU/mL in PSM. Lactic acid production increased, and 12 hours were required to reach a pH value of 4.3. In general, the strains were more efficient in the use of available carbohydrates and release of metabolites in co-cultured than in single culture fermentations. An average of 58% and 78% of available carbohydrates was consumed when single and co-cultures were evaluated, respectively. Higher lactic acid contents were found in a binary culture of *P. acidilactici* (UFLA BFFCX 27.1) and *L. acidophilus* (LACA 4), and by co-culture of *P. acidilactici* (UFLA BFFCX 27.1), *L. acidophilus* (LACA 4) and *S. cerevisiae* (UFLA YFFBM 18.03) (9.03 and 8.51 g/L, respectively). The final content of ethanol was 0.03% (v/v) or less, which classified the final beverage as non-alcoholic.

Keywords: Vegetable fermentation. Lactic acid bacteria. Yeasts. Functional beverage. Soymilk. Peanut milk.

[7] PSM – Peanut-soy milk

1 INTRODUCTION

The demand for alternatives to dairy products is growing due to problems with lactose intolerance, cholesterol content, allergenic milk proteins and desire for vegetarian alternatives (GRANATO et al., 2010). These are the major drawbacks related to the intake of dairy products, which makes the development of new non-dairy probiotic essential foods (HEENAN et al., 2005; RIVERA-ESPINOZA; GALLARDO-NAVARRO, 2010; YOON; WOODAMS; HANG, 2004). Probiotic food contains high populations of probiotic microorganisms in its formulation. According to Fao (2001), probiotics are live microorganisms that confer benefits to the health of the host when administered in adequate amounts.

Non-dairy probiotic products represent a huge growth potential for the food industry, and may be widely explored through the development of new ingredients, processes, and products. There are a wide variety of traditional non-dairy fermented beverages produced around the world. Much of them are non-alcoholic beverages manufactured with cereals as the main raw material (PRADO et al., 2008). Nevertheless, fruit juices, desserts and legume based products can also be used featuring probiotics (CARGILL, 2008).

Many studies indicated that soy is a good substrate for probiotic bacteria (CHAMPAGNE et al., 2009; CODA et al., 2012; FARNWORTH; MAINVILLE; DESJARDINS, 2007; HOU; YU; CHOU, 2000; WANG et al., 2003). Peanuts have also been described as a potential substrate for this use (SCHAFFER; BEUCHAT, 1986, MUSTAFA et al., 2009). Over the years, peanut milk has been successfully converted into low cost edible products with high nutritional value (DIARRA; NONG; JIE, 2005). In this area, researchers have focused on peanut milk fermented foods, such as yogurt, buttermilk, ripened cheese analogs, and curd or “tofu” (BEUCHAT; NAIL, 1978;

DIARRA; NONG; JIE, 2005; ISANGA; ZHANG, 2009; LEE; BEUCHAT, 1991; SCHAFFER; BEUCHAT, 1986; YADAV et al., 2010).

The use of two substrates at once may enhance the nutrient availability for the microbial population and affect their growth and metabolism (GRANATO et al., 2010). Research related with cereals, fruits and vegetables fermentation for production of functional foods utilize mainly single substrates for potentially probiotic LAB growth (ANGELOV et al., 2006; CHARALAMPOPOULOS; PANDIELLA; WEBB, 2003; HELLAND; WICKLUND, 2004).

It is known that the use of probiotic cultures requires a large fermentation period to reach low pH values. However, the food industries always prefer short fermentation periods in order to increase plant output and reduce microbial contamination. The potential solution to this problem is the use of mixed cultures or co-cultures (MACEDO; SOCCOL; FREITAS, 1998). Mixed culture fermentations provide complex growth patterns that can also considerably affect the organoleptic and functional properties of the food. No information is available regarding the growth of probiotic bacteria in mixed cultures and how mixed LAB, and yeasts with probiotics will behave in PSM.

The development of a fermented PSM containing probiotic microorganisms in co-culture requires a strain selection showing ability to grow in the substrate, ability to compete, and/or establish a synergistic growth between strains. The aim of this work was to study the growth and acidification of PSM by *Lactobacillus rhamnosus* (LR 32), *Lactobacillus acidophilus* (LACA 4), *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB 340), *Pediococcus acidilactici* (UFLA BFFCX 27.1), *Lactococcus lactis* (CCT 0360), and *Saccharomyces cerevisiae* (UFLA YFFBM 18.03); to select strains better adapted and with best characteristics for the fermented mixed milk; and to

describe the development of an inoculum that rapidly ferments peanut and soy maintaining significant probiotic yields after refrigerated storage.

2 MATERIALS AND METHODS

2.1 Peanut-soy milk preparation

Peanut milk was prepared as previously described (SALUNKHE; KADAM, 1989) with minor modifications. The seeds were sorted to remove discolored grains and any foreign material. Selected peanut seeds were roasted at 130 °C for 20 min in a forced air circulation oven. The seeds were peeled and weighed before being soaked in 0.5 g/100 mL $NaHCO_3$ for at least 12 h. The shelled seeds were then washed with running water and mixed with distilled water in a ratio of 1:5 [peanuts (g):water (mL)] and transferred to a stainless steel blender (Cemaf, São Paulo, Brazil) for 5 min. The resulting paste was filtered through a double-layered cheese cloth.

For the soymilk preparation, 250 g of selected beans were initially soaked in 1 L of distilled water for 16 h at 25 °C. Subsequently, 190 g of these moistened beans were soaked in 500 mL water in a stainless steel blender and mixed for 3 minutes. Then, the resulting slurry was filtered through double-layered cheese cloth and boiled for 5 min (CHAMPAGNE et al., 2009).

The peanut and soybean milk were mixed in the 2:1 – peanut milk:soymilk ratio. The heat treatment was carried out by pasteurization at 90 °C/20min, followed by immediate cooling to 4 °C.

2.2 Preparation of cultures

The commercial probiotic cultures *L. rhamnosus* (LR 32) and *L. acidophilus* (LACA 4) were acquired from Danisco (Flora Fit, USA) and from Danisco (Yo Mix, Deutschland), respectively. The *L. delbrueckii* subsp. *bulgaricus* (LB 340) yogurt starter culture was purchased from Danisco (Yo

Mix, France). The *P. acidilactici* lactic acid bacteria (UFLA BFFCX 27.1) was isolated from *caxiri* indigenous beverage (SANTOS et al., 2012). The *S. cerevisiae* (UFLA YFFBM 18.03) yeast was isolated from cocoa beans fermentations (PEREIRA et al., 2012). These strains belong to the culture collection of Microbial Physiology Laboratory/Department of Biology, Federal University of Lavras (UFLA), Brazil. *Lc. lactis* was isolated from cocoa fermentations, which belong to the André Toselo Tropical Culture Collection (Campinas, SP, Brazil).

The commercial cultures activation was performed according to the manufacturer's instructions. Stock cultures for bacteria were prepared by mixing culture medium Man Rogosa Sharp broth (MRS, Merck Whitehouse Station, USA) and 40% glycerol (w/v) in the 1:1 proportion placing 1 mL in cryovials and freezing at - 20 º C. For yeast, YPD agar (10 g/L yeast extract (Merck, Darmstadt, Germany), 10 g/L peptone (Himedia, Mumbai, India), 20 g/L glucose (Merck, Darmstadt,Germany), 20 g/L agar (Merck, Darmstadt, Germany) pH 3.5 were mixed with 40% glycerol (w/v) in the 1:1 proportion placing 1 mL in cryovials and freezing at - 20 º C. The glycerol solution was previously sterilized at 121 °C for 15 minutes (CHAMPAGNE et al., 2009).

The preparation of inoculum for single and co-culture fermentation was performed by culturing 100 µL of each strain separately in 5 mL of sterile MRS broth (bacteria) and YPD pH 3.5 (yeast), then incubating at 37 ºC and 30 °C, respectively. After 16 h and 24 h, the supernatant was removed and the cells were transferred to 50 mL MRS and YPD, respectively. The flasks were again incubated for 16 h (bacteria) and 24 h (yeast), the cells were subsequently transferred to 500 mL of the respective medium and then incubated in the same conditions described above. The cells were centrifuged for 7 min at 7,000 rpm at 4 °C and the supernatant was removed. Cells were washed two times with sterile distilled water and inoculated in PSM with a population of 7 log

CFU/mL. To perform the inoculation, OD growth kinetics were constructed by plotting the OD (600 nm) of suspensions minus the OD of non-inoculated media vs the time of incubation (RUIZ-MOYANO et al., 2008). To calibrate the ODs against the cell concentration of the cultures, the viable counts (VC) of a strain for each broth used were determined between readings, by the method of Miles, Misra and Irwin (1938).

2.2.1 Single fermentations

The washed cells were inoculated in 400 mL of the pasteurized peanut-soy milk (2:1). The suspension with inoculated cells was fractionated in 25 mL portions, placed into 50 mL conical tubes. These tubes were incubated at 37 °C for up to 24 h and afterwards, the fermented PSM remained for another 24 h at $\pm$ 4 °C. The experiments were performed in three independent assays. For each repetition two samples were taken at each time (duplicate).

2.2.2 Co-culture fermentations

Four co-culture fermentations were performed with: (1) *S. cerevisiae* (UFLA YFFBM 18.03) and *P. acidilactici* (UFLA BFFCX 27.1); (2) *S. cerevisiae* (UFLA YFFBM 18.03) and *L. acidophilus* (LACA 4); (3) *P. acidilactici* (UFLA BFFCX 27.1) and *L. acidophilus* (LACA 4); and finally, (4) *S. cerevisiae* (UFLA YFFBM 18.03), *P. acidilactici* (UFLA BFFCX 27.1) and *L. acidophilus* (LACA 4) strains. An initial population of 7 log CFU/mL of each strain was inoculated in pasteurized PSM. The suspension with inoculated cells was fractionated in 25 mL portions, and placed into 50 mL conical tubes. These tubes were incubated at 37 °C for up to 24 h and afterwards, the fermented PSM remained for 24 h at $\pm$ 4 °C. The experiments were performed in three

independent assays. For each repetition two samples were taken at each time (duplicate).

2.3 Viable count of inoculated strains (VC)

A one milliliter sample was taken from each fermentation flask. Serial tenfold dilutions was prepared in a solution of 0.9% NaCl (w/v) and 0.1% (w/v) bacto peptone (Difco). Viable counts of LAB and probiotic bacteria in mixed cultures were obtained by spreading 0.1 mL sample of an appropriate dilution onto the surface of MRS plates containing 0.1% (w/v) cysteine-HCl and 0.01% (w/v) aniline blue under anaerobic conditions during incubation (37º C for 2 days) (EVANGELISTA; GHISELLI; FILHO, 2012; WANG; YU; CHOU, 2002). The colonies presented different shades of color (white and blue) allowing differential count. Yeasts were grown on YPD agar pH 3.5 at 28 ºC for 5 days. Colony forming units (CFU) were enumerated in plates containing 30 to 300 colonies, and cell concentration was expressed as log CFU/mL of fermented PSM.

2.5 Analytical methods

2.5.1 Determination of pH and titratable acidity

The pH of the fermenting PSM samples was measured with a pH meter (Tecnal, Tec-3MT, São Paulo, Brazil). Estimation of titratable acidity was performed by a previously reported method Aoac (2000), in which a sample of 5 mL was titrated against 0.1 or 0.01 M NaOH using phenolphthalein as indicator.

2.5.2 Organic acids, alcohol and carbohydrates

Samples were prepared by adding 3mL of n-hexan (Sygma-Aldrich) to 3 ml of samples. The mixture was vortexed for 30 seconds and the non-hexanic phase was pipetted into another tube. The procedure was again performed. Subsequently, the samples were centrifuged two times at 7,000 rpm for 10 minutes at 4 °C, and then supernatant was filtered through 0.22 μm filters, and analyzed. Organic acids (acetic, lactic, and succinic acid), alcohol (ethanol) and carbohydrates (glucose, sucrose, maltose, fructose, raffinose, and stachyose) were identified according to the methodology proposed by Duarte et al. (2010). The analyses were carried out using a high performance liquid chromatography system (HPLC) (Shimadzu, model LC-10A*i*, Shimadzu Corp., Japan), equipped with a dual detection system consisting of a UV-Vis detector (SPD-10A*i*) and a refractive index detector (RID-10A*i*). A Shimadzu ion exclusion column (Shim-pack SCR-101 H, 7.9 mm x 30 cm) was used operating at 50 ºC, using 100 Mm of perchloric acid as the eluent at a flow rate of 0.6 mL/min. The acids were detected via UV absorbance (210 nm), while alcohols were detected via RID. For carbohydrates, the Supelcosil LC-NH_2 column (4.6mm x 25 cm) was used, operating at 30 ºC with a mobile phase (acetonitrile:water (75:25)) in a flow rate of 1 mL/min. The sugars were detected via RID. Individual compounds were identified based on the retention time of standards injected in the same conditions, and their concentrations were determined using the external calibration method. All samples were examined in triplicate.

2.5.3 Amino acids

The determinations of total amino acids were performed by High Performance Liquid Chromatography (HPLC) as described by White, Hart and

Fry (1986). After a 24 h hydrolysis in 6 M HCl at 100 °C, the amino acids were reacted with phenyl isothiocyanate, and the derivatives separated using a Luna C-18, 100 Å; 5 µ, 250 mm × 4.6 mm (00G-4252-E0, Torrance, CA, USA) column, at 50 °C. The detection was by ultraviolet absorption measurements using a fixed wavelength (254 nm) detector. Quantification was carried out by comparison with a standard mixture (Thermo Scientific, Rockford IL, USA) and DL-2-aminobutyric acid was used as an internal standard from Sigma–Aldrich Corp., St. Louis, MO; (HAGEN; FROST; AUGUSTIN, 1989).

2.6 Data presentation and statistical analyses

All the results are expressed as means ± standard deviation (SD). In all of analyses, the assumption of normality was tested using Shapiro and Wilk test. The assumption of homogeneity of variances was tested using Barlett test. As both assumptions of normality and homogeneity of variances were satisfied, parametric testing was performed. The data discussed are the averages from three independent assays and were subject to one-way analysis of variance (ANOVA), followed by Fisher's Least Significant Difference (LSD) test (GRANATO; ARAÚJO CALADO; JARVIS, 2014). Differences in values were considered significant when the *P* value was less than 0.05. The statistical analyses were performed using the Sisvar 5.3 software (FERREIRA, 2010), except the Bartlett test, which was performed using the Assistat software (SILVA; AZEVEDO, 2009).

3 RESULTS AND DISCUSSION

3.1 Growth and acidification profiles

The success of new probiotic formulations do not only rely on the ability to provide enough probiotic cells that may survive the human gastrointestinal tract. An appropriate selection of substrate composition and strains are necessary to efficiently control the distribution of the metabolic end products (DE VUYST, 2000). Santos, Libeck and Schwan (unpublished results 2014) developed a formulation using peanut milk mixed with soy milk, with high nutritional value, as a suitable novel substrate to fermentation. However, the present study is the first to report the behavior of different single and co-cultures, including probiotic ones in this novel PSM.

Results of cell growth and acidification profiles of single cultures are presented in Fig. 1. The *P. acidilactici* (UFLA BFFCX 27.1) LAB showed the highest increase in viable population ($p < 0.05$) during PSM fermentation, followed by *L. rhamnosus* (LR 32) probiotic bacteria and the *L. delbrueckii* subsp. *bulgaricus* (LB 340) yogurt starter, that do not differ statistically ($p > 0.05$). All these strains reached populations above 8 log CFU/mL. Although these bacteria were effective in growing, they did not acidify the PSM, and the pH remained above 6. This poor ability of lowering the pH of the soy-based substrates by *L. rhamnosus* and *L. delbrueckii* subsp. *bulgaricus* has also been reported by Champagne et al. (2009) and Farnworth, Mainville and Desjardins (2007).

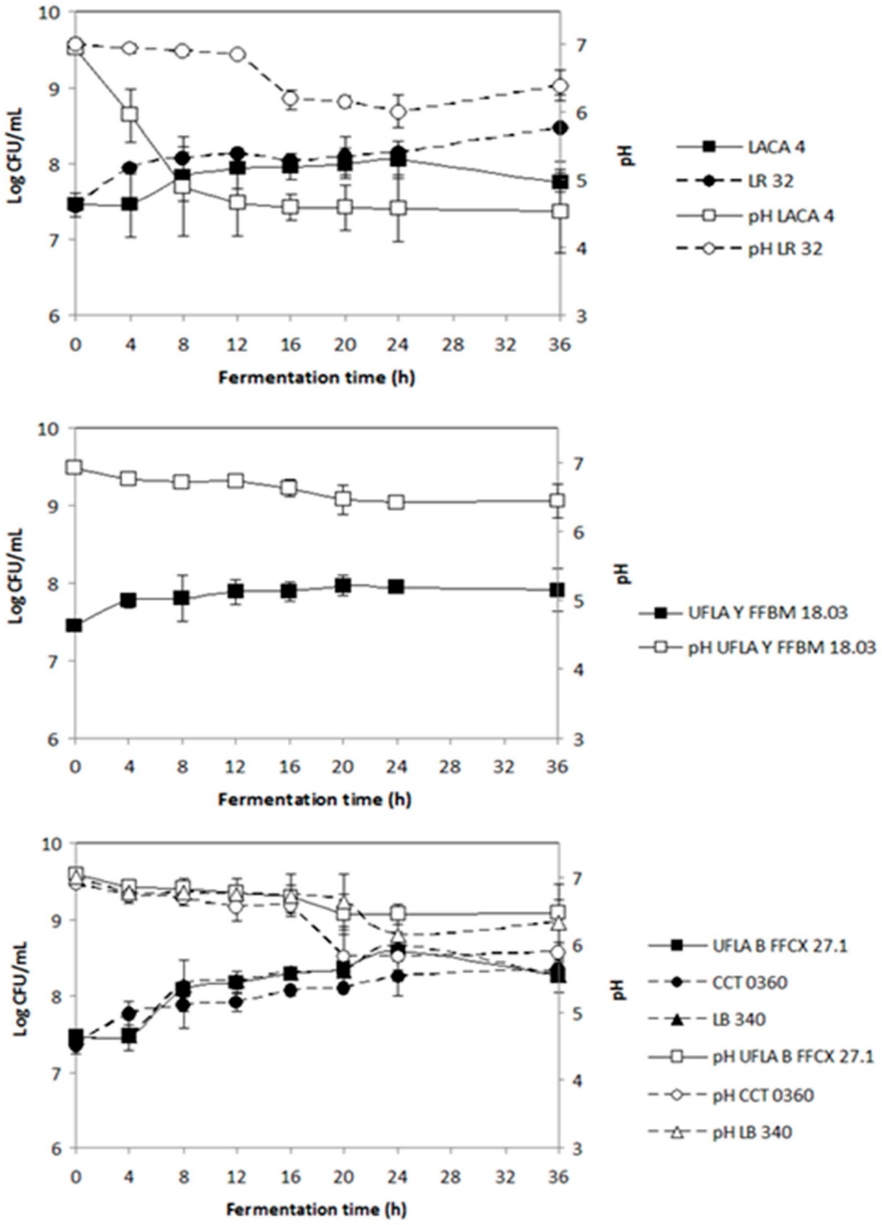

Fig. 1 Results of cell growth and acidification profiles of single cultures

A rapid pH value drop in the PSM substrate was observed by *L. acidophilus* (LACA 4), reaching 4.6 at 12 h of fermentation. *L. acidophilus* is an obligatory homofermentative bacterium that produces a single end product (lactic acid) from the glycolysis of carbohydrates (EM pathway) (RATHORE; SALMERÓN; PANDIELLA, 2012). High viable counts are necessary to get the desired acid production and reduction in pH, which affects organoleptic properties, shelf-life, and prevents products contamination (RATHORE; SALMERÓN; PANDIELLA, 2012). The cell populations of *L. acidophilus* (LACA 4) increased rapidly within the first 8 hours of fermentation and remained stable at 7.9 log CFU/mL. After 24 h under refrigerated conditions the viable cell counts were about 7.75 Log CFU/mL (Fig. 1).

The growth of *Lc. lactis* (CCT 0360) over 24 h was slow and constant reaching 8.25 log CFU/mL at 24 h. The slow growth reflected in the acidification curve showing an equally slow acidification rate in peanut-soy beverage, with a pH of 5.8 eventually reached after 24 h. Therefore, the PSM provided appropriate growth and fermentation conditions for the pure microbial cultures. *L. acidophilus* (LACA 4) and *P. acidilactici* (UFLA BFFCX 27.1) presented populations above 8 log CFU/mL and may preserve the low pH of beverage. The use of *S. cerevisiae* (UFLA YFFBM 18.03) may give rise to a new class of products, where the addition of yeast may serve as a source of protein and vitamin B (STEINKRAUS, 1996). Thus, these strains were selected to be co-cultured to obtain short fermentation periods and larger populations of the probiotic bacteria.

The evolution of cell populations and acidification during co-culture fermentations of PSM are shown in Fig. 2. It should be noted that fermentations carried out in co-culture were more efficient in substrate acidification. Twelve hours were required to reach a pH value of 4.3 in both fermentations with *L. acidophilus* (LACA 4) and *P. acidilactici* (UFLA BFFCX 27.1), and with *L.*

acidophilus (LACA 4), *P. acidilactici* (UFLA BFFCX 27.1) and *S. cerevisiae* (UFLA YFFBM 18.03). The co-culture of *L. acidophilus* and the *S. cerevisiae* yeast also reached pH 4.3, but required a longer time of about 20 h.

The pH value reached was below 4.5 in all co-cultured fermentations, which demonstrated that 7 log CFU/mL of inoculum was sufficient to decrease the pH. The pH around 3.5-4.5 has been reported for food formulations aids the pH increase of the gastrointestinal tract, and thus enhances the stability and benefits of probiotic strains consumed (KAILASAPATHY; CHIN, 2000).

It has been suggested that fermented products required probiotic bacteria at 7 log CFU/mL in order to give health effects in the gastro-intestinal tract when consumed (OUWEHAND; SALMINEN, 1998). Data from this study showed that high levels (8 log CFU/mL) of the *L. acidophilus* (LACA 4) probiotic could be reached when this strain is inoculated with *P. acidilactici* (UFLA BFFCX 27.1), and *S. cerevisiae* (UFLA YFFBM 18.03) at the beginning of fermentation of PSM. When these three selected strains were co-incubated, the probiotic bacteria had their growth favored reaching the population of 8 log CFU/mL within 8 h of fermentation. *P. acidilactici* populations remained above 8 log CFU/mL after 12 h of fermentation. *S. cerevisiae* population ranged from 7.3 to 7.5 log CFU/mL at 24 h. At the end of 24 h, the population decreased slightly, but it was not statistically significant ($p > 0.05$).

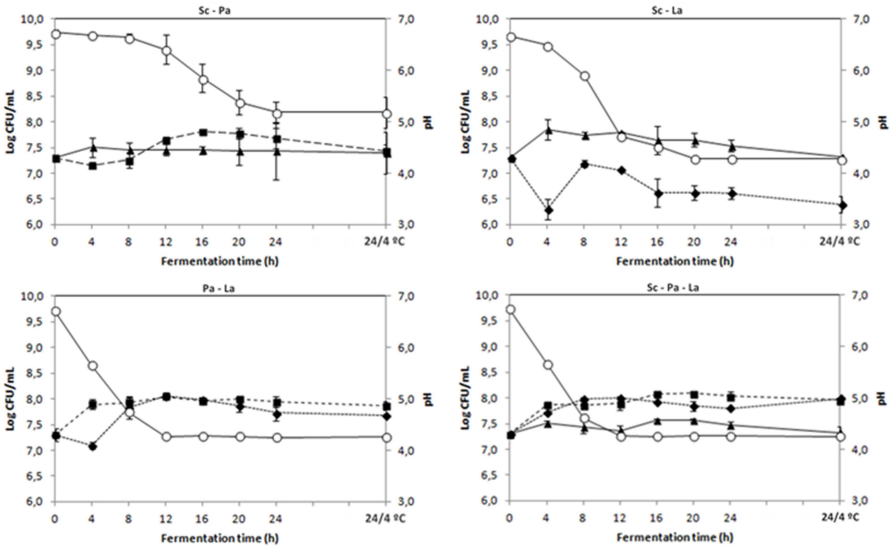

Fig. 2 Evolution of cell populations and acidification during co-culture fermentations of PSM.

The co-cultured organism may compete for nutrients or may produce metabolic products that may stimulate or inhibit each other's growth. It has been reported that yeast may produce vitamins that enhance the growth of LAB (MARSHALL, 1987). Growth of lactic acid bacteria is believed to be promoted when co-cultured with yeasts, mainly due to the excretion of specific amino acids and small peptides by the latter, either during growth, and regardless of the antagonism for the main carbon source (GOBBETTI; CORSETTI; ROSSI, 1994) or as a consequence of an accelerated autolysis (ZAMBONELLI et al., 2000).

The yeast when co-cultured with the *L. acidophilus* probiotic was more competitive than in mono culture, regarding the final population reached, showing the increase of 1 log CFU/mL. The yeast probably caused a significant ($p < 0.05$) reduction in the population of bacteria in 4 h of fermentation. After a reduction in the *L. acidophilus* (LACA 4) population, these LAB increased their

population and stabilized at 6.6 log CFU/mL. In the co-culture of *S. cerevisiae* and *P. acidilactici*, both presented one log higher than when cultivated in pure culture. Despite the slow growth start, the LAB reached viable counts of about 8 log CFU/mL after 16 h, and remained constant ($p>0.05$) until the end of the fermentative process.

The cultures showed a trend to decrease their populations when they were kept at 4 ºC. Fermentations conducted with *L. acidophilus* (LACA 4) and *P. acidilactici* (UFLA BFFCX 27.1), and the fermentation involving all strains were the ones that showed smaller effect of low temperature on viable populations ($p < 0.05$). The fermentation involving all cultures led to an increase of *L. acidophilus* (LACA 4) viable count during 24 h under refrigeration.

In general, results of the pH values followed an opposite trend to that observed for titratable acidity measurements, i.e., as the acidity increased, the pH decreased. Fig. 3a shows clearly that *L. acidophilus* (LACA 4), in pure culture, was the only strain able to acidify significantly the substrate ($p < 0.05$). Efficient acidification profiles were generally achieved in co-culture fermentation (Fig. 3b).

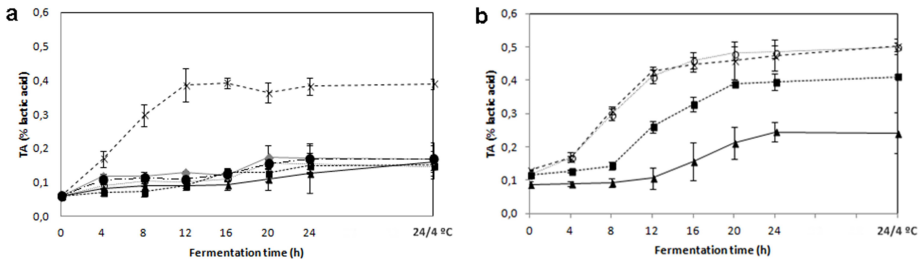

Fig. 3 L. acidophilus (LACA 4), in pure culture, as the only strain able to acidify significantly the substrate; efficient acidification profiles generally achieved in co-culture fermentation.

The titratable acidity of PSM increased from 0.06% to 0.48% after 24 h of fermentation with the co-cultivation of all microorganisms. After 24 h at ± 4 ºC, the titratable acidity increased to 0.50% of lactic acid with UFLA BFFCX 27.1 and LACA 4, and with the three mixed cultures. Isanga and Zhang (2007) developed fermented peanut milk with the mixed cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus*, and *Streptococcus salivarius* ssp. *thermophilus*. In accordance, the authors reported that fermentation with combined cultures had higher titratable acidity values (0.39%) than with the single cultures of these strains.

3.2 Consumption of sugars and produced metabolites

Fig. 4 and 5 illustrate the metabolic behaviors of the strains either in pure cultures or in co-cultures. They were monitored in fermented PSM by determination of the main metabolic products (lactic, acetic, succinic acids, and ethanol) and consumed sugars. As shown in Fig. 4, the L. acidophilus (LACA 4), P. acidilactici (UFLA BFFCX 27.1), and S. cerevisiae (UFLA YFFBM 18.03) strains in single cultures produced 3.26, 0.26 and 0.03 g/L, respectively, of lactic acid after 24 h of fermentation. The highest values (9.03 and 8.51 g/L) were found in fermentations after 24 h at 4 ºC in a binary culture of P. acidilactici and L. acidophilus, and in combination of the three strains, respectively. The marked lactic acid increase in co-culture fermentations could be due to interactions between the different species used. These results highlighted the importance of the selection of the substrate composition and inocula in the development of the organoleptic properties of these fermented products.

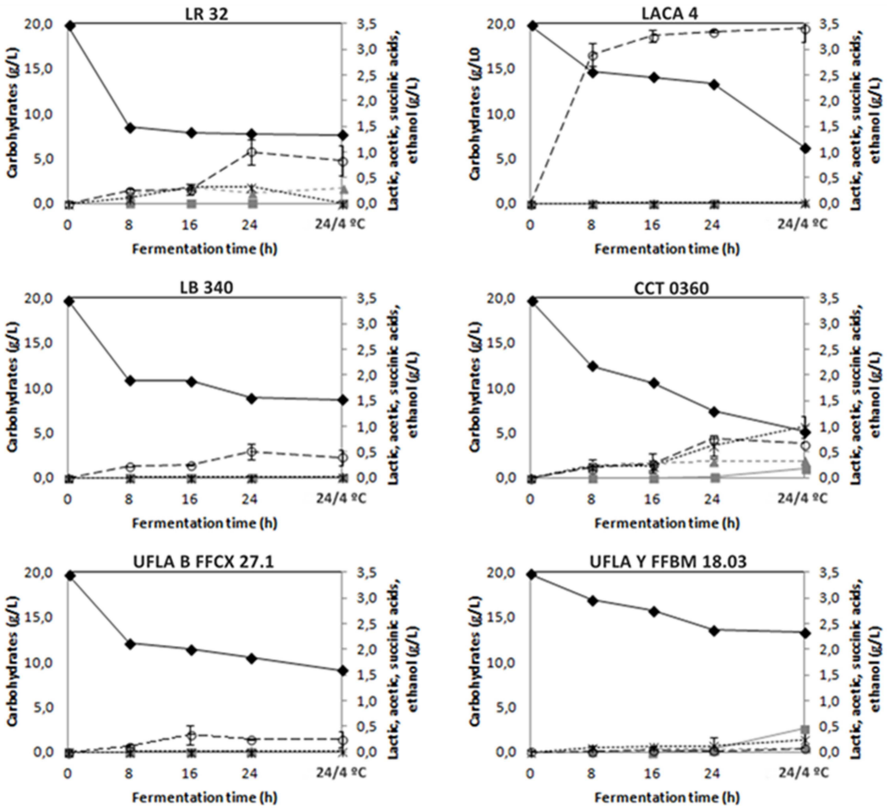

Fig. 4. Metabolic behaviors of the strains in pure cultures

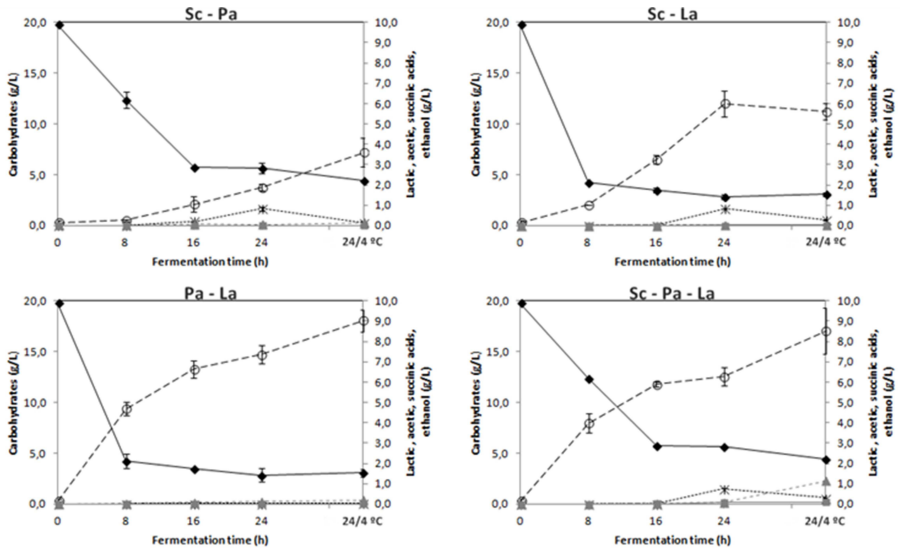

Fig. 5. Metabolic behaviors of the strains in co-cultures

Acetic acid was produced in three co-culture fermentations, and amounts of 1.41 g/L after 24 h at 4 °C were detected. The *S. cerevisiae* yeast (UFLA YFFBM 18.03) also produced succinic acid, reaching 0.46 g/L as the maximum content in pure culture. In co-culture, this acid was produced from 0.02 to 0.10 g/L after 24 h.

The *S. cerevisiae* yeast produced low ethanol content (0.24 g/L after 24 h under refrigeration) in pure culture. This value was smaller than those obtained for *L. rhamnosus* (0.32 g/L). It may be due to non-complete substrate adaptation, inhibition of growth by some medium components, or lack of required nutrients in the raw material. The highest ethanol (0.84 and 0.75 g/L) concentrations were found after 24 h of fermentation in the co-culture of *S. cerevisiae* and *L. acidophilus*, followed by *S. cerevisiae, P. acidilactici* and *L. acidophilus*, respectively. A decrease in these values was noticed during storage under refrigeration. It was probably due to the ethanol evaporation. The final content of ethanol was 0.03% (v/v) or less, which classifies the beverage as a

non-alcoholic beverage (BRASIL, 2009). It seems that *L. acidophilus, P. acidilactici,* and *S. cerevisiae* may influence each other's metabolism, which can lead to different profiles of important organoleptic compounds. There might also be other interactions among the produced metabolites, where a compound produced by one organism may be further metabolized by another (AXELSSON, 1998; RATHORE; SALMERÓN; PANDIELLA, 2012).

The content of sugars in PSM is due to raw materials and the breakdown of starch during the heat treatment. The available carbohydrates contributed to the cell viability and to the production of metabolites. In our work, sucrose and stachyose decreased during all fermentations (Table 1). Our results are in agreement with Beuchat and Nail (1978). These authors reported that sucrose is the major fermentable carbohydrate found in peanut milk. Champagne et al. (2009) stated that, for soy beverages, the available carbohydrates, by order of importance, are sucrose, stachyose, raffinose, glucose and fructose.

The reduction in the contents of raffinose and stachyose and the increase in the contents of monosaccharide may be attributed to the hydrolytic reaction catalyzed by α- and β-galactosidases produced by both LAB species studied here (DONKOR et al., 2007). Raffinose and stachyose are α-galactosides of sucrose, comprising three and four monomeric units, respectively, and are non-digestible in the gut due to the absence of α-galactosidase in the human intestinal mucosa. Consequently, intact oligosaccharides pass directly into the lower intestine, where they are

metabolized by bacteria that possess this enzyme, resulting in the production of gases (TSANGALIS; SHAH, 2004).

The α-galactosides are sources of carbon for the growth of *Lactobacillus* species, such as *Lactobacillus acidophilus* (SCALABRINI et al., 1998). Our data showed that binary co-culture of *L. acidophilus* and *P. acidilactici*, and co-culture of *L. acidophilus*, *P. acidilactici* and *S. cerevisiae* may be a practical approach to overcome the flatulence factor in PSM.

The utilization of sugars in PSM was different than the data reported by Farnworth, Main and Desjardins (2007), who showed that fructose was the most utilized sugar, while glucose, raffinose, and stachyose were much less used. These authors studied the growth of *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. johnsonii*, several *Bifidobacterium* sp. and *Streptococcus termophilus* in a soy-yogurt formulation. These discrepancies in the results suggested that the initial concentration of the carbohydrates may alter the dynamics of consumption thereof.

Table 1 Carbohydrate content in fermented peanut-soy milk with different combinations of *L. acidophilus* (LACA 4), *P. acidilactici* (UFLA BFFCX 27.1), and *S. cerevisiae* (UFLA YFFBM 18.03)

Time (h)	Fructose	Glucose	Sucrose	Maltose	Raffinose	Stachyose
0	1.26±0.04 [a]	Nd	12.16±0.33 [a]	Nd	0.77±0.01 [a]	5.52±0.02 [a]
L. acidophilus (LACA 4) + *P. acidilactici* (UFLA B FFCX 27.1)						
8	0.63±0.02 [b]	0.43±0.03 [a]	1.08±0.04 [b]	Nd	Nd	2.09±0.74 [b]
16	0.43±0.00 [c]	0.10±0.04 [b]	Nd	0.94±0.02 [a]	Nd	2.00±0.00 [b]
24	0.29±0.00 [d]	Nd	Nd	0.46±0.04 [b]	0.25±0.09 [b]	1.84±0.65 [b]
24 h/4 ºC	0.74±0.01 [b]	Nd	Nd	0.43±0.03 [b]	0.80±0.06 [a]	1.10±0.35 [b]
L. acidophilus (LACA 4) + *S. cerevisiae* (UFLA Y FFBM 18.03)						
8	0.70±0.01 [bc]	0.64±0.14 [a]	6.87±1.74 [b]	Nd	0.65±0.00 [bc]	4.80±0.02 [b]
16	0.43±0.00 [d]	0.21±0.01 [b]	2.21±0.06 [c]	0.65±0.01 [a]	Nd	3.97±0.22 [c]
24	0.76±0.02 [b]	0.14±0.00 [b]	0.44±0.04 [d]	0.42±0.00 [b]	0.61±0.06 [bc]	3.76±0.25 [c]
24 h/4 ºC	0.49±0.07 [cd]	0.12±0.01 [b]	0.33±0.01 [d]	0.39±0.01 [b]	0.51±0.00 [c]	3.61±0.16 [c]
P. acidilactici (UFLA B FFCX 27.1) + *S. cerevisiae* (UFLA Y FFBM 18.03)						
8	0.84±0.05 [c]	0.36±0.05 [a]	7.84±0.48 [b]	Nd	0.41±0.15 [ab]	2.89±0.79 [b]
16	Nd	Nd	2.83±0.78 [c]	0.64±0.01 [a]	Nd	2.25±0.00 [b]
24	0.35±0.00 [c]	0.19±0.02 [b]	1.48±0.29 [c]	0.62±0.03 [a]	0.86±0.22 [a]	2.19±0.52 [b]
24 h/4 ºC	0.31±0.01 [c]	0.24±0.03 [ab]	0.68±0.15 [c]	0.74±0.10 [a]	0.47±0.09 [ab]	2.00±0.07 [b]
L. acidophilus (LACA 4) + *P. acidilactici* (UFLA B FFCX 27.1) + *S. cerevisiae* (UFLA Y FFBM18.03)						
8	0.63±0.00 [c]	0.29±0.00 [a]	5.43±0.13 [b]	Nd	0.48±0.03 [b]	3.78±0.09 [b]
16	0.96±0.08 [b]	Nd	Nd	Nd	0.02±0.10 [c]	3.04±0.10 [c]
24	0.58±0.01 [c]	Nd	Nd	0.51±0.18 [ab]	Nd	2.51±0.03 [d]
24 h/4 ºC	0.60±0.02 [c]	0.26±0.04 [a]	Nd	1.03±0.24 [a]	Nd	2.45±0.15 [d]

[a] Mean±SD. Means followed by the same letters in each column do not differ significantly ($p > 0.05$). Nd – not detected.

In general, when co-cultured, the strains were more efficient in the use of available carbohydrates and in the release of metabolites in the fermentation medium. In single cultures, the strains consumed an average of 58% and in co-cultures the consumption of available carbohydrates was approximately 78%. The fermentations carried out in binary culture of the probiotic bacteria *L. acidophilus* (LACA 4) and the LAB *P. acidilactici* (UFLA BFFCX 27.1), were the most efficient in the consumption of sugars. At the end of the fermentation, there were 3.07 g/L of available carbohydrates (15.58%).

3.3 Total amino acids

As shown in Table 2, the unfermented and fermented PSM contain almost all the essential amino acids and non-essential amino acids, though in varying amounts. According to Blandino et al. (2003) the effect of fermentation on the protein and amino acids levels is a topic of controversy. It appears that the effect of fermentation on the nutritive value of foods is variable, although the evidence for improvements is substantial. Certain amino acids may be synthesized and the availability of B group vitamins may be improved. Yeast extract provides growth stimulants such as amino acids and nucleotides (NAGODAWITHANA, 1992) for cheese starter which results in increased activity (NSOFOR et al., 1996). In fact, the *S. cerevisiae* yeast (UFLA YFFBM 18.03) cultivated in pure culture was the strain that provided the greatest increase in the amounts of amino acids in PSM.

S. cerevisiae (UFLA YFFBM 18.03), *L. rhamnosus* (LR 32), *L. acidophilus* (LACA 4), and *P. acidilactici* (UFLA BFFCX 27.1) promoted the release of amino acids into the PSM. The percentages of total amino acids in PSM fermented for 24 h at 37 ºC were about 48.02%, 47.32%, 46.21%, and 44.07%, respectively for the above pure cultures (Table 2). However, when co-

cultured, the strains consumed the amino acids, this probably contributed to growth increase, higher cell viability, greater production of lactic acid, and consequent fast drop in pH value.

PSM, fermented with pure cultures, was found to be richer in some essential amino acids (such as threonine, histidine, phenylalanine, tyrosine, and lysine) than unfermented milk. However, unfermented milk was richer in other amino acids (like cysteine, glutamic acid, arginine, and serine) than in pure culture fermentations.

Table 2 Total amino acids (g/100 g of sample, on dry basis) in unfermented peanut-soy milk and after 24 h of fermentation (pure and co-cultures)

Amino acid	Unfermented P-S Milk	UFLA Y FFBM 18.03	LACA 4	LR 32	LB 340	UFLA B FF CX 27.1	CCT 0360	Sc – Pa	Sc - La	Pa - La	Sc - Pa - La
Histidine (Hys)	1.12	1.32	1.15	1.2	1.09	1.31	1.07	1.21	1.16	1.07	0.97
Isoleucine (Ile)	1.78	2.10	1.97	2.03	1.77	1.84	1.82	1.81	1.74	1.67	1.71
Leucine (Leu)	2.90	3.27	3.13	3.01	2.88	2.92	2.87	2.90	2.78	2.72	2.77
Lysine (Lys)	1.75	2.10	2.09	1.82	1.90	1.82	1.81	1.84	1.72	1.65	1.78
Methionine (Met)	0.50	0.63	0.60	0.67	0.53	0.56	0.53	0.58	0.55	0.55	0.51
Cysteine (Cys)	0.67	0.72	0.76	0.8	0.20	0.65	0.67	0.49	0.38	0.45	0.54
Phenylalanine (Phe)	1.54	1.67	1.66	1.6	1.61	1.67	1.59	1.38	1.51	1.35	1.37
Tyrosine (Tyr)	1.61	1.80	1.67	1.67	1.57	1.67	1.60	1.55	1.48	1.50	1.45
Threonine (Thr)	1.71	1.92	1.89	1.97	1.66	1.71	1.61	1.65	1.60	1.52	1.57
Valine (Val)	2.01	2.31	2.12	2.42	2.00	2.07	2.00	2.05	1.94	1.89	1.90
Total essential amino acids	15.58	17.84	17.04	17.19	15.21	16.22	15.57	15.46	14.86	14.37	14.57
Arginine (Arg)	4.80	5.07	4.65	4.68	4.33	4.96	4.33	4.57	4.61	4.49	4.50
Alanine (Ala)	1.92	2.10	2.07	2.19	1.85	1.97	1.85	1.94	1.86	1.78	1.83
Aspartic acid (Asp)	5.13	5.62	5.41	5.52	5.06	5.10	5.00	5.00	4.87	4.78	4.77
Glutamic acid (Glu)	9.44	10.37	10.19	10.4	9.24	9.34	9.24	9.02	8.87	8.91	8.92
Glycine (Gly)	1.93	2.09	1.98	2.06	1.88	1.90	1.87	1.89	1.81	1.76	1.77
Proline (Pro)	2.40	2.61	2.64	2.65	2.35	2.44	2.27	2.35	2.26	2.16	2.21
Serine (Ser)	2.20	2.33	2.25	2.63	2.09	2.13	2.06	2.07	2.03	2.07	2.07
Total amino acids	43.37	48.02	46.21	47.32	42.01	44.07	42.19	42.30	41.15	40.31	40.64

P-S Milk –peanut-soy milk. Pure cultures: UFLA Y FFBM 18.03 - *S. cerevisiae;* LACA 4 - *L. acidophilus;* LB 340 - *L. rhamonsus;* LB 340 - *L. delbrueckii* subsp. *delbrueckii;* UFLA B FFCX 27.1 - *P. acidilactici;* and CCT 0360 - *L. lactis.* Co-cultures: *S. cerevisiae – P. acidilactici* (Sc – Pa); *S. cerevisiae - L. acidophilus* (Sc – La); *P. adicilaciti – L. acidophilus* (Pa – La) and *S. cerevisiae – P. acidilactici – L. acidophilus* (Sc – Pa – La).

In general, the lysine content of PSM prepared in this study was close to the range observed in the similar studies referred to here on peanut-based beverages. The ranges of lysine content in experimental fermentations were 1.65-2.10 % in PSM. According to Rubico, Resurreccion and Beuchat (1988), the amino acid profiles of various peanut beverages treatments indicated that raw peanut extracts had a lysine content of 4.31 %; and the lysine content in homogenized peanut milk was within the range of 2.88–3.81 %.

4 CONCLUSION

The main aim for the development of this product was the utilization of two important vegetable protein sources, peanut and soy, which are readily available in abundance and at a reasonable cost. Both peanut and soy protein are comparable with protein content from animal based food and it is suitable for all ages (DESHPANDE; CHINNAN; MCWATTERS, 2008). This study is the first report of the potential of *L. acidophilus* as probiotics in fermented peanut-soy beverage in mixed cultures. This strain showed higher viability when cultivated with *P. acidilactici* and *S. cerevisiae* in PSM. Further studies are needed to evaluate the survival of these cultures over a longer period of post-acidification and also, the sensory acceptation of the final beverage.

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CAPÍTULO 4 Development of a potential probiotic peanut-soy milk fermented in co-culture of lactic acid bacteria and yeast: Optimization by response surface methodology

Claudia Cristina Auler do Amaral Santos[8], Bárbara da Silva Libeck[9], Rosane Freitas Schwan[10]

[8] Department of Biology, Federal University of Lavras (UFLA), CP 3037 - Campus Universitário, CEP 37.200-000 Lavras, MG, Brazil

[9] Department of Food Science, , Federal University of Lavras (UFLA), CP 3037 - Campus Universitário, CEP 37.200-000 Lavras, MG, Brazil

[10] Department of Food Science, Federal University of Lavras (UFLA), CP 3037 - Campus Universitário, CEP 37.200-000 Lavras, MG, Brazil. Corresponding author. [telephone (+55) 35 3829 1614; fax (+55) 35 3829 1100; e-mail: rschwan@dbi.ufla.br

ABSTRACT

The aim of this study was the development and optimization of a potentially probiotic beverage based on peanut-soy milk (PSM) using lactic acid bacteria (LAB) and yeast as a novel and value-added product. The composition and process conditions were defined using a Plackett & Burman factorial design in which the independent variables were fermentation time and temperature, PSM formulation, concentrations of fructooligosaccharides (FOS) and the initial inocula of LAB *Pediococcus acidilactici* (UFLA B FFCX 27.1), of probiotic *Lactobacillus acidophilus* (LACA 4) and of yeast *Saccharomyces cerevisiae* (UFLA Y FFBM 18.03). *P. acidilactici* and *L. acidophilus* culturesand PSM formulationwere the significant variables on probiotic viability, pH, lactic acid, acetic acid and ethanol contents during fermentation.The optimal conditions to develop the potentially probiotic PSM were obtained by response surface methodology. They were found to be 61.2 % of peanut milk in PSM formulation inoculated with 8.00 and 6.70 Log UFC/mLof *P. acidilacticiand L. acidophilus* strains, respectively. Optimized beverage was characterized by using physicochemical, microbiological and sensorial analysis. The fresh fermented probiotic PSM presented a pH of 4.29, 11.99 and 0.19 g/L of lactic and acetic acids, respectively. The PSM stored for 35 days at 4 ºC allowed the starters survival above 7.20 Log CFU/mL, and showed significant post-acidification reaching 3.80 of pH and 15.53 g/L of lactic acid. Finally, sensory analysisshowed goodacceptance,demonstrating the potential for commercialization of the developed probiotic PSM.

Keywords: Functional food. Probiotic bacteria. Symbiotic beverage. Co-culture. Plackett-Burman. Central Composite Rotatable Design.

1 INTRODUCTION

Protein foods especially animal sources are scarce in the diets consumed by a large segment of the population in developing countries. Generally, legumes are low priced source of protein, important in alleviating proteic foodstuffs scarcity (MUREVANHEMA; JIDEANI, 2013). Vegetable milk has a great potential to be a feasible alternative to animal milk in the food industry, due to its functionality and health claims (CHAMPAGNE et al., 2009; HINDS; BEUCHAULT; CHINNANL, 1997; KPODO et al., 2013; RATHORE; SALMERON, 2012; SUNNY-ROBERTS; OTUNOLA; IWAKUN, 2004).

It has been proven that peanuts (*Arachis hypogaea* L.) have the potential for lactic acid bacteria (LAB) growth (ISANGA; ZHANG, 2007; ISANGA; ZHANG, 2009, SUNNY-ROBERTS, OTUNOLA; IWAKUN, 2004, YADAV et al., 2010), including probiotic ones (BEUCHAT; NAIL, 1978, SCHAFFNER; BEUCHAT, 1986). Previous works reported that soymilk fermented with lactic acid bacteria can provide unique probiotic foods for human nutrition (GRANATO et al., 2010a; HELLER, 2001; FARNWORTH et al., 2007; OLIVERIA et al., 2001). However, there is scarce information about the potential of the mixture of these two oilseeds milks as a probiotic beverage. The essence of combining aqueous extract from two vegetable sources is guided by the fact that no single vegetable milk can adequately resemble milk from a dairy source in its nutritional and physico-chemical attributes (KPODO et al., 2013).

Santos, Libeck and Schwan (unpublished results 2014a) developed a formulation using a PSM with high nutritional value, containing 4.00% of protein, 3.17% of fat content, 1.62% of carbohydrate, and 15.59 g/100g of essential amino acids (on dry basis). In a subsequent work, the same authors used different pure and co-cultures of LAB and yeast to ferment PSM. The

authors showed that when *P. acidilactici* (UFLA B FFCX 27.1) LAB,*L. acidophilus* (LACA 4) commercial probiotic culture and the yeast*S. cerevisiae* (UFLA Y FFBM 18.03) were co-incubated, the probiotic bacteria had its growth favored reaching the population of 8 log CFU/mL (SANTOS; LIBECK; SCHWAN, submitted paper 2014b). In sight of this, lactic acid fermented peanut-soy milk (PSM) may be suitable probiotic bacterial strain carrier to the host.

New product development is a challenge for both scientific and applied research, and it has been observed that food design is essentially a problem of optimization to generate the best formulation (ELLENDERSEN et al., 2012; JOUSSE, 2008; KPODO et al., 2013). The determination of optimum levels of key ingredients is necessary to obtain suitable sensory and physicochemical characteristics, extended shelf life, chemical stability and reasonable price. Moreover, it is important that the formulation maintains the activity and viability of the probiotic for extended periods of time (GRANATO et al., 2010a; SHAH, 2007).

Substrate optimization by the classical method of changing one variable while fixing the others at a certain level is laborious and time-consuming, especially when the number of variables is large. An alternative and more efficient approach in microbial systems is the use of statistical methods. Plackett–Burman design allows testing of the largest number of factor effects with the least number of observations, and provides random error variability estimation and testing of the statistical significance of the parameters (PLACKETT; BURMAN, 1946). Response surface methodology (RSM) is a commonly used method to assess the optimal fermentation conditions and also an efficient statistical technique for optimization of multiple variables with minimum number of experiments. This method has been successfully applied to optimize new probiotic food and beverages (ERDEM et al., 2014; BERNAT et

al., 2014, SHILPI; KUMAR, 2013). Central composite rotatable design (CCRD) allows to fit a first or second order polynomial by a least significance technique. An equation is used to describe how the test variables affect the response, determine the interrelationship among the test variables, and describe the combined effect of all the test variables in the response (RASTOGI; RAJESH; SHAMALA, 1998).

The objectives of this study were to: (1) investigate the effect of PSM formulation, fructooligosaccharides (FOS) concentration, temperature, time, inocula of LAB *Pediococcus acidilactici* (UFLA B FFCX 27.1), of probiotic LAB *Lactobacillus acidophilus* (LACA 4) and of yeast *Saccharomyces cerevisiae* (UFLA Y FFBM 18.03) on the quality of PSM in terms of probiotic viability, pH, lacticacid,acetic acid and ethanol content; (2) optimize PSM fermentation as a potentially probiotic beverage with a view to developing value-added product; (3) evaluate the quality and consumer acceptability of PSM fermented beverage; (4) evaluate the probiotic viability, pH, lactic and acetic acids and ethanol contents under refrigerated storage.

2 MATERIALS AND METHODS

2.1 PSM preparation

Peanut milk was prepared as previously described (SALUNKHE; KADAM, 1989) with minor modifications. The seeds were sorted to remove discolored grains and any foreign material. Selected peanut seeds were roasted at 130 °C for 20 min in a ventilated oven. The seeds were peeled and weighed before being soaked in 0.5 g/100 mL $NaHCO_3$ for at least 12 h. The shelled seeds were then washed with water and mixed with distilled water in a ratio of 1:5 [peanuts (g):water (mL)] and transferred to a stainless steel blender (Cemaf, São Paulo, Brazil) for 5 min. The resulting paste was filtered through a double layered cheese cloth.

For the soymilk preparation, 250 g of selected beans were initially soaked in 1 L of water for 16 h at 25 °C. Subsequently, 190 g of these moistened beans were soaked in 500 mL water in a stainless steel blender and mixed for 3 minutes. Then, the resulting slurry was filtered through double layered cheese cloth and boiled for 5 min (CHAMPAGNE et al., 2009).

The peanut and soybean milks were mixed in different ratios, according PB and CCRD designs. The heat treatment was carried out by pasteurization at 90 °C/20min, followed by immediate cooling to 4 °C.

2.2 Preparation of cultures

The commercial probiotic culture *L. acidophilus* (LACA 4) was acquired from Danisco (Yo Mix, Deutschland). The *P. acidilactici* lactic acid bacteria (UFLA BFFCX 27.1) was isolated from *caxiri* indigenous beverage (SANTOS et al., 2012). The *S. cerevisiae* (UFLA YFFBM 18.03) yeast was

isolated from cocoa beans fermentations (PEREIRA et al., 2012). These strains belong to the culture collection of Microbial Physiology Laboratory/Department of Biology, Federal University of Lavras (UFLA), Brazil.

The commercial cultures activation was performed according to the manufacturer's instructions. Stock cultures forbacteria were prepared by mixing culture medium Man Rogosa Sharp broth (MRS, Merck Whitehouse Station, USA) and 40% glycerol (w/v) in the 1:1 proportion placing 1 mL in cryovials and freezing at - 20 º C. For yeast, YPD agar (10 g/L yeast extract (Merck, Darmstadt, Germany), 10 g/L peptone (Himedia, Mumbai, India), 20 g/L glucose (Merck, Darmstadt,Germany), 20 g/L agar (Merck, Darmstadt, Germany) pH 3.5 were mixed with 40% glycerol (w/v) in the 1:1 proportion placing 1 mL in cryovials and freezing at - 20 º C. The glycerol solution was previously sterilized at 121 °C for 15 minutes (CHAMPAGNE et al., 2009).

The preparation of inoculum for single and co-culture fermentation was performed by culturing 100 µL of each strain separately in 5 mL of sterile MRS broth (bacteria) and YPD pH 3.5 (yeast), then incubating at 37 ºC and 30 °C, respectively. After 16 h and 24 h, the supernatant was removed and the cells were transferred to 50 mL MRS and YPD, respectively. The flasks were again incubated for 16 h (bacteria) and 24 h (yeast), the cells were subsequently transferred to 500 mL of the respective medium and then incubated in the same conditions described above. Cells were washed two times with sterile distilled water and inoculated in PSM with population according PB and CCRD designs. To perform the inoculation, OD growth kinetics were constructed by plotting the OD (600 nm) of suspensions minus the OD of non-inoculated media vs the time of incubation (RUIZ-MOYANO et al., 2008). To calibrate the ODs against the cell concentration of the cultures, the viable counts (VC) of a strain for each broth used were determined between readings, by the method of Miles, Misra and Irwin (1938).

2.3 Experimental design

A Plackett and Burman (PB) experimental design was used, with 12 trials and three central points, totaling 15 trials (PLACKETT; BURMAN, 1946; RODRIGUES; IEMMA, 2009) with seven independent variables: PSM formulation; FOS concentration as prebiotic (Raftilose 95®, Orafti-Belgium), time, temperature, and inocula of *L. acidophilus*, *P. acidilactici* and *S. cerevisiae*. Table 1 shows the conditions of the assays with coded and real variables.

Table 1 Coded variables and real values (in brackets) of the Plackett & Burman design

Assays	Independent variables						
	PSM formulation PM (%)*	**FOS (%)**	**Time (h)**	**Temp. (ºC)**	***S. cerevisiae* (Log UFC/mL)**	***P. acidilactici* (Log UFC/mL)**	***L. acidophilus* (Log UFC/mL)**
1	-1 (60)	1 (5)	1 (12)	-1 (34)	1 (7.00)	1 (9.00)	1 (8.00)
2	-1 (60)	-1(3)	-1 (8)	-1 (34)	-1 (5.00)	-1 (7.00)	1 (8.00)
3	1 (72)	1 (5)	-1 (8)	-1 (34)	-1 (5.00)	1 (9.00)	-1 (6.00)
4	1 (72)	-1 (3)	-1 (8)	-1 (34)	1 (7.00)	-1 (7.00)	1 (8.00)
5	-1(60)	-1 (3)	-1 (8)	1 (40)	-1 (5.00)	1 (9.00)	1 (8.00)
6	1 (72)	-1 (3)	1 (12)	1 (40)	1 (7.00)	-1 (7.00)	-1 (6.00)
7	-1 (60)	1 (5)	-1 (8)	1 (40)	1 (7.00)	-1 (7.00)	1 (8.00)
8	0 (66)	0 (4)	0 (10)	0 (37)	0 (6.00)	0 (8.00)	0 (7.00)
9	-1 (60)	1 (5)	1 (12)	1 (40)	-1 (5.00)	1 (9.00)	-1 (6.00)
10	1 (72)	1 (5)	1 (12)	-1 (34)	-1 (5.00)	1 (9.00)	-1 (6.00)
11	1 (72)	1 (5)	-1 (8)	1 (40)	1 (7.00)	1 (9.00)	-1 (6.00)
12	1 (72)	-1 (3)	1 (12)	1 (40)	-1 (5.00)	1 (9.00)	1 (8.00)
13	-1 (60)	-1 (3)	1 (12)	-1 (34)	1 (7.00)	1 (9.00)	-1 (6.00)
14	0 (66)	0 (4)	0 (10)	0 (37)	0 (6.00)	0 (8.00)	0 (7.00)
15	0 (66)	0 (4)	0 (10)	0 (37)	0 (6.00)	0 (8.00)	0 (7.00)

PSM – peanut-soy milk; PM – peanut milk. *The preparation of the PSM was performed using the percentage of PM mentioned in the table and the volume was completed with soymilk.

Furthermore, a Central Composite Rotatable Design (CCRD) was performed using the independent variables selected as significant in the screening design (PB). The independent variables and the outline of experimental design (17 runs) with the coded levels are given on Table 2. The experiment was carried out in randomized order. The experimental data was fitted to a polynomial regression model for predicting individual responses.

Table 2 Central Composite Rotatable Design (CCRD) arrangement for PSM beverage fermentation

Test run	**% PSM* PM (%)**	***P. acidilactici* (Log CFU/mL)**	***L. acidophilus* (Log CFU/mL)**
1	-1 (60)	-1 (7.00)	-1 (5.68)
2	-1 (60)	-1 (7.00)	+1 (7.68)
3	-1 (60)	+1 (9.00)	-1 (5.68)
4	-1 (60)	+1 (9.00)	+1 (7.68)
5	+1 (72)	-1 (7.00)	-1 (5.68)
6	+1 (72)	-1 (7.00)	+1 (7.68)
7	+1 (72)	+1 (9.00)	-1 (5.68)
8	+1 (72)	+1 (9.00)	+1 (7.68)
9	-1.68 (56)	0 (8.00)	0 (6.68)
10	+1.68 (76)	0 (8.00)	0 (6.68)
11	0 (66)	-1.68 (6.32)	0 (6.68)
12	0 (66)	+1.68 (9.68)	0 (6.68)
13	0 (66)	0 (8.00)	-1.68 (5.00)
14	0 (66)	0 (8.00)	1.68 (8.36)
15 (C)	0 (66)	0 (8.00)	0 (6.68)
16 (C)	0 (66)	0 (8.00)	0 (6.68)
17 (C)	0 (66)	0 (8.00)	0 (6.68)

PSM – peanut-soy milk; PM – peanut milk. *The preparation of the PSM was performed using the percentage of PM mentioned in the table and the volume was completed with soymilk. The real values are in brackets.

Statistical significance of the terms in the regression equation was examined by analysis of variance (ANOVA) for each response. Lack of fit and adequate precision was used to judge adequacy of model fit. The fitted model was used to navigate the design space and search for optimum initial inocula and PSM composition for optimum PSM potentially probiotic beverage using

numerical optimization tool in STATISTICA™, Version 7.0 for Windows (StatSofts). The software Statistica 7 was used to estimate desirability, an objective function that ranges from zero outside of the limits to one at the goal. The numerical optimization found a point that maximizes the desirability function. The fermentation of PSM probiotic beverage was carried out at the estimated optimal conditions to validate the optimization process. The validation experiments were carried out in three independent assays.

2.4 Fermentation

The suspension with inoculated cells was fractionated in 25 mL portions, placed into 50 mL conical tubes. Samples were taken periodically for analysis of pH, probiotic viability, determination of ethanol, acetic acid and lactic acid content.

2.5 Analytical methods

2.5.1 Viability of inoculated strains and pH

A one milliliter sample was taken from each fermentation flask. Serial tenfold dilutions were prepared in a solution of 0.9% NaCl (w/v) and 0.1% (w/v) bacto peptone (Difco). Viable counts of LAB and probiotic bacteria in mixed cultures were obtained by spreading 0.1 mL sample of an appropriate dilution onto the surface of MRS plates containing 0.1% (w/v) cysteine-HCl and 0.01% (w/v) aniline blue under microaerophilic conditions during incubation (37º C for 2 days) (EVANGELISTA; GHISELLI; FILHO, 2012; WANG; YU; CHOU, 2002). The colonies presented different shades of color (white and blue) allowing differential count. Yeasts were grown on YPD agar pH 3.5 at 28 ºC for

5 days. Violet Red Bile Agar with glucose (VRBG) (Oxoid, Hampshire, England) was used for bile-tolerant Gram-negative *Entorobacteriaceae* detection, the plates were incubated at 37°C for 24-48 h. Colony forming units (CFU) were enumerated in plates containing 30 to 300 colonies, and cell concentration was expressed as log CFU/mL of fermented PSM.

The pH of the fermenting PSM samples was measured with a pH meter (Tecnal, Tec-3MT, São Paulo, Brazil).

2.6 HPLC analysis

2.6.1 Organic acids and ethanol

Samples were prepared by adding 3mL of n-hexan (Sygma-Aldrich) to 3 ml of samples. The mixture was vortexed for 30 seconds and the non-hexanic phase was pipetted into another tube. The procedure was performed again. Subsequently, the samples were centrifuged two times at 7,000 rpm for 10 minutes at 4 °C, and then supernatant was filtered through 0.22 μm filters, and analyzed. Organic acids (acetic, lactic, and succinic acid), alcohol (ethanol) and carbohydrates (glucose, sucrose, maltose, fructose, raffinose, and stachyose) were identified according to the methodology proposed by Duarte et al. (2010). The analyses were carried out using a High Performance Liquid Chromatography system (HPLC) (Shimadzu, Shimadzu Corp., Japan), equipped with a dual detection system consisting of a UV-Vis detector (SPD-10A*i*) and a refractive index detector (RID-10A*i*). A Shimadzu ion exclusion column (Shim-pack SCR-101 H, 7.9 mm x 30 cm) was used operating at 50 ºC, using 100 Mm of perchloric acid as the eluent at a flow rate of 0.6 mL/min. The acids were detected via UV absorbance (210 nm), while alcohols were detected via RID. For carbohydrates, the Supelcosil LC-NH_2 column (4.6mm x 25 cm) was used,

operating at 30 ºC with a mobile phase (acetonitrile:water (75:25)) in a flow rate of 1 mL/min. The sugars were detected via RID. Individual compounds were identified based on the retention time of standards injected in the same conditions, and their concentrations were determined using the external calibration method. All samples were examined in triplicate.

2.6.2 Amino acids

The determinations of total amino acids were performed by High Performance Liquid Chromatography (HPLC) as described by White, Hart and Fry (1986). After a 24 h hydrolysis in 6 M HCl at 100 °C, the amino acids reacted with phenyl isothiocyanate, and the derivatives separated using a Luna C-18, 100 Å; 5 µ, 250 mm × 4.6 mm (00G-4252-E0, Torrance, CA, USA) column, at 50 °C. The detection was by ultraviolet absorption measurements using a fixed wavelength (254 nm) detector. Quantification was carried out by comparison with a standard mixture (Thermo Scientific, Rockford IL, USA) and DL-2-aminobutyric acid was used as an internal standard from Sigma–Aldrich Corp., St. Louis, MO (HAGEN; FROST; AUGUSTIN, 1989).

2.7 Sensory evaluation

After fermentation, 1.5 L of PSM symbiotic beverage was sweetened using the dietary powder sweetener sucralose 100% in a proportion of 5 g/L of the beverage, followed by homogenization. The optimized beverage was evaluated by 53 non trained panelists, between 18 and 65 years of age (staff and students of the Federal University of Lavras - UFLA). Refrigerated (10 ºC) sample of approximately 25 mL was served in clear transparent plastic glass with a volume of 100 mL. Cold mineral water was provided for rinsing of the

palate during the testing. Evaluations took place in the mornings between 9:00 and 10:00 a.m. and were conducted at room temperature (20-22 ºC) under white light. The PSM potentially probioticbeverage were evaluated for appearance, aroma, taste and overall aspect according to the hedonic scale (MORAES, 1993). In this affective testing, the subject is told to decide how much the panelist likes or dislikes the product and to mark a scale of nine points (9 = like extremely; 5= neither like, nor dislike; and 1= dislike extremely).For determination of the purchase intention parameter a scale of 5 points was used (1 = definitely would not buy the product; 5 = definitely would buy the product).

2.8 Refrigerated storage

PSM beverage after fermentation was stored at 4ºC for 35 days. Probiotic viability, pH, lactic acid, acetic acid and ethanol contents were evaluated according to the methods above described.

3 RESULTS AND DISCUSSION

3.1 Screening of independent variables that enhance PSM fermentation

Probiotics have been usually incorporated into dairy products and supplied to consumers. However, due to the lactose intolerance, probiotics have recently been supplied within several non-traditional food matrices, including oilseeds, cereals, chocolate and beverages (CHAMPAGNE et al., 2009; GAWKOWSKI; CHIKINDAS, 2013; RATHORE; SALMERON; PII, 2012; RIVERA-ESPINOZA; GALLARDO-NAVARRO, 2010). In this context, the development of new functional food products turns out to be increasingly challenging, as it has to fulfill the consumer's expectations for products that are simultaneously palatable and healthy (GRANATO et al., 2010b; SHAH, 2007).

PB design-based experiments were performed to evaluate the effect of time, temperature, *L. acidophilus*, *P. acidilactici* and *S. cerevisiae* inocula, the concentration of FOS, the PSM formulation and select the significant variables. Table 3 shows the experimental data for the distinct response variables: pH, lactic acid, acetic acid and ethanol concentrations after fermentation time and after 24 h under refrigerated storage (4 ºC).

As can be seen in Table 4, regression analysis showed that the models for: probiotic viability after fermentation, pH under refrigerated storage, lactic and acetic acids contents after 24 h/4 ºC, ethanol content (after fermentation and 24 h/4 ºC, respectively) were adequate. The regression analysis for pH after fermentation, for lactic acid content after fermentation time also were adequate, since the R^2adjusted$\geq$70%. While, the model for probiotic viability at 24 h/4 ºC and acetic acid content after fermentation time were not satisfactory. The significance of each coefficient was determined by student's *t*-test. The *p*-Value was used as indicator of the statistical significance.

Table 3 Placket & Burman design results

Experiments (h)	Probiotic viability (Log CFU/mL)		pH		Lactic acid (g/L)		Acetic acid (g/L)		Ethanol (g/L)	
	AF	24/4ºC	AF	24/4ºC	AF	24/4ºC	AF	24/4ºC	AF	24/4ºC
1 (12)	8.52	8.40	4.11	4.08	11.39	10.87	0.15	0.14	0.53	0.43
2 (8)	8.67	8.63	4.21	4.11	9.98	10.76	0.01	0.02	0.28	0.28
3 (8)	7.48	7.60	5.07	5.04	1.28	3.40	0.06	0.07	0.17	0.17
4 (8)	8.74	8.61	4.20	4.11	8.51	9.59	0.01	0.02	0.44	0.47
5 (8)	8.49	8.54	4.04	4.12	11.23	10.71	0.22	0.23	0.18	0.36
6 (12)	7.40	7.53	4.32	4.28	7.64	7.62	0.26	0.17	0.50	0.45
7 (8)	8.72	8.66	3.93	3.86	13.04	13.16	0.07	0.09	0.68	0.76
8 (10)	8.35	8.32	4.10	4.06	8.41	8.45	0.11	0.11	0.26	0.26
9 (12)	7.41	7.51	4.38	4.33	7.96	8.97	0.17	0.18	0.57	0.32
10 (12)	8.48	8.72	4.08	4.06	9.36	10.37	0.01	0.04	0.39	0.38
11 (8)	7.60	7.90	4.89	4.71	4.20	4.43	0.06	0.12	0.49	0.43
12 (12)	8.04	8.00	4.09	3.91	9.73	10.89	0.23	0.23	0.17	0.18
13 (12)	7.48	8.45	5.07	4.87	4.06	4.61	0.06	0.10	0.32	0.33
(10)	8.23	8.26	4.27	4.20	7.86	8.64	0.11	0.11	0.28	0.29
15 (10)	8.51	8.28	4.15	4.11	7.58	8.22	0.11	0.10	0.24	0.25

AF – after fermentation time (8, 10 or 12 h).

Table 4 Main effects of variable estimates on parameters during fermentation of PSM in the Plackett and Burman experimental design

Term	**Average**	**Curvature**	**R^2**	**R^2 adj.**	**Effect**	**SE**	***t*-Value**	***p*-Value**
Probiotic viability after fermentation								
(1) PSM (v/v)					**-0.592**	**0.173**	**-3.416**	**0.014**
(2) FOS (%)					**-0.436**	**0.173**	**-2.513**	**0.046**
(3) Time (h)					-0.062	0.173	-0.357	0.733
(4) Temp. (ºC)	**7.919**	0.549	0.931	0.838	-0.283	0.173	-1.633	0.154
(5) *S. cerevisiae**					-0.020	0.173	-0.117	0.910
(6) *P. acidilactici**					**-0.635**	**0.173**	**-3.663**	**0.010**
(7) *L. acidophilus**					**1.157**	**0.173**	**6.674**	**0.000**
Probiotic viability after 24h/4ºC								
(1) PSM (v/v)					-0.304	0.174	-1.747	0.131
(2) FOS (%)					-0.164	0.174	-0.940	0.384
(3) Time (h)					-0.227	0.174	-1.302	0.241
(4) Temp. (ºC)	**8.213**	0.145	0.780	0.488	-0.377	0.174	-2.166	0.073
(5) *S. cerevisiae**					0.092	0.174	0.531	0.615
(6) *P. acidilactici**					-0.128	0.174	-0.734	0.490
(7) *L. acidophilus**					**0.552**	**0.174**	**3.169**	**0.019**
pH after fermentation								
(1) PSM (v/v)					0.152	0.111	1.368	0.220
(2) FOS (%)					0.088	0.111	0.797	0.456
(3) Time (h)					-0.048	0.111	-0.436	0.678
(4) Temp. (ºC)	**4.366**	-0.385	0.886	0.734	-0.182	0.111	-1.638	0.152
(5) *S. cerevisiae**					0.108	0.111	0.977	0.366
(6) *P. acidilactici**					**0.358**	**0.111**	**3.232**	**0.018**
(7) *L. acidophilus**					**-0.582**	**0.111**	**-5.246**	**0.002**

"to be continued"

Table 4 “continuation”

Term	Average	Curvature	R^2	R^2 adj.	Effect	SE	*t*-Value	*p*-Value
pH after 24 h/4ºC								
(1) PSM (v/v)					0.123	0.112	1.103	0.312
(2) FOS (%)					0.113	0.112	1.014	0.350
(3) Time (h)					-0.070	0.112	-0.626	0.554
(4) Temp (ºC)	**4.290**	-0.333	0.866	0.700	-0.177	0.112	-1.580	0.165
(5) *S. cerevisiae**					0.057	0.112	0.507	0.630
(6) *P. acidilactici**					**0.330**	**0.112**	**2.952**	**0.025**
(7) *L. acidophilus**					**-0.533**	**0.112**	**-4.771**	**0.003**
Lactic acid after fermentation								
(1) PSM (v/v)					**-2.822**	**0.916**	**-3.081**	**0.022**
(2) FOS (%)					-0.651	0.916	-0.711	0.504
(3) Time (h)					0.317	0.916	0.346	0.741
(4) Temp. (ºC)	**8.198**	-.0493	0.885	0.732	1.536	0.916	1.677	0.145
(5) *S. cerevisiae**					-0.118	0.916	-0.129	0.902
(6) *P. acidilactici**					**-2.434**	**0.916**	**-2.657**	**0.038**
(7) *L. acidophilus**					**4.694**	**0.916**	**5.125**	**0.002**
Lactic acid after 24 h/4ºC								
(1) PSM (v/v)					**-2.131**	**0.758**	**-2.813**	**0.031**
(2) FOS (%)					-0.496	0.758	-0.655	0.537
(3) Time (h)					0.212	0.758	0.279	0.789
(4) Temp. (ºC)	**8.782**	-0.692	0.902	0.772	1.029	0.758	1.358	0.223
(5) *S. cerevisiae**					-0.804	0.758	-1.061	0.330
(6) *P. acidilactici**					**-2.597**	**0.758**	**-3.427**	**0.014**
(7) *L. acidophilus**					**4.301**	**0.758**	**5.676**	**0.001**

“to be continued”

Table 4 “continuation”

Term	Average	Curvature	R^2	R^2 adj.	Effect	SE	*t*-Value	*p*-Value
Acetic acid after fermentation								
(1) PSM (v/v)					-0.008	0.035	-0.223	0.831
(2) FOS (%)					-0.0434	0.035	-1.253	0.257
(3) Time (h)					0.076	0.035	2.203	0.070
(4) Temp. (ºC)	**0.109**	0.003	0.771	0.466	**0.120**	**0.035**	**3.464**	**0.013**
(5) *S. cerevisiae**					-0.015	0.035	-0.449	0.669
(6) *P. acidilactici**					0.042	0.035	1.202	0.275
(7) *L. acidophilus**					0.011	0.035	0.326	0.755
Acetic acid after 24 h/4ºC								
(1) PSM (v/v)					-0.020	0.015	-1.397	0.212
(2) FOS (%)					-0.024	0.015	-1.672	0.145
(3) Time (h)					**0.0529**	**0.015**	**3.605**	**0.011**
(4) Temp. (ºC)	**0.119**	-0.025	0.937	0.854	**0.105**	**0.015**	**7.190**	**0.000**
(5) *S. cerevisiae**					-0.021	0.015	-1.443	0.199
(6) *P. acidilactici**					**0.060**	**0.015**	**4.063**	**0.007**
(7) *L. acidophilus**					0.015	0.015	1.050	0.334
Ethanol after fermentation								
(1) PSM (v/v)					**-0.067**	**0.017**	**-3.909**	**0.008**
(2) FOS (%)					**0.157**	**0.017**	**9.068**	**0.000**
(3) Time (h)					0.0370	0.017	2.139	0.076
(4) Temp. (ºC)	**0.392**	-0.258	0.985	0.965	**0.077**	**0.017**	**4.445**	**0.004**
(5) *S. cerevisiae**					**0.200**	**0.017**	**11.561**	**0.000**
(6) *P. acidilactici**					**-0.1637**	**0.017**	**-9.461**	**0.000**
(7) *L. acidophilus**					0.0103	0.017	0.595	0.573

“to be continued”

Table 4 “conclusion”

Term	**Average**	**Curvature**	**R^2**	**R^2 adj.**	**Effect**	**SE**	***t*-Value**	***p*-Value**
Ethanol after 24 h/4ºC								
(1) PSM (v/v)					**-0.066**	**0.027**	**-2.45**	**0.049**
(2) FOS (%)					**0.070**	**0.027**	**2.609**	**0.040**
(3) Time (h)					-0.064	0.027	-2.369	0.056
(4) Temp. (ºC)	**0.380**	-0.221	0.955	0.895	**0.072**	**0.027**	**2.656**	**0.038**
(5) *S. cerevisiae**					**0.198**	**0.027**	**7.335**	**0.000**
(6) *P. acidilactici**					**-0.123**	**0.027**	**-4.544**	**0.004**
(7) *L. acidophilus**					**0.100**	**0.027**	**3.705**	**0.011**

R^2 adj. - Regression analysis adjusted, SE - Standard Error * Viable counts(Log CFU/mL); Variables in bold have a significant effect ($p < 0.05$).

The independent variables with significant effect in pH and lactic acid are the initial *L. acidophilus* (LACA 4) and *P. acidilactici* (UFLA B FFCX 27.1) concentrations. The *P. acidilactici* strain had a positive effect on pH, indicating that this LAB was able to grow, but did not acidify the PSM. *L. acidophilus* has negative effect on pH, consequently, the probiotic culture contributed significantly to enhancing lactic acid production during PSM fermentation.

PSM formulation had a significant positive effect on the probiotic final cell count. Therefore, the higher the peanut milk (72%) concentration in PSM, lower was the probiotic viability, usually below 8 Log CFU/mL. The negative effect of greater peanut milk concentration in the PSM can also be observed for the lactic acid production. This result indicated that a probiotic viabilitylowerthan 8 LogCFU/mLgenerate under 8 g/L of lactic acid o fermented beverage.

FOS concentration was fixed at 3% (w/v). The addition of FOS, despite being a substrate for probiotics and aiding in its growth, did not contribute to an increase in microbial population, and presented a positive effect in ethanol production. It has been observed that usage of high concentrations of FOS results in greater production of acetic and lactic acids, which quickly decreases the pH and consequently may negatively influence the final probiotic count (YEO; LIONG, 2010, EVANGELISTA; GHISELLI; FILHO, 2012).

Fermentation time did not influence the probiotic viability, pH, lactic acid and ethanol responses. The exception was for acetic acid production under refrigeration, where a positive influence was observed ($p < 0.05$). Thus fermentation time was kept at lowest level (8 h). Furthermore, industrial short fermentation times are preferable in order to increase plant output as well as to reduce unwanted contaminating microorganisms.

The temperature during fermentation interfered positively in the acetic acid and ethanol final contents.However, no significant effect of temperature was observed on the probiotic counts, pH and lactic acid content. In order to avoid excessive ethanol and acetic acid production the temperature at the central point (37 ºC) was selected for further study.

As expected, the *S. cerevisiae* showed a positive effect on ethanol production. Thus, as the purpose was to obtain a non-alcoholic product, the initial population of this yeast was fixed at the minimum value studied (5 log CFU / mL).

3.2 Optimization of conditions for PSM potentially probiotic beverage production

It is difficult to demonstrate that two or more agents act synergistically or antagonistically on the metabolism of acids and growing of microbial cultures. Only by very careful experimental design can such interactions be assessed. Response surface is one way of detecting interactions between two or more agents (NARENDRANATH; THOMAS; OMGLEDEW, 2001). A five-level three-factor CCRD was performed with PSM formulated with different combinations of the variables (*P. acidilactici* and *L. acidophilus* inocula and PSM formulation) that were selected by the PB design as significant to enhance the probiotic viability, lactic acid production and, consequently, reducing the pH of PSM beverage.

Table 5 shows the experimental data. In all assays probiotic *L. acidophilus* growth was observed. pH varied from 4.33 to 4.85. According to Yoon, Woodams and Hang (2004) pH values between 4.0 and 4.5 is adequate for probiotic products. The pH in this range suppresses the development of contaminating microorganisms and on the other hand, is organoleptically appropriate (MCMASTER et al., 2005). Indeed, in our work, the fermentations

with pH of 4.5 or below, were kept free of Gram-negative *Entorobacteriaceae*. Minor changes in pH (less than 0.12) occurred after 24 h under refrigeration, tending to decrease, due topost-acidification.At different combinations of the variables, the lactic acid content ranged from 5.43 to 13.83 g/L, reflecting the importance of optimization to obtain higher yields of the desired product.

Table 5 Response variables as affected by probiotic *L. acidophilus*, LAB *P. acidilactici* inocula and PSM formulation on PSM potentially probiotic beverage during fermentation

Test run	**Probiotic count* 8 h**	**Probiotic count* 24/4ºC**	**pH 8 h**	**pH 24/4ºC**	**Lactic acid (g/L) 8 h**	**Lactic acid (g/L) 24/4ºC**
1	7.90	7.48	4.83	4.81	6.85	7.08
2	8.60	8.04	4.36	4.35	12.96	13.15
3	8.20	8.52	4.73	4.75	7.40	7.24
4	8.46	8.32	4.44	4.44	10.06	9.54
5	7.85	7.95	4.89	4.77	5.43	7.45
6	8.49	8.15	4.37	4.33	5.94	8.62
7	8.78	8.34	4.75	4.63	6.77	9.33
8	8.04	8.69	4.45	4.39	6.59	10.34
9	8.45	8.51	4.63	4.58	9.79	10.53
10	8.60	8.30	4.64	4.63	7.15	8.02
11	7.85	8.20	4.69	4.68	7.60	7.83
12	8.53	8.32	4.63	4.64	8.86	9.15
13	7.78	8.04	4.85	4.82	6.48	6.63
14	8.79	8.77	4.26	4.21	12.81	13.83
15 (C)	7.36	7.38	4.64	4.57	7.71	8.32
16 (C)	7.32	7.30	4.66	4.64	8.11	8.73
17 (C)	7.45	7.40	4.65	4.65	8.25	8.35

* Viable counts (Log CFU/mL).

Data presented in Table 6 was fitted to the quadratic models giving the probiotic viability, lactic acid production and pH (Equations (1), (2) and (3)) as functions of PSM formulation (X1), *P. acidilactici* (X2) and *L. acidophilus* (X3) inocula, respectively:

Probiotic viability 24 h/4 ºC (Log CFU/mL) = $7.38 + 0.64X_1^2 + 0.53\ X_2^2 + 0.63\ X_3^2$ (1)

pH value 24 h/4 ºC = $4.61 - 0.36X_3 - 0.08X_3^2 + 0.09\ X_{2*}X_3$ (2)

Lactic acid 24 h (g/L) = $8.48 + 3.32\ X_3 + 1.76\ X_{1*}X_2 – 1.55\ X1_*X_3$ (3)

The reparametrized models contain only the statistically significant terms. The mathematical model for the optimization of the process was generated by using data of PSM fermented and cooled for 24 hours, since this product is closer to the end product to be consumed. The analysis of variance (ANOVA) of the quadratic regression model indicated that all models were statistically significant at 95% of confidence level.

Table 6 Estimated effects of initial *L. acidophilus* and *P. acidilactici* counts and PSM formulation on the studied responses

Independent variables	**Probiotic count* (8 h)**						**Probiotic count* (24h/4 ºC)**					
	R^2	R^2 adj.	Effect	SE	*t*-Value	*p*-Value	R^2	R^2 adj.	Effect	SE	*t*-Value	*p*-Value
Mean	0.852	0.761	**7.373**	**0.137**	**66.170**	**0.000**	0.825	0.724	**7.375**	**0.168**	**43.802**	**0.000**
(1) PSM (L)			0.036	0.137	0.343	0.742			0.061	0.158	0.386	0.711
PSM (Q)			0.129	0.137	1.122	0.299			**0.636**	**0.174**	**3.656**	**0.008**
(2) *P. acidilactici*(L)			0.132	0.137	1.266	0.246			0.359	0.158	2.271	0.057
P. acidilactici (Q)			**-0.296**	**0.137**	**-2.573**	**0.037**			**0.534**	**0.174**	**3.067**	**0.018**
(3) *L. acidophilus* (L)			**0.3745**	**0.137**	**3.583**	**0.009**			0.313	0.158	1.980	0.088
L. acidophilus (Q)			-0.042	0.137	-0.369	0.723			**0.636**	**0.174**	**3.656**	**0.008**
1L by 2L			0.080	0.137	0.588	0.575			-0.097	0.207	-0.472	0.651
1L by 3L			-0.262	0.137	-1.915	0.097			0.047	0.207	0.230	0.825
2L by 3L			**-0.456**	**0.137**	**-3.334**	**0.012**			-0.152	0.207	-0.738	0.484
	pH (8 h)						pH 24/4ºC (24h/4 ºC)					
Mean	0.994	0.987	**4.651**	**0.012**	**390.417**	**0.000**	0.973	0.939	**4.609**	**0.025**	**181.299**	**0.000**
(1) PSM (L)			0.017	0.011	1.529	0.170			-0.021	0.024	-0.895	0.400
PSM (Q)			-0.0160	0.012	-1.299	0.235			-0.016	0.026	-0.615	0.558
(2) *P. acidilactici* (L)			**-0.026**	**0.011**	**-2.368**	**0.049**			-0.017	0.024	-0.719	0.495
P. acidilactici (Q)			0.002	0.012	0.136	0.895			0.023	0.026	0.864	0.416
(3) *L. acidophilus* (L)			**-0.377**	**0.011**	**-33.669**	**0.000**			**-0.363**	**0.024**	**-15.186**	**0.000**
L. acidophilus (Q)			**-0.0726**	**0.012**	**-5.893**	**0.001**			**-0.080**	**0.026**	**-3.037**	**0.019**
1L by 2L			-0.010	0.015	-0.684	0.516			-0.027	0.031	-0.881	0.407
1L by 3L			-0.015	0.015	-1.026	0.339			0.022	0.031	0.721	0.494
2L by 3L			**0.100**	**0.015**	**6.841**	**0.000**			**0.087**	**0.0312**	**2.805**	**0.026**
	Lactic acid (g/L) (8 h)						Lactic acid (g/L)24/4ºC (24h/4 ºC)					
Mean	0.937	0.856	**8.451**	**0.530**	**15.950**	**0.000**	0.907	0.788	**8.485**	**0.527**	**16.098**	**0.000**
(1) PSM (L)			-0.962	0.498	-1.933	0.094			-0.803	0.495	-1.622	0.149
PSM (Q)			0.680	0.548	1.241	0.254			0.442	0.545	0.810	0.444
(2) *P. acidilactici* (L)			-0.205	0.498	-0.413	0.692			0.348	0.495	0.703	0.505
P. acidilactici (Q)			-0.061	0.548	-0.111	0.915			-0.113	0.545	-0.207	0.841

"to be continued"

Tabel 6 “conclusion”

Independent variables	**Probiotic count* (8 h)**						**Probiotic count* (24h/4 ºC)**					
	R^2	R^2 adj.	Effect	SE	*t*-Value	*p*-Value	R^2	R^2 adj.	Effect	SE	*t*-Value	*p*-Value
	Lactic acid (g/L) (8 h)						Lactic acid (g/L)24/4ºC (24h/4 ºC)					
(3) *L. acidophilus* (L)			**4.681**	**0.498**	**9.407**	**0.000**			**3.317**	**0.495**	**6.701**	**0.000**
L. acidophilus (Q)			0.944	0.548	1.724	0.128			1.119	0.545	2.054	0.079
1L by 2L	0.937	0.856	-0.094	0.650	-0.145	0.889	0.907	0.788	**1.762**	**0.647**	**2.725**	**0.030**
1L by 3L			0.555	0.650	0.854	0.421			**-1.547**	**0.647**	**-2.391**	**0.048**
2L by 3L			**-1.705**	**0.650**	**-2.622**	**0.034**			-0.981	0.647	-1.517	0.173

Since the calculated F values (27.57 for Eq. (1), 99.93 for Eq. (2), and 14.50 for Eq. (3)) were higher than the listed F value ($F_{3;13} = 3.41$) ($p < 0.05$). Good correlation coefficients were also obtained, except for probiotic viability ($R^2 = 0.72$).

P. acidilactici and *L. acidophilus* effects on pH, as well as their interaction, were statistically significant at 95% of confidence level. Accordingly, the probiotic culture and the interaction of the two LAB strains were statistically significant on lactic acid production at 8 h of fermentation. Interaction means that the effect produced on pH and lactic acid content in PSM beverage by a change in probiotics population depends on the inoculation level of *P. acidilactici*.

The PSM formulation with higher content of peanut milk had a negative effect on the production of lactic acid after 24 hours under refrigeration. This fact corroborates with our previous findings that show the peanut milk negatively affect the lactic acid production by the inoculated cultures. Soymilk generally has higher carbohydrate content than peanut milk. Values between 1.5 to 2.0% for soymilk and 0.53 to 0.95% for peanut milk (GATADE; RANVEER; SAHOO, 2009; ISANGA; ZHANG, 2009; SANTOS; LIBECK; SCHWAN, unpublished results 2014a; SCHAFFNER; BEUCHAT, 1986) have been reported. It is interesting to note that *L. acidophilus* can ferment raffinose (BUCHANAN; GIBBONS, 1974), a sugar present at higher concentration in soybeans than in peanuts (11.6 mg/g and 3.3 mg/g, respectively) (KUO; VANMIDDLESWORTH; WOLF, 1988). The interaction of PSM formulation and *L. acidophilus* also had a negative effect on the lactic acid production.

The quadratic models for probiotic viability, pH and lactic acid content in PSM potentially probiotic beverage were utilized to generate the three-dimensional response surface plots (**Fig. 1, 2** and **Fig. 3**). Figure 1 is the response surface for the probiotic viability in the PSM beverage as influenced

by the PSM proportions (X_1), *P. acidilactici* (X_2) and *L. acidophilus* initial inoculum (X_3). The positive linear term for *L. acidophilus* and the negative quadratic interaction between the probiotic strain and *P. acidilactici* suggests that the effect of *L. acidophilus* inoculum resulted in: an increase in probiotic viability, lactic acid content and in pH decrease. Figure 2 is the response surface for the pH in the PSM potentially probiotic beverage as influenced by the *P. acidilactici* (X_2) and *L. acidophilus* initial inoculum (X_3).

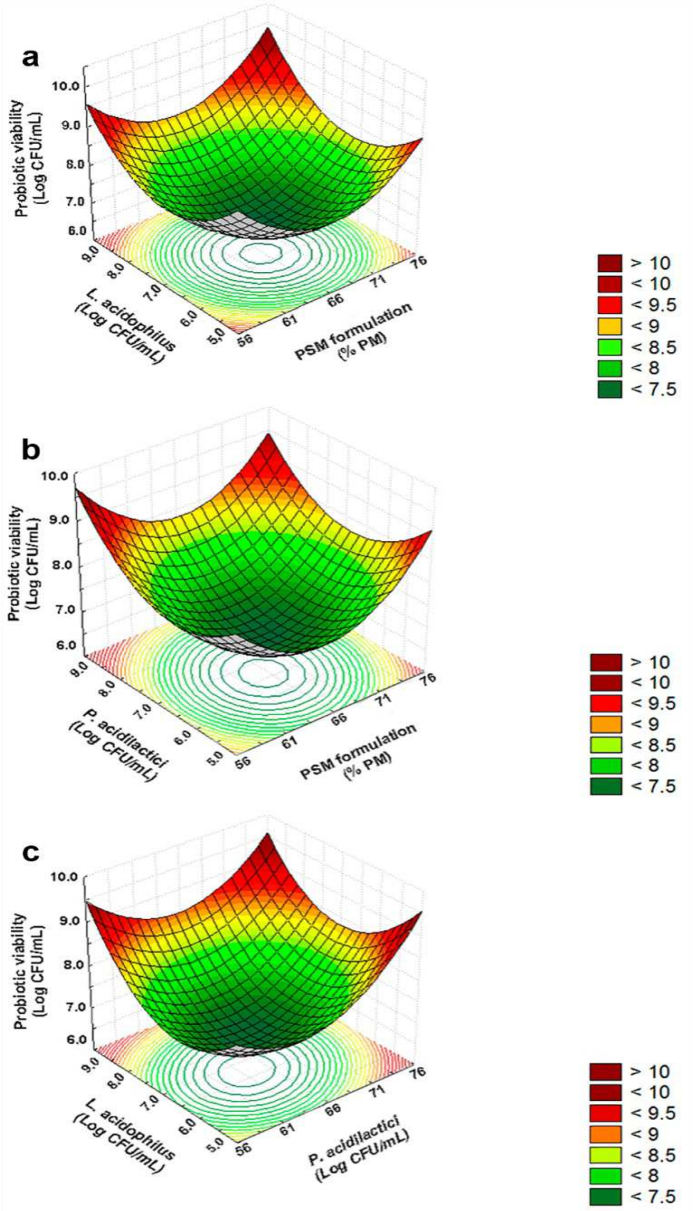

Figure 1 Effect of: A) *L. acidophilus* inoculum and PSM formulation;B) *P.acidilactici* inoculum and PSM formulation; C) *L. acidophilus* and *P.acidilactici* inocula on the probiotic viability of PSM symbiotic beverage after 24 under refrigeration storage

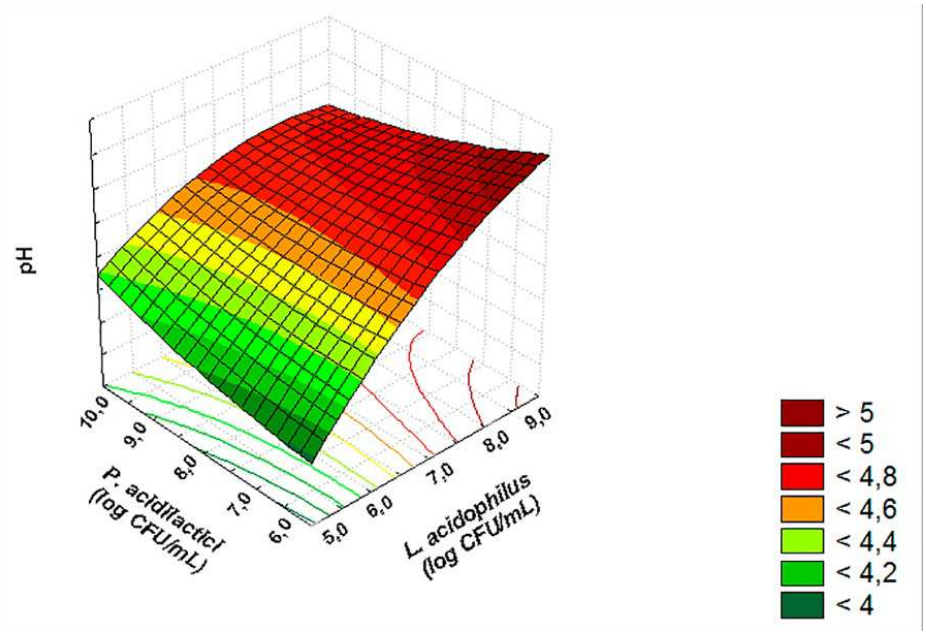

Figure 2 Effect of *L. acidophilus* and *P. acidilactici* inocula on the pH of PSM beverage after 24 under refrigeration storage

The interaction between PSM formulationand *L. acidophilus* decreased the lactic acid content of the PSM beverage after 24 h under refrigeration. The figure 3A shows the negative quadratic effect of PSM formulation on probiotic viability. Figure 3B displays the positive interaction between *P. acidilactici* and PSM formulation, causing an increase in the lactic acid concentration in PSM beverage under refrigeration.

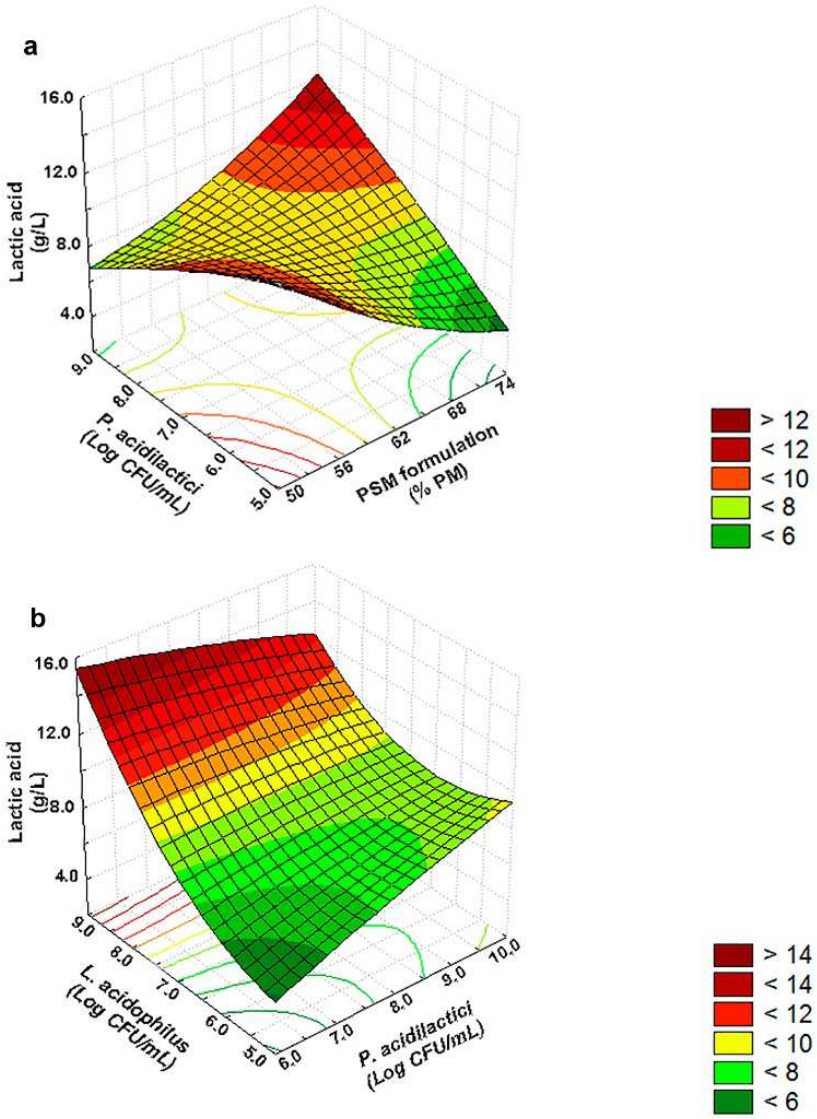

Figure 3 Effect of: a) *L. acidophilus* inoculum and PSM formulation; b) *P. acidilactici* and PSM formulation on the lactic acid content of PSM beverage after 24 under refrigeration storage

The content of lactic acid in PSM potentially probiotic beverage can reach values above 18 g/L by increasing the initial population of *L. acidophilus*

and reducing peanut milk content in PSM formulation. However, several studies on the survival of the probiotic microorganisms have shown that products with high acidity (for example yogurt) lead to further loss of viability than products with low acidity (for example frozen yogurt, ice cream cheese and traditional) (CHAMPAGNE; GARDNER, 2008; DONKOR et al., 2007; NAJI; HASHEMI; HOSEINI, 2014; WANG; YU; CHOU, 2002). So it is necessary to make a careful selection of strains to be used,beyond the amount to be inoculated.

The aim of the optimization was to minimize pH value while maximizing probiotic viability and lactic acid content, in order to obtain a product with physical-chemical and sensorial acceptable characteristics. The optimal condition for the production of PSM potentially probiotic beverage was estimated to be initial population of 6.68 Log CFU/mL of *L. acidophilus*, 8.00 Log CFU/mL of *P. acidilactici* and 61.2% of PM on PSM formulation with a desirability of 0.929974. The other conditions used were fixed after the PB experimental design, namely: 3 % of FOS, 5.0 Log CFU/mL of *S. cerevisiae*, 8 h of fermentation at 37 ºC.

Three independent fermentation runs at the above optimized conditions were carried out. The predicted values according to the CCRD were: 8.09 Log CFU/mL, 4.35, and 11.95 g/L for probiotic viability, pH and lactic acid content, respectively. The obtained values for these responses in the validation experiment were: 8.30 Log CFU/mL, 4.29 and 11.99 g/L, respectively. The good agreement between the predicted and experimental results confirmed the validity of the model.

3.3 Quality assessment and refrigerated storage

The total amino acid composition of a food protein, particularly of the essential amino acids, has classically been considered a measure of its

biological adequacy (SANTOS; LIBECK; SCHWAN, unpublished results 2014a). The total amino acid composition of PSM potentially probiotic beverage is presented in Table 7. These values are compared to the official amino acid profile of an ideal protein as established by WHO (2007). The conversion of PSM into a fermented beverage brought about significant decreases in the levels of histidine, isoleucine, leucine, lysine, cysteine, tyrosine, threonine and valine essentials aminoacids. While, the concentration of methionine remained fairlyconstant in both fermented and unfermented samples and phenylalanine increased from 1.54 to 1.90 g/100g of PSM beverage.

In refrigerated storage there was a decrease in the probiotic viability (Fig. 4). The *L. acidophilus* was above 8 Log CFU/mL until day 7 of storage. After 35 days, the number of *L. acidophilus* cells decreased to 7.26±0.11 Log CFU/mL. *P. acidilactici* populations remained stable throughout the storage period. While the yeast *S. cerevisiae* showed slight growth from day 14, reaching 5.95 log CFU/mL in the thirty-fifth day. PSM beverage obtained enough population to act as probiotic microorganisms in the human intestine, providing also a good sensory quality and texture.

Table 7 Total amino acid composition of the PSM symbiotic beverage, compared with the WHO standard (2007)

Amino acid	Requirements WHO/FAO/ONU (g/100g of protein, dry basis)			PSM (2:1)* (g/100g of PSM, dry basis)	PSMPB (g/100g of PSM symbiotic beverage, dry basis)
	Children (age)		Adults		
	1-2	3-10			
Histidine (Hys)	-	-	1.5	1.12	0.72
Isoleucine (Ile)	-	-	3.0	1.78	1.44
Leucine (Leu)	-	-	5.9	2.90	2.09
Lysine (Lys)	5.2	4.8	4.5	1.75	1.55
Methionine (Met)	-	-	1.6	0.50	0.44
Cysteine (Cys)	-	-	0.6	0.67	0.28
Phenylalanine (Phe)	-	-	-	1.54	1.90
Tyrosine (Tyr)	-	-	-	1.61	1.03
Threonine (Thr)	2.7	2.5	2.3	1.71	1.05
Tryptophan (Trp)	7.4	6.6	0.6	nd	nd
Valine (Val)	-	-	3.9	2.01	1.43
Total essential amino acids	15.3	13.9	23.9	15.59	11.93
Arginine (Arg)				4.80	3.17
Alanine (Ala)	-	-	-	1.92	1.39
Aspartic acid (Asp)	-	-	-	5.13	5.11
Glutamic acid (Glu)	-	-	-	9.44	6.23
Glycine (Gly)	-	-	-	1.93	1.19
Proline (Pro)	-	-	-	2.40	1.32
Serine (Ser)	-	-	-	2.20	1.61
Total amino acid	-	-	-	43.37%	31.95%

* PSM – peanut-soy milk in ratio (2:1 – peanut:soy) developed by Santos et al (2014a, submitted paper). Nd – not determined.

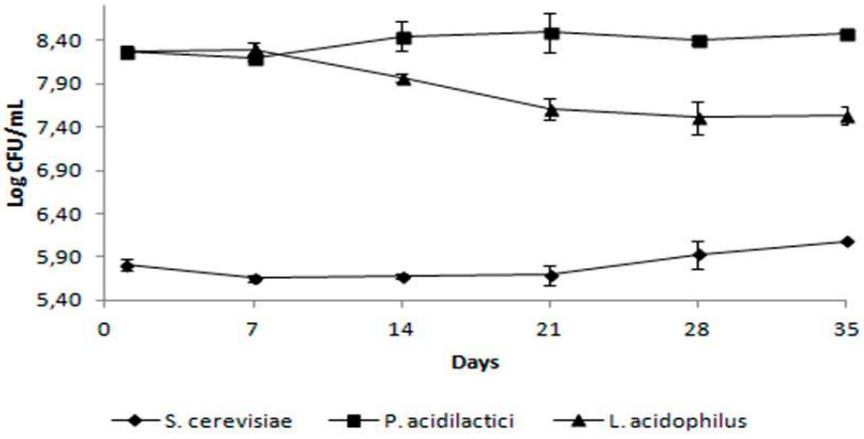

Figure 4 Cell number (Log CFU/mL) of inoculated microorganisms of peanut-soy milk beverage fermented for 8 h at 37 ºC and further stored for 35 days at 4 ºC. *S. cerevisiae* (♦), *L. acidophilus* (■) and *P. acidilactici* (▲). Data are means of three independent experiments. Bars on data points represent standard errors

The final pH and lactic acid content of the fermented PSM beverage felt within an acceptable range (4.0 to 4.5; 12 to 22 g/L) for a good yoghurt (TAMIME; DEETH, 1980). Table 8 shows the main physical-chemical changes observed in PSM beverage during the 35 days under refrigeration. The pH decreased to 3.80 and lactic acid content increased, reaching 15.53 g/L in 35 days of storage.

Glucose, sucrose, maltose and raffinose contentsdecreasedduring fermentation, probably being mainly converted intolactic acid by the probiotic culture. Instead, thefructosecontentincreasedduring storage, this mayindicatethat reduction in the contents of raffinose may be attributed to the hydrolytic reaction catalyzed by α- and β-galactosidases produced by both LAB (*P. acidilactici* and *L. acidophilus*) species studied here (DONKOR et al., 2007).

Table 8 Physico-chemical characteristics of PSM symbiotic beverage during refrigerated storage

Days	pH	Lactic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)	Fructose (g/L)	Glucose (g/L)	Sucrose (g/L)	Maltose (g/L)	Stachyose (g/L)	Raffinose (g/L)
1	4.29 ± 0.02	11.99 ±0.35	0.19 ± 0.01	0.00 ± 0.00	0.60±0.02	Nd	0.29±0.00	0.51±0.18	3.83 ± 0.21	0.48±0.03
7	3.99 ± 0.02	11.69 ± 1.41	0.46 ± 0.06	0.17 ± 0.00	0.57 ± 0.10	Nd	0.17 ± 0.03	0.11 ± 0.00	3.65 ± 0.36	0.45 ± 0.15
14	3.91 ± 0.04	13.27 ± 0.22	0.52 ± 0.01	0.18 ± 0.00	0.93 ± 0.06	0.04 ± 0.01	0.10 ± 0.00	0.03 ± 0.01	3.58 ± 0.12	0.42 ± 0.04
21	3.87 ± 0.00	14.44 ± 0.12	0.50 ± 0.27	0.18 ±0.02	1.07 ± 0.07	0.02 ± 0.00	0.06 ± 0.01	0.04 ± 0.01	3.46 ± 0.04	0.38 ± 0.04
28	3.84 ± 0.02	15.58 ± 0.25	0.47 ± 0.01	0.22 ± 0.01	1.40 ± 0.21	0.03 ± 0.00	Nd	0.05 ± 0.01	3.15 ± 0.45	0.30 ± 0.04
35	3.80 ± 0.02	15.53 ± 1.47	0.45 ± 0.06	0.19 ± 0.04	1.52 ± 0.15	0.02 ± 0.01	Nd	0.05 ± 0.02	3.04±0.10	0.28 ± 0.02

Data are the means of three independent assays; Nd - Not detected; ± Standard deviation

3.4 Sensorial analysis

After the chemical and microbiological analyses, the optimized beverage was subjected to sensory analysis to assess its acceptance among the consumers. Fig. 5 presents the percentage of acceptance attributed to the beverage by 53 untrained tasters, designated based upon the nine-point hedonic scale. The appearance reached a higher frequency (28 tasters, 53% of the total) in point 8 of the hedonic scale, averaging 7.7, meaning that the tasters "liked very much" this attribute of the PSM beverage.

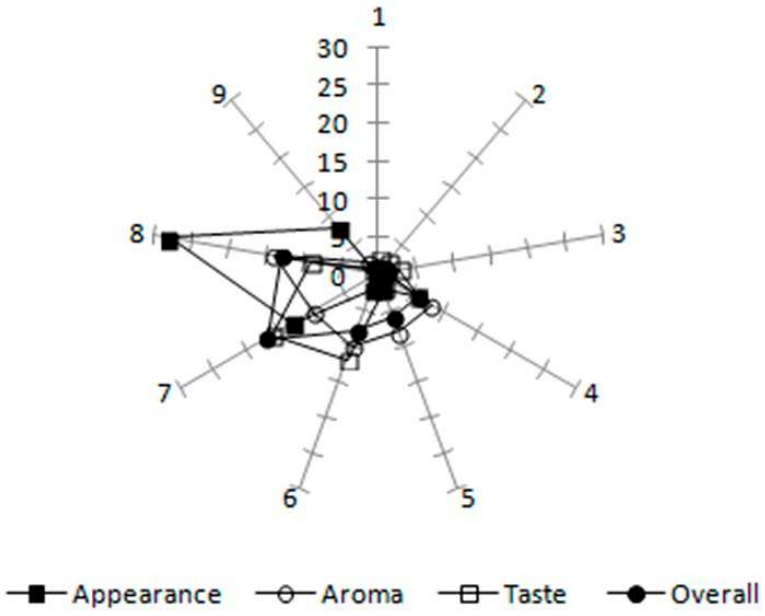

Figure 5 Distribution of the number of panelists in the sensory analysis. Numbers 1 to 9 range from disliked extremely (1) to liked extremely (9).

Similar averages were recorded for other two evaluated attributes, where the overall aspects achieved a slightly higher value, followed by aroma with respective scores of 6.4 and 6.3. The taste of the beverage reached an

average of 5.9, indicating that the tasters had “slightly liked” this attribute of the PSM beverage.

The PSM potentially probiotic beverage was sweetened with sucralose. Artificial sweeteners have been used to replace carbohydrate in the management of diabetes and obesity (AMERICAN DIETETIC ASSOCIATION, 2004). Furthermore, sucralose is a noncaloric sweetener derived from sucrose and is approximately 600 times sweeter.The sweetness level was also evaluated by the 53 panelists. Among them, 45% felt the ideal sweetness of the product and 24% thought the product was slightly less sweet than the ideal. Moreover, 41% of the panelists stated that would probably buy the product if it was available and 24% reported having doubts whether buy the product developed. Therefore, sensory analysis showed good acceptance of the product, taking into account the fact that the tasters were not familiar with the PSM beverage, demonstrating the potential for commercialization of the developed product.

4 CONCLUSION

Optimal PSM potentially probiotic beverage could be produced by fermentation with a combination of the LAB *P. acidilactici*, probiotic culture *L. acidophilus* and the yeast *S. cerevisiae* with addition of 3% FOS at a temperature of 37 °C for 8 h. The possibility of producing a vegetable milk from peanut and soy is beneficial as (a) the product is lactose-free; (b) will contain all the goodness in the seeds especially essentials aminoacids, which may contribute to nutritional well-being; (c) will contain a population of probiotics above the minimum recommended by law until 35 days of storage; and (d) give rise to a new class of products, where the addition of yeast may provide greaterviability of theprobiotic culture, and serve as a source of protein and vitamin B. It was observed that, from the sensorial acceptability of the beverage, this technology can be an alternative use for peanut and soybeans and may provide a new industrial outlet for these oilseeds.

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