Universidade Estadual de Campinas
Faculdade de Engenharia de Alimentos

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Probiotic *Bacillus*: behavior during food processing, *in vitro* and *in vivo* functionality, modulation of gut microbiota and health impacts

Bacillus probióticos: respostas ao processamento de alimentos, funcionalidade *in vitro* e *in vivo*, modulação da microbiota intestinal e impactos à saúde

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Supervisor/Orientador: Anderson de Souza Sant'Ana.

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Resumo

Micro-organismos dos gêneros *Lactobacillus* e *Bifidobacterium* são os mais utilizados como culturas probióticas em alimentos. No entanto, algumas cepas de bactérias formadoras de esporos, tais como *Bacillus coagulans*, *Bacillus subtilis*, *Bacillus polyfermenticus* e *Bacillus pumilus* têm sido recentemente caracterizadas como probióticas. A principal vantagem das cepas probióticas de *Bacillus* frente à *Lactobacillus* e *Bifidobacterium*, reside no fato de sobreviverem melhor às condições gástricas e intestinais adversas em animais em virtude da formação de esporos. Apesar disto, o estudo da aplicação dos micro-organismos probióticos esporulados, *Bacillus*, em alimentos são incipientes e também faltam informações sobre o comportamento destes micro-organismos durante o processamento/estocagem de diferentes alimentos, impacto sobre a microbiota intestinal e, benefícios à saúde influenciados por diferentes matrizes. Desta forma, o presente estudo objetivou caracterizar a resistência de diversas cepas de *Bacillus* probióticos frente à diferentes processos/condições de produção de alimentos e elucidar o impacto de diferentes matrizes alimentares (lácteas e vegetais) na sobrevivência/funcionalidade de cepas probióticas de *Bacillus* no trato gastrointestinal, na modulação da microbiota intestinal e benefícios à saúde em ratos machos da linhagem Wistar. Em nosso estudo, *Bacillus* mostrou sobrevivência/resistência a todos os processos tecnológicos. Entretanto, o comportamento/resistência das cepas de *Bacillus* variou de processo para processo. Além disso, a sobrevivência e a especificidade do *Bacillus* no processo tecnológico e no alimento, respectivamente, variaram de cepa para cepa, assim a resposta pode ser considerada como cepa-dependente. Estudos complementares devem ser feitos para entender o mecanismo de ação e as diferenças protéicas envolvidas na resistência e especificidade da cepa ao processo e alimento em particular, respectivamente. Portanto, nosso estudo está trazendo grandes contribuições para a diversificação no mercado de alimentos probióticos. Ainda, no geral, *Bacillus coagulans* GBI-30, 6086 apresentou elevada resistência ao processo de forneamento e também durante o armazenamento. Portanto, ambos os pães de forma tradicionais e integrais são matrizes alimentares promissoras como transportadoras de probióticos. No entanto, o pão de forma integral mostrou maior capacidade para proteger a cepa probiótica, possivelmente pela presença de fibras na sua composição. Adicionalmente, percebeu-se que é evidente a necessidade de testes *in vitro* que precedem experiências *in vivo*. De acordo com a elevada sobrevivência de *B. coagulans* GBI-30, 6086 observadas em duas matrizes alimentares, tanto o suco como o iogurte provaram, possivelmente, ser excelentes matrizes alimentares para funcionarem como transportadores probióticos durante a passagem

através do trato gastrointestinal (*in vitro*). O desenvolvimento de produtos alimentares contendo *B. coagulans* GBI-30, 6086 é muito promissor para o crescimento da área de alimentos funcionais. O estudo *in vivo* mostrou que o iogurte apresenta vantagens frente ao suco. Uma vez que o consumo diário de iogurte contendo o probiótico *B. coagulans* GBI-30, 6086 durante 21 dias foi capaz de diminuir significativamente os níveis de glicose e triglicerídeos e modular a microbiota intestinal. Assim, o iogurte pode ser uma matriz alimentar mais eficiente para o fornecimento de probióticos.

Palavras-Chave: *Bacillus*, microbiota intestinal, probióticos, processos tecnológicos, trato gastrointestinal.

Abstract

Microorganisms of the genus *Lactobacillus* and *Bifidobacterium* are the most used as probiotic cultures in foods. However, some strains of spore-forming bacteria, such as *Bacillus coagulans*, *Bacillus subtilis*, *Bacillus polyfermenticus* and *Bacillus pumilus* have recently been characterized as probiotic. The main advantage of *Bacillus* probiotic strains over *Lactobacillus* and *Bifidobacterium* lies in the fact that they better survive adverse gastric and intestinal conditions in animals due to the formation of spores. Despite this, the study of the application of sporulated probiotic microorganisms, *Bacillus*, in foods are incipient and also lack information on the behavior of these microorganisms during the processing/storage of different foods, impact on intestinal microbiota and health benefits influenced by different matrix. Thus, the present study aimed to characterize the resistance of several strains of *Bacillus* probiotics to different food production processes/conditions and elucidate the impact of different food matrix (dairy and vegetables) on the survival/functionality of *Bacillus* probiotic strains in the gastrointestinal tract, intestinal microbiota modulation and health benefits in male Wistar rats. In our study, *Bacillus* showed survival/resistance to all technological processes. However, the behavior/resistance of *Bacillus* strains varied from process to process. In addition, the survival and specificity of *Bacillus* in the technological process and in the food, respectively, varied from strain to strain, so the response could be considered as strain-dependent. Complementary studies should be done to understand the mechanism of action and the protein differences involved in the resistance and specificity of the strain to the particular process and food, respectively. Therefore, our study is bringing great contributions to the diversification in the probiotic food market. Furthermore, in general, *Bacillus coagulans* GBI-30, 6086 showed high resistance to the delivery process and also during storage. In addition, both wheat and whole wheat pan breads are promising food matrix as probiotic carriers. However, whole wheat pan bread showed greater capacity to protect the probiotic strain, possibly due to the presence of fibers in its composition. Additionally, it has noticed that the need for *in vitro* tests preceding *in vivo* experiments is evident. According to the high survival of *B. coagulans* GBI-30, 6086 observed in two food matrix, both juice and yogurt proved to be excellent food matrices to function as probiotic transporters during passage through the gastrointestinal tract (*in vitro*). The development of food products containing *B. coagulans* GBI-30, 6086 is very promising for the growth of

functional food area. The *in vivo* study showed that yogurt has advantages over juice. Thus, yogurt may be a more efficient food matrix for the delivery of probiotics. Since the daily consumption of yogurt containing the probiotic *B. coagulans* GBI-30, 6086 for 21 days was able to significantly decrease glucose and triglyceride levels and to modulate the intestinal microbiota.

Key words: *Bacillus*, intestinal microbiota, probiotics, technological processes, gastrointestinal tract.

SUMÁRIO

1. INTRODUÇÃO

1.1 Esporos Bacterianos

Determinadas espécies bacterianas passam, pelo menos parte de sua vida, como estruturas celulares dormentes, compactas e não reprodutivas, chamadas de esporos. Os esporos bacterianos são estruturas altamente resistentes à agentes químicos e físicos de esterilização e desinfecção, sendo produzidos como uma resposta à condições ambientais adversas (Moir, 2006; Setlow, 2003).

O esporo é resultado da desidratação da célula bacteriana, sendo composto do DNA bacteriano, ribossomos e normalmente por até 10% de ácido dipicolínico (Nicholson et al., 2000). Através da desidratação, os esporos suprimem sua atividade metabólica e, conseguem sobreviver sob condições desfavoráveis, como altas temperaturas, radiação UV, dessecação, exposição a solventes, peróxido de hidrogênio, enzimas, dentre outros (Nicholson et al., 2000). Sob condições favoráveis, os esporos bacterianos podem germinar e posteriormente, retomar o crescimento vegetativo.

Os micro-organismos esporulados desempenham papel fundamental em diversas áreas, porém eles são particularmente importantes na área de alimentos, onde estão grandemente associados à deterioração de alimentos processados ou como causadores de toxinfecções alimentares (Heyndrickx, 2011). Dentre os diversos gêneros de bactérias formadoras de esporos, *Clostridium* e *Bacillus* destacam-se como os de maior importância para a preservação dos alimentos (Augustin, 2011; Caspers et al., 2011; Peck, Stringer, Carter, 2011). Apesar da grande preocupação com o impacto destes micro-organismos sobre os alimentos, sabe-se que algumas cepas tem encontrado aplicações diversas em biotecnologia (Ricca, Cutting, 2003; Sanders, Morelli, Tompkins, 2003; Wolken, Tramper, van der Werf, 2003). Dentre estas, destacam-se as aplicações de cepas de *Bacillus* spp. como micro-organismos probióticos, tais como suplementos dietéticos em humanos, promotores de crescimento e agentes de exclusão competitiva em animais e melhoria de crescimento e resistência a doenças em aqüicultura (Cutting, 2011).

1.2 Bacillus como Micro-organismos Probióticos

Probióticos são micro-organismos vivos que quando ingeridos em quantidades adequadas conferem benefícios a saúde do hospedeiro (Hill et al, 2014). Micro-organismos dos gêneros *Lactobacillus* e *Bifidobacterium* têm sido os mais utilizados como probióticos. No entanto, algumas cepas de bactérias formadoras de esporos, tais como *Bacillus coagulans*,

Bacillus subtilis, *Bacillus polyfermenticus* e *Bacillus pumilus* têm sido recentemente caracterizadas como probióticas (Cutting, 2011).

Bacillus spp. são micro-organismos bastonetes Gram-positivos, catalase positivos, aeróbios ou anaeróbios facultativos, móveis e ubíquos (Nakano, Zuber, 1998; Vos et al., 2009). Apesar do solo ser mais frequentemente considerado como fonte primária de *Bacillus* spp., sabe-se que estes micro-organismos deveriam ser considerados como comensais do trato gastrointestinal, já que tem sido isolados do trato gastrointestinal de humanos (Cutting, 2011; Fakhry et al., 2008; Hong et al., 2009).

Nos últimos anos, diversas espécies de *Bacillus* tem sido reportadas como probióticas. Dentre elas, destacam-se: *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans*, *Bacillus mojavensis* KJS-3, *Bacillus flexus* Hk1, *Bacillus licheniformis* Me1, *Bacillus subtilis* Bn1, *Bacillus subtilis* natto, *Bacillus subtilis* KD1, *Bacillus subtilis* PY79 e *Bacillus indicus* HU36 (Cutting, 2011; Hong et al., 2008; Kim et al., 2011; Nithya, Halami, 2013; Riazi *et al.*, 2009; Ripamonti et al., 2009; Sanders, Morelli, Tompkins, 2003; Sun, Wang, Deng, 2013; Wu et al., 2011; Zhou et al., 2010).

Algumas cepas probióticas de *Bacillus* têm sido amplamente empregadas como suplementos dietéticos para humanos, como promotores de crescimento e agentes de exclusão competitiva em animais e como agentes de exclusão competitiva e promotores de crescimento em aqüicultura (Cutting, 2011). No entanto, devido à propriedades fornecidas pelos esporos, como *i)* maior resistência à condições adversas aplicadas no processamento de alimentos, ou seja, maior resistência à etapas e processos tecnológicos que normalmente culminam com o estresse (secagem, por exemplo), perda da viabilidade e potencial probiótico de micro-organismos não esporulados como *Lactobacillus* e *Bifidobacterium, ii)* melhor viabilidade no trato gastrointestinal (sobreviverem melhor às condições gástricas e intestinais adversas em animais) (Tam et al., 2006), devido à maior resistência dos esporos aos sucos gástricos e biliar (Hong, Duc, Cutting, 2005), *iii)* necessidade de menor dose efetiva para ser usada como probiótico, devido à capacidade de sobrevivência de *Bacillus* probióticos (Durkee, 2010) o interesse pelo uso de *Bacillus* probióticos em alimentos tem se expandido rapidamente, já que tais características fornecem vantagens competitivas frente a outros gêneros que não formam esporos, como *Lactobacillus* e *Bifidobacterium* (Nithya, Halami, 2013). Desta forma, sugere-se que as cepas probióticas de *Bacillus*, podem permanecer viáveis por um longo tempo e/ou sobreviver à processos de fabricação mais drásticos, possibilitando a sua aplicação e veiculação através de diversas matrizes alimentícias até então pouco exploradas como potencialmente probióticas.

1.3 Impacto das Matrizes Alimentícias sobre a Eficácia dos Probióticos

No que concerne especificamente à sua aplicação nos alimentos, sabe-se que as matrizes alimentícias têm impacto fundamental na eficácia dos probióticos (Ranadheera, Baines, Adams, 2010). Tal importância deve-se ao fato do substrato alimentar transportar, entregar e ajudar a tamponar o probiótico no trato gastrointestinal, regular sua colonização, além de poder conter outros ingredientes funcionais, que combinados com metabólitos dos micro-organismos podem atuar sinergisticamente impactando beneficamente sobre a saúde do hospedeiro (Ranadheera, Baines, Adams, 2010).

Por um lado, sabe-se que combinações de metabólitos dos micro-organismos probióticos com componentes dos alimentos ingeridos na dieta, juntamente com a capacidade de tamponamento, pH do alimento e propriedades nutricionais (teor de vitaminas, por exemplo), conteúdo de gordura, concentração e tipo de proteínas e açúcares, são fatores que influenciam na sobrevivência e funcionalidade dos probióticos como *Lactobacillus* e *Bifidobacterium* (Ranadheera, Baines, Adams, 2010). Diversos estudos têm reportado efeito variado da matriz alimentar sobre a funcionalidade de cepas não esporuladas de micro-organismos probióticos, como: uma maior atividade metabólica com níveis mais altos de ácido lático e acético, maior número de peptídeos potencialmente bioativos liberados e redução da população de *Listeria monocytogenes* em queijo cottage adicionado de *Lactobacillus casei cremoris* ou *Lactobacillus rhamnosus* GG quando comparados com queijo sem probiótico (Abadia-Garcia et al., 2013), iogurte com gordura completa mostrou efeito mais inibitório na sobrevivência de cepas probióticas (*Bifidobacterium bifidum* BBI e *Lactobacillus acidophilus* LAI) do que iogurte com gordura reduzida (Vinderola, Bailo, Reinheimer, 2000), a viabilidade de *Lactobacillus acidophilus* LA-5 foi maior em iogurte de frutas do que em iogurte natural (Ranadheraa et al., 2012) e o chocolate preto foi capaz de manter a viabilidade de *Lactobacillus plantarum* durante três meses de estocagem (Foong et al., 2013), dentre outros.

Se permanecerem viáveis nos alimentos, os probióticos podem resultar em diversos efeitos positivos, como: *i)* melhorar o sabor e valor nutritivo dos alimentos (Nogueira, Gonçalves, 2011), *ii)* fornecer vários benefícios a saúde, como modulação da microbiota intestinal (Sun et al., 2011), *iii)* tratamento de doenças intestinais (Hun, 2009), *iv)* proteção contra patógenos (Vidyalaxme et al., 2012), *v)* melhoria do sistema imune (Sun, Wang, Zhang, 2010) e *vi)* redução de sintomas associados com obesidade (Paik, Park, Park, 2005).

Apesar da grande disponibilidade de dados sobre a funcionalidade e impacto da matriz alimentícia sobre os probióticos não esporulados, há uma escassez de dados sobre tais aspectos quando se consideram cepas probióticas esporuladas, como *Bacillus* spp. Assim, sabendo-se as vantagens da aplicação de cepas probióticas de *Bacillus* spp. em alimentos, torna-se patente investigar-se o impacto da dieta e do tipo de matriz alimentar na funcionalidade dos probióticos esporulados e sobre a ecologia microbiana (*in vivo*). Adicionalmente, são escassas as informações sobre o impacto de diferentes métodos de conservação de alimentos sobre a eficácia de micro-organismos probióticos esporulados. Como sugere-se que os efeitos benéficos dos micro-organismos probióticos esporulados podem ser dependentes da presença ou dominância dos esporos ou células vegetativas quando no trato gastrointestinal (Sanders, Morelli, Tompkins, 2003), tais aspectos tornam-se extremamente relevantes, pois existe a possibilidade de diferentes métodos de conservação/processamento dos alimentos resultarem em maior ou menor porcentagem de germinação dos esporos no trato gastrointestinal.

1.4 Efeitos à Saúde Advindos do Consumo de Alimentos Probióticos

O consumo regular de alimentos probióticos está associado à uma variedade de benefícios à saúde. Existem evidências que comprovam as aplicações clínicas dos probióticos na manutenção do equilíbrio da microbiota intestinal (Sarkar, 2013), na prevenção e tratamento de doenças, como gastrointestinais (Kajander et al., 2008), respiratórias (Wang et al., 2004) e mentais (Yeon et al., 2010), bem como no combate de infecções por patógenos (Castillo et al., 2012) e na promoção do bem-estar (Messaoudi et al., 2011). Outros importantes efeitos são a prevenção de hipertensão (Jauhiainen et al., 2005) e diabetes (Tabuchi et al., 2003), redução dos níveis de colesterol (Huang et al., 2010) e da intolerância a lactose (He et al., 2008), atividade anti carcinogênica (Thirabunyanon, Boonprasom, Niamsup, 2009), benefícios no tratamento de ansiedade e depressão (Bravo et al., 2011), modulação do sistema imune (O'Mahony et al., 2005) e prevenção de alergias alimentares e doenças atópicas, como asma, eczema e dermatite atópica (Kirjavainen, Salminen, Isolauri, 2003). Entre outras aplicações incluem tratamento de diarréia em adultos e crianças (Allen et al., 2004), prevenção de cáries dentárias (Krasse et al., 2005), controle de doenças inflamatórias intestinais e síndrome do intestino irritado (Nobaek et al., 2000). Recentemente, incluem indicações de uso de probióticos em controle de queda capilar e acne (Foligné, Daniel, Pot, 2013).

Apesar de muito já se conhecer sobre os efeitos benéficos à saúde, advindos do consumo de alimentos contendo cepas probióticas não esporuladas, dados sobre os efeitos benéficos à saúde de cepas probióticas esporuladas são ainda limitados. Os principais efeitos benéficos à saúde associados à cepas probióticas são:

i) *Bacillus polyfermenticus* SDC como suplemento da dieta de ratos: benefícios significativos para a saúde através da modulação de funções fisiológicas, incluindo perfil lipídico e antioxidante em ratos hipercolesterolêmicos (Paik, Park, Park 2005);

ii) *Bacillus subtilis* administrado à peixes como suplemento da dieta: melhoria do desempenho reprodutivo em termos de maior índice gonadossomático, fecundidade, sobrevivência e características morfométricas (Ghosh, Sinha, Sahu, 2007);

iii) *Bacillus coagulans* GBI-30, 6086 ministrado em pacientes humanos na forma de cápsulas: melhoria nos sintomas de síndrome do intestino irritado, como inchaço e dores abdominais (Dolin, 2009; Hun, 2009);

iv) *Bacillus coagulans* GBI-30, 6086: por meio de teste em animais (camundongos, ratos e coelhos) foi considerado atóxico, não patogênico e seguro para uso humano (Endres et al., 2009).

v) *Bacillus clausii* UBBC07, *Bacillus coagulans* SANK70258, *Bacillus licheniformis* Me1 e *Bacillus subtilis* CU1 por meio de testes *in vitro* e/ou *in vivo* foram considerados seguros para aplicação em alimentos, mostrando ausência de efeitos indesejáveis com o consumo (Akagawa et al., 2016; Lakshmi et al., 2017; Lefevre et a., 2017; Nithya, Muthukumar, Halami, 2012).

vi) *Bacillus polyfermenticus* em camundongos: redução da mortalidade, da gravidade da colite (perda de peso, diarréia e danos na mucosa), da expressão de moléculas inflamatórias, incluindo fator de necrose tumoral-α e aumento da expressão da citocina anti-inflamatória IL-10 no cólon do camundongo inflamado, suprimiu a apoptose tanto *in vivo* como *in vitro* e promoveu a proliferação das células epiteliais (Im et al., 2009);

vii) *Bacillus coagulans* GBI-30, 6086: modulação imune e ainda efeitos anti-inflamatórios (*in vitro)* (Jensen et al., 2010);

viii) *Bacillus coagulans* GBI-30, 6086 administrados em pacientes humanos na forma de cápsulas: aumentou os níveis de marcadores imunológicos, como citocinas (IL -6, IL -8, e INF-γ) e células CD3CD69 (Kimmel et al., 2010);

ix) *Bacillus coagulans* GBI -30, 6086: digestão de lactose, frutose e proteína do leite, proporcionando alívio nos sintomas de intolerância a lactose (*in vitro)* (Maathuis, Keller, Farmer, 2010);

x) *Bacillus subtilis* natto em leite ministrado à bezerros leiteiros na fase de desmame: aumento no desempenho de crescimento, melhorando a média de ganho de peso e a eficiência alimentar, além de estimular a secreção de IgG sérico e níveis de citocinas Th1, incluindo IFN-γ (ativação do sistema imunológico e aumento da imunidade) (Sun, Wang, Zhang, 2010);

xi) *Bacillus pumilus* SE5 administrado à peixes como suplemento na dieta: modulação da microbiota intestinal (Sun et al., 2011);

xii) *Bacillus coagulans* ATCC 7050 como suplementos na dieta de frangos: melhoria do equilíbrio da microflora intestinal (Hung et al., 2012);

xiii) *Bacillus subtilis* natto RG4365: efeito sinérgico com *Leuconostoc mesenteroides* em malte e aumento da quantidade de ácido linoléico e linolênico, conteúdo de minerais (ferro e zinco) e atividade antimicrobiana contra *Vibrio cholerae in vitro* (Vidyalaxme et al., 2012);

xiv) *Bacillus polyfermenticus* KU3: efeito profilático contra células cancerígenas, pela inibição de células cancerígenas (HeLa, LoVo, HT-29, AGS e MCF-7) e atividade anti-inflamatória, pela redução do óxido nítrico e das citocinas pró-inflamatórias (TNF-α, IL-10, TGF-P2, e COX-2) (*in vitro*) (Lee et al., 2015);

xv) *Bacillus subtilis* NC11: atividade protetora contra infecção por *Salmonella* Enteritidis (*in vitro*) (Thirabunyanon, Thongwittaya, 2012).

1.5 Abordagem Metagenômica no Estudo da Ecologia Microbiana

Metagenômica é a análise genômica aplicada a todos os micro-organismos de um ecossistema microbiano sem identificação anterior. O campo é emergente, engloba estudos de cultura independente das estruturas, funções e suas interações com os habitats que ocupam (Lepage et al., 2013). O processo é dividido em duas áreas: *a)* alvos individuais são amplificados utilizando a reação em cadeia da polimerase (PCR) e, em seguida, os produtos são sequenciados (estudo metagenômico focado de um único gene ambiental); *b)* DNA total é isolado a partir de uma amostra e, em seguida, sequenciado (estudo metagenômico aleatório de todos os genes ambientais) (Gilbert, Dupont, 2011).

Anteriormente, o conhecimento sobre a composição e desenvolvimento da microbiota se baseava na utilização de métodos tradicionais dependentes de cultura. No entanto, com o surgimento de técnicas moleculares independentes de cultura se observou que somente uma minoria dos membros da microbiota seriam cultiváveis e, portanto, a maior parte dos micro-organismos presentes numa amostra não eram identificados pelos métodos tradicionais (Gerritsen et al., 2011; Gueimonde, Collado, 2012).

O desenvolvimento de métodos rápidos e de custo mais reduzido de seqüenciamento de DNA tem permitido estudos metagenômicos extensivos sobre a ecologia microbiota em diversos substratos e ambientes. Tais fatos têm permitido o aumento do conhecimento sobre a composição e atividade da microbiota característica de determinada amostra, e no caso específico da microbiota intestinal, pode permitir a identificação de micro-organismos relacionados à doenças e assim o desenvolvimento de probióticos direcionados a atuar sobre a microbiota, modulando-a (Gueimonde, Collado, 2012). De fato, estudos recentes têm mostrado como os probióticos podem modular a microbiota intestinal e resultar em efeitos benéficos à saúde dos hospedeiros. Por exemplo, *Lactobacillus reuteri* DSM 17938 pode acelerar o desenvolvimento natural do microbioma intestinal neonatal (simples) ao estado adulto (complexo) e assim reforçar a capacidade de um recém-nascido em resistir à perturbações microbianas, incluindo doenças infecciosas (Preidis et al., 2012). Em outro estudo, em camundongos suplementados com probióticos (*Lactobacillus rhamnosus* GG) por 6 a 8 semanas, houve uma prevenção das alterações negativas na microbiota intestinal causadas quando da ingestão de álcool por 6-8 semanas (Bull-Otterson et al., 2013). Tal resultado sugere que a prevenção nas alterações na microbiota intestinal observadas associadas aos probióticos podem resultar em estratégias terapêuticas para a prevenção e/ou tratamento de disfunção intestinal (Bull-Otterson et al., 2013). Em comum, os estudos mencionados anteriormente aplicaram a abordagem metagenômica para elucidar o impacto da suplementação da dieta com probióticos na microbiota intestinal e correlacionar tais mudanças com indicadores específicos relacionados ao estado de saúde dos hospedeiros. Neste contexto, a abordagem metagenômica e o sequenciamento de alto desempenho possuem vantagens importantes, como o fato de permitirem a realização da triagem de várias amostras ao mesmo tempo e identificação da maioria dos micro-organismos presentes (Ercolini, 2013). Apesar de todas as vantagens desta técnica, inexistem estudos cujos objetivos tenham sido avaliar a metagenoma intestinal em função da suplementação da dieta com alimentos produzidos com *Bacillus* probióticos e o impacto de sua associação com uma dieta variada em termos de teor de calorias, fibras e gordura. Desta forma, estudos envolvendo metagenômica e alimentos probióticos ainda são escassos e fica patente a necessidade de sua realização para melhor elucidação dos mecanismos envolvidos com efeitos benéficos do consumo de probióticos.

Considerando o exposto anteriormente, o estudo da aplicação dos micro-organismos probióticos esporulados, *Bacillus*, em alimentos são incipientes. Desta forma, considerando-se as potenciais aplicações das cepas de *Bacillus* probióticos em diferentes matrizes alimentícias

e a falta de informações sobre o comportamento destes micro-organismos durante o processamento/estocagem de diferentes alimentos, impacto sobre a microbiota intestinal e, benefícios à saúde influenciados por matrizes diversas, tem-se o presente trabalho.

2. OBJETIVOS

- ✓ Avaliar a resistência de diversas cepas de *Bacillus* probióticos frente à diferentes operações unitárias;
- ✓ Avaliar a resistência de *Bacillus* probióticos frente ao processamento e durante estocagem de pão de forma tradicional e integral;
- ✓ Avaliar o impacto de diferentes matrizes alimentares (lácteas e vegetais) na persistência/funcionalidade de cepas probióticas de *Bacillus* no trato gastrointestinal (*in vitro*);
- ✓ Avaliar o impacto de diferentes matrizes alimentares (lácteas e vegetais) adicionadas de *Bacillus* probióticos na modulação da microbiota intestinal de ratos machos da linhagem Wistar;
- ✓ Determinar os benefícios à saúde, de ratos machos da linhagem Wistar, advindos da administração de alimentos adicionados de *Bacillus* probióticos.

3. ARTIGOS

- ✓ **Characterization of the intestinal microbiota and its interaction with probiotics and health impacts**

- ✓ **Resistance characterization of eight probiotic *Bacillus* strains in different foods matrices to eight unit operations applied in food industry**

- ✓ **Whole wheat bread is a good matrix for delivering probiotic spore-forming bacteria**

- ✓ **In vitro evaluation of survival/functionality of the *Bacillus coagulans* GBI-30, 6086 spores in juice and yogurt in simulated gastrointestinal tract conditions**

- ✓ ***Bacillus coagulans* GBI-30, 6086 spores modulates intestinal microbiota and changes health parameters in rats**

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MINI-REVIEW

Characterization of the intestinal microbiota and its interaction with probiotics and health impacts

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Abstract The gastrointestinal tract (GIT) is a dynamic microecosystem containing a diversified microbiota of about 500–1000 different microbial species. Humans depend on their intestinal microbiota to carry out vital functions, and thus, equilibrium among intestinal groups of microorganisms is essential. In this review article, the use of traditional and molecular methods is discussed for the characterization of the intestinal microbiota, as well as its interaction with probiotics and their effects on health. An improved knowledge on intestinal microbiota composition and diversity and how changes in this microecosystem can cause or are associated with diseases remains far from being completely understood. Therefore, a better understanding of the GIT microbial populations is crucial, which will certainly contribute to the development of new strategies for the prevention and/or treatment of several diseases. The manipulation of the GIT microbiota by probiotics consumption is an interesting approach to maintain and restore human health.

Keywords Gut · Microbiota · Diseases · Probiotics · Immune system

Introduction

Microorganisms colonize practically the whole surface of the human body exposed to the external environment, including the skin, oral cavity, respiratory and urogenital membranes, and the gastrointestinal tract (GIT) (Gerritsen et al. 2011). The GIT is a complex and dynamic microecosystem containing a high microbial diversity, estimated at 500–1000 microbial species, which are in equilibrium (Collado et al. 2009). These microorganisms can be permanent residents of the intestinal microbiota or transient, environmentally acquired, for example, by food consumption (Gerritsen et al. 2011).

The intestinal microbiota has an important role in the improvement of the bioavailability of nutrients and degradation of nondigestible components of the diet, production of new nutrients, removal of toxic compounds, metabolism of carbohydrates and proteins, intestinal barrier, protection against diseases, boost of the immune system, and the development, maturation, and maintenance of motor and sensory functions of the GIT (Guarner and Malagelada 2003; Barbara et al. 2005; Rajilic-Stojanovic 2013). Therefore, the intestinal microbiota can be recognized as an active organ (Collado et al. 2009). The GIT microbiota composition is not homogenous since it varies in terms of spatial and temporal perspectives (Fig. 1a–c) (Sekirov et al. 2010). Due to the presence of acids, pancreatic and bile secretions, oxygen gradients, and the ileum motor activity, a stable colonization of the stomach and duodenum is not easy to be reached for the majority of the microorganisms. As a result, the small intestine only houses a few species and a reduced number of microorganisms. The bacterial populations increase along the GIT, reaching the highest numbers and diversity in the colon. Apart from variations in the microbiota composition, the luminal microbial populations also differ in terms of the ratios between anaerobic and aerobic microorganisms, which are lower at the epithelial surface of the mucosa than at the intestinal lumen (O'Hara and Shanahan 2006; Espey 2013).

The microbial colonization of the human intestine starts at the moment of birth. During the first year of life, its composition is simple but greatly varies among individuals and over time. After 1 year of age, the intestinal microbiota of children

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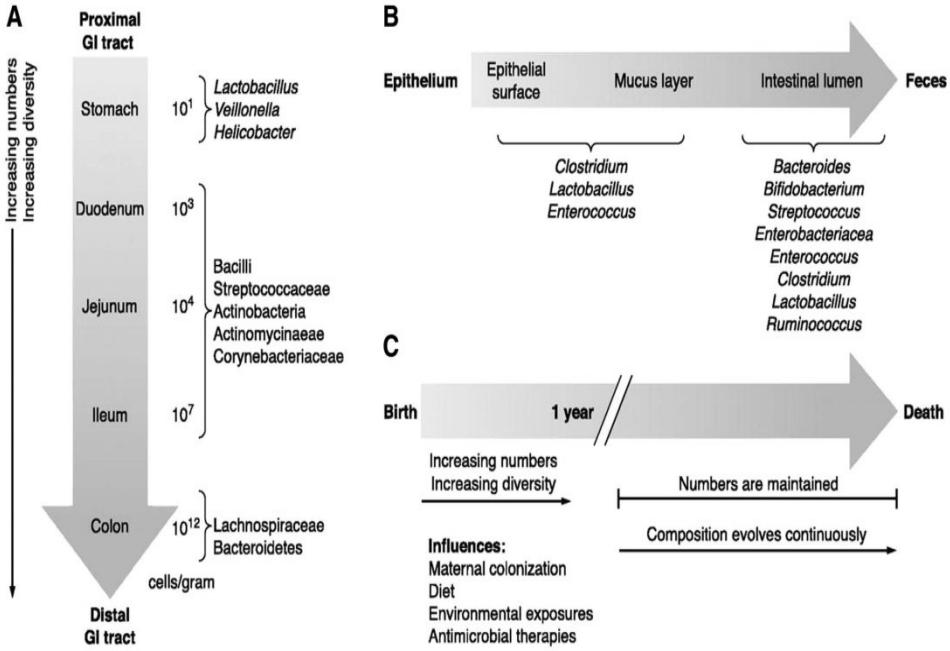

Fig. 1 Composition of the intestinal microbiota: spatial and temporal aspects. **a** Variations in microbial numbers and composition throughout the gastrointestinal (*GI*) tract. **b** Longitudinal variations in microbial composition in the intestine. **c** Temporal aspects of microbiota establishment and maintenance, and factors influencing microbial composition (Sekirov et al. 2010, with permission)

starts to become similar to that of a young adult and finally stabilizes (Fig. 1c) (Sekirov et al. 2010). The human GIT is sterile on birth and first colonized by fecal and vaginal microbiota acquired from the mother, which is affected by the type of delivery (natural or cesarean) and hygienic practices. Later, the intestinal microbiota is influenced by feeding habits and environmental microorganisms, being stable and unique for every individual throughout his/her adult life (Salminen et al. 2005).

Some factors can influence the host microbiota composition including the mother's microbiota composition, diet, environmental exposition, and use of antimicrobial therapies (Fig. 1c). Other aspects, including the host's inflammatory state and the genetic background, also impact the microbiota and, therefore, are able to contribute to the individual's health state; however, their exact roles remain mostly unknown (Gerritsen et al. 2011). Despite this multiplicity of factors, the composition of the human intestinal microbiota is stable, with main groups dominating the microecosystem, although variations in the proportions of these groups are common (Sekirov et al. 2010). The dominant genera in the microbiota of an adult human being are *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Lactobacillus*, *Fusobacterium*, and various Gram-positive cocci, while *Enterococcus* and *Enterobacteriaceae* are considered subdominant genera (Fig. 1a, b) (Guarner and Malagelada 2003). Thus, variations in the intestinal microbiota composition among individuals do not compromise the maintenance of an adequate function.

Normally, the microorganisms and their hosts have a symbiotic relationship, where the host offers a nutrient-rich environment, and on the other hand, the diversified intestinal microbiota exerts beneficial effects upon the host (Lutgendorff et al. 2008). However, the equilibrium among the intestinal microbial groups (nonpathogenic versus pathogenic microorganisms) is essential for health maintenance, and once disrupted, the relationship between host and microorganisms can culminate in a pathologic condition (Collado et al. 2009; Prakash et al. 2011) (Fig. 2). Perturbations of the microbiota composition, also known as dysbiosis (Gerritsen et al. 2011), have been associated with a greater risk for specific diseases, including chronic GIT inflammatory diseases (Joossens et al. 2011), diarrheas (Young and Schmidt 2004), irritable bowel syndrome (Maukonen et al. 2006; Malinen et al. 2010), allergies (Suzuki et al. 2007), diabetes (Wu et al. 2010), and obesity (Turnbaugh et al. 2006).

Functional foods not only satisfy hunger and provide the basic nutrients, but also improve the host's well-being (Vergari et al. 2010). Among them, foods with additional new nutrients or components, such as probiotics, are a highly profitable market niche for food industries because of their valuable health potential (Bigliardi and Galati 2013).

Given the above, it is comprehensible that probiotics consumption seems to be an interesting and feasible approach to modulate the intestinal microbiota and to maintain or restore human health (FAO/WHO 2002). Therefore, the goal of this review article is to discuss probiotics interactions with the

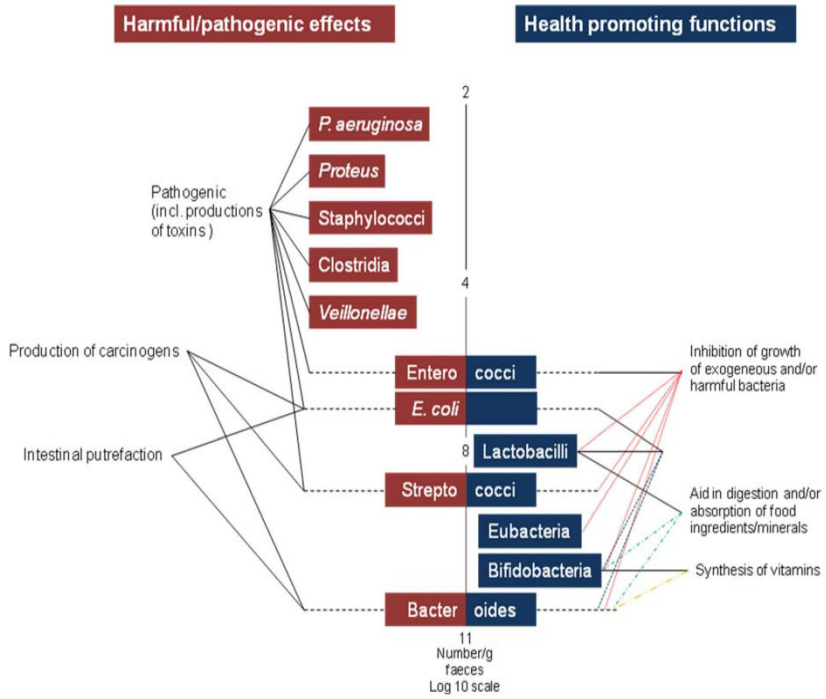

Fig. 2 The composition and health effects of predominant human fecal microbiota. Estimated numbers of genera present in feces are shown. Microorganisms can be divided into three groups based on their pathogenic potential or benefic effects on host: (i) bacteria that cause pathogenic effects, (ii) bacteria that exert beneficial effects, and (iii) bacteria that may lead to either harmful or beneficial effects (Gibson and Roberfroid 1995, with permission)

intestinal microbiota and their impacts on human health. Firstly, the techniques used to analyze the intestinal microbiota are presented, followed by an overview of the characteristics of probiotics and their action mechanisms. Finally, studies that correlate probiotics, the intestinal microbiota, and health interactions/impacts are discussed.

Techniques used to characterize the intestinal microbiota

The intestinal microbiota can be characterized according to its richness (number of species) and regularity (relative abundance of each species), representing its microbial diversity (Gerritsen et al. 2011). In Table 1, the main methods used for the characterization of the intestinal microbiota in the presence or not of probiotics are shown, which are discussed in detail in the succeeding subsections.

Culture-dependent method

This technique evaluates the microbiota composition by the use of different selective culture media for specific bacterial populations. Among the most widely used culture media for the evaluation of the main intestinal microbial populations that are worth mentioning are as follows: Wilkins-Chalgren agar for total anaerobic bacteria (Collado et al. 2007); de Man, Rogosa & Sharp (MRS) for total lactobacilli (Palomar et al. 2013); modified MRS for *Bifidobacterium* (Montesi et al. 2005); kanamycin-esculin agar for *Enterococcus* (Collado et al. 2007); Bacteroides bile esculin agar for *Bacteroides* (Carroll et al. 2010); reinforced clostridial agar (RCA) for *Clostridium* (Collado et al. 2007); and McConkey for enterobacteria (Palomar et al. 2013). Although culture-based methods for bacterial enumeration are highly reproducible, they are limited to distinguish between different bacterial groups. Despite this, it should be taken into account that the study of the intestinal microbial community is difficult since the majority of the microorganisms are strictly anaerobes. In addition, about 80 % of the intestinal microbiota cannot be cultivated under standard laboratory conditions (Eckburg et al. 2005).

The human GIT microbial diversity is markedly influenced by the approach used (Fig. 3). Firmicutes represents the most diverse phylotype both with respect to culture-dependent and culture-independent techniques. Bacteroidetes is a more diverse group when analyzed by molecular methods, in comparison to culture-based approaches. However, proteobacterial diversity has been better determined by the use of molecular techniques (Rajilic-Stojanovic et al. 2007).

Table 1 Methods used for characterization of the intestinal microbiota under different conditions

Method	Source	Application	Probiotic microorganism (s) tested	Detected microbiota	References
Culture-dependent	Feces	Assessment of the relationship between intestinal microbiota and the development of allergies in children[a]	–[b]	*S. aureus*, enterococci, lactobacilli, bifidobacteria, *Bacteroides* and clostridia	Björkstén et al. (2001)
Culture-dependent and FISH	Feces	Characterization of the intestinal microbiota composition as a prospective treatment target in infant atopic dermatitis[a]	–[b]	Culture-dependent (enterococci, *S. aureus*, streptococci, citrobacteria, enterobacteria, *E. coli*, *Klebsiella*, *Proteus mirabilis* and *C. albicans*); FISH (bifidobacteria, *Bacteroides*, lactobacilli/enterococci and *Clostridium histolyticum*)	Kirjavainen et al. (2001)
Culture-dependent, FISH and DGGE	Feces	Assessment of fecal microbiota of humans after consumption of dried milk containing probiotic (*L. rhamnosus* DR20)[a]	*L. rhamnosus* DR20	Lactobacilli, enterococci, enterobacteria, total bacteria, anaerobic microorganisms, total aerobic bacteria, bifidobacteria, clostridia, and *Bacteroides*	Tannock et al. (2000)
Culture-dependent and 16S rRNA sequencing	Feces and stomach and intestine contents	Comparison of fecal microbiota from healthy and autistic children[a]	–[b]	*Clostridium* spp. and *Ruminococcus* spp.	Finegold et al. (2002)
Culture-dependent	Feces	Characterization of fecal probiotic *Bacillus* (*B. cereus*, *B. clausii*, *B. pumilus*)[c]	*B. cereus* IP 5832, *B. clausii* and *B. pumilus*	–[f]	Duc et al. (2004)
Culture-dependent and DGGE	Feces	Comparison of composition and stability of intestinal microbiota of healthy individuals patients with irritable bowel syndrome[a]	–[b]	Culture-dependent (*Bacteroides*, bifidobacteria, lactobacilli, total aerobes, total anaerobes, coliforms and spore-forming bacteria); DGGE (*E. rectale*, *Eubacterium ventriosum*, *Ruminococcus*, *Clostridium* sp., *Clostridium glycolicum*, *Abiotrophia adiacens*, *Granulicatella elegans*, *Bifidobacterium* sp., and *Enterobacteriaceae*)	Mättö et al. (2005)
Culture-dependent	Large intestine	Evaluation of the effects of probiotic bacterium (*L. casei* CRL 431) on immune system[c]	*L. casei* CRL 431	Anaerobes, lactobacilli, and *Enterobacteriaciae*	Palomar et al. (2013)
Culture-dependent and qPCR	Feces and colonic mucosa	Comparison of fecal and mucosa-associated microbiota of healthy individuals and patients with irritable bowel syndrome[a]	–[b]	*Bacteroides*, *Clostridium*, bifidobacteria, *E. coli*, and *Lactobacillus*	Carroll et al. (2010)
Culture-dependent and FISH	Feces	Study of the differences between the composition of fecal microbiota in celiac and healthy children[a]	–[b]	Culture-dependent (total anaerobes, *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Enterobacteriaceae*,	Collado et al. (2007)

Table 1 (continued)

Method	Source	Application	Probiotic microorganism (s) tested	Detected microbiota	References
				Staphylococcus, and yeast); FISH (total microbial counts, *Bacteroides/Prevotella*, *C. histolyticum*, *Clostridium lituseburense*, *Eubacterium rectale-Clostridium coccoides*, *Bifidobacterium*, *Lactobacillus-Enterococcus*, *E. coli*, *Atopobium*, *Coriobacterium*, and sulfate-reducing bacteria)	
Culture-dependent and DGGE	Tissue and cecum content (first section of large intestine)	Evaluation of the effects of diet supplementation with prebiotic (fructooligosaccharides) or probiotic (*B. lactis* and *S. thermophilus*) in rats[d]	*B. lactis* BL and *S. thermophilus*	Culture-dependent (anaerobes, lactobacilli, clostridia, bifidobacteria, *Bacteroides*, and coliforms); DGGE (lactobacilli and *B. animalis* and *B. lactis*)	Montesi et al. (2005)
Culture-dependent and DGGE	Intestinal content	Study of the effect of probiotic bacterium (*Pediococcus acidilactici*) on intestinal microbiota and immune status of red tilapia (*Oreochromis niloticus*)[e]	*P. acidilactici*	Total aerobic and anaerobic bacteria, LAB	Ferguson et al. (2010)
Culture-dependent and DGGE	Feces	Assessment of the probiotic potential of *L. paracasei* A added to yogurt in children[a]	*L. paracasei* A	Culture-dependent (clostridia, bifidobacteria, total aerobes, enterococci, total anaerobes, *Bacteroides*, enterobacteria, lactobacilli); DGGE (*L. amylovorus*, *L. brevis*, *L. casei/paracasei*, *L. curvatus*, *L. bulgaricus*, *L. fermentum*, *L. fuchuensis*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. ruminis*, *L. sakei*, *L. salivarius*, *L. mesenteroides*, and *S. thermophilus*)	Marzotto et al. (2006)
Culture-dependent and FISH	Feces	Evaluation of probiotic bacteria supplementation (*B. lactis animalis* subsp. Bb-12) on intestinal microbiota of premature children (gestational age of 37 weeks)[a]	*B. lactis animalis* subsp. Bb-12	Culture-dependent (*Enterobacteriaceae*, *Enterococcus* and *Streptococcus* spp., *Staphylococcus* spp., anaerobic bacteria, *C. albicans*, *Clostridia*, *Bacteroides*, *Bifidobacterium* spp., and aerobic bacteria); FISH (total bacteria, *E. rectale*, *Bacteroides* and *Prevotella*, *Bifidobacterium* spp., *Lactobacillus* and *Enterococcus* spp., *Veillonellae*, *Streptococcus* and *Lactococcus* spp., *Enterobacteriaceae*, *Staphylococcus* spp., *C. histolyticum*, and	Mohan et al. (2006)

Table 1 (continued)

Method	Source	Application	Probiotic microorganism (s) tested	Detected microbiota	References
				C. lituseburense)	
Culture-dependent and qPCR	Feces	Evaluation of a probiotic-fermented oat-based beverage (*B. longum* 46 and *B. longum* 2C) on modulation of microbiota in the elderly[a]	*B. longum* 46 and *B. longum* 2C	Culture-dependent (*Bifidobacterium*); qPCR (*B. adolescentis*, *B. animalis*/*lactis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. infantis*, and *B. longum*)	Lahtinen et al. (2009)
Culture-dependent and TRFLP	Feces	Study of the effects of probiotics (*B. lactis* Bl-04, *B. lactis* Bi-07, *L. acidophilus* NCFM, *L. paracasei* Lpc-37, and *B. bifidum* Bb-02) on antibiotic-induced gut microbiota alterations[a]	*B. lactis* Bl-04, *B. lactis* Bi-07, *L. acidophilus* NCFM, *L. paracasei* Lpc-37, and *B. bifidum* Bb-02	Culture-dependent (*Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Clostridium*, and *Enterobacteriaceae*); TRFLP (*Bifidobacterium*, *Lactobacillus*, *Enterobacteriaceae*, *Bacteroides*, and *Prevotella*)	Engelbrektson et al. (2009)
Sequencing based on 16S rRNA (Sanger sequencing) and pyrosequencing	Feces	Evaluation of ciprofloxacine on human intestinal microbiota[a]	–[b]	Bifidobacteria, *Bacteroides*, *Parabacteroides*, *Alistipes*, *Oscillospira*, *Dialister*, *Clostridium*, *Dorea*, *Faecalibacterium*, *Subdoligranulum*, *Clostridiaceae*, *Eubacterium*, *Anaerostipes*, *Coprococcus* and *Lachnospira*, *Roseburia*, *Ruminococcus*, *Lachnospiraceae*, Clostridiales, Firmicutes, *Sutterella*, and *Akkermansia*	Dethlefsen et al. (2008)
DGGE and TRFLP	Feces and intestinal tissue	Study of the impact of probiotic administration (*L. casei* and *L. plantarum*) on endogenous microbial population[c]	*L. casei* and *L. plantarum*	Lactobacilli (*Lactobacillus* spp., *L. johnsonii*, *L. murinus*, *L. reuteri*, *L. gasseri*, *L. psittaci*, *L. plantarum*, and *L. helveticus*)	Fuentes et al. (2008)
DGGE and qPCR	Feces	Evaluation of prebiotic (galactooligosaccharide), probiotic (*B. animalis* Bb-12), and potentially probiotic (*Lactobacillus amylovorus* DSM 16698) on colonic human and pig microbiota[a, g]	*B. animalis* Bb-12 and *L. amylovorus* DSM 16698	DGGE (total lactobacilli, total bifidobacteria, and total bacteria); qPCR (*L. amylovorus*, total lactobacilli, total bifidobacteria, and total bacteria)	Martínez et al. (2013)
DGGE and qPCR	Feces	Evaluation of prebiotic (lactulose), probiotic (*S. boulardii*) and the symbiotic combination on human fecal microbiota[a]	*S. boulardii*	*Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Enterococcus*	Vanhoutte et al. (2006)
DGGE and qPCR	Feces	Qualitative and quantitative evaluation of the effects of yogurt on intestinal microbiota of healthy individuals[a]	–[b]	Lactic acid bacteria (LAB), *C. coccoides*, *C. perfringens*, and *Bacteroides*	García-Albiach et al. (2008)
DGGE	Feces	Study of galactooligosaccharide and/or probiotic (*B. lactis* Bb-12) effects on qualitative composition of *B. indigena* populations in human microbiota[a]	*B. lactis* Bb-12	*Bifidobacterium (B. adolescentis*, *B. ruminantium*, *B. angulatum*, *B. infantis*, *B. adolescentis*, and *B. pseudocatenulatum)*	Satokari et al. (2001)

Table 1 (continued)

Method	Source	Application	Probiotic microorganism (s) tested	Detected microbiota	References
qPCR	Feces	Qualitative and quantitative determination of fecal microbiota in elderly and characterization of the impact of probiotic oat-based beverage on endogenous microbiota and the relation to inflammatory responses[a]	*B. longum* 2C (DSM 14579) and 46 (DSM 14583) and *B. lactis* Bb-12	*B. longum*, *B. adolescentis*, *B. bifidum*, *B. catenulatum*, *B. breve*, *B. animalis*, and *B. dentium*	Ouwehand et al. (2008)
FISH, microarray, and 454-pyrosequencing	Feces	Evaluation of the effect of probiotic bacteria (*L. reuteri* DSM 17938 and ATCC PTA 6475) on enterocytes migration and microbial diversity in the intestine[c]	*L. reuteri* DSM 17938 and ATCC PTA 6475	FISH and microarray (*L. reuteri*); 454-pyrosequencing (*L. murinus*, *Klebsiella*, *Staphylococcus*, *Parabacteroides*, *Bryantella*, *Lachnospiraceae*, *Enterobacteriaceae*, *Ruminococcaceae*, *Anaerotruncus*, and *Erysipelotrichaceae Incertae sedis*)	Preidis et al. (2012)
qPCR and 454-pyrosequencing	Feces	Study of the effects of probiotic (*B. lactis* DN-173 010) fermented milk consumption on intestinal inflammation[c]	*B. lactis* DN-173 010	qPCR (*Enterobacteriaceae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *B. lactis*, *L. lactis*, *L. bulgaricus*, and *S. thermophilus*); 454-pyrosequencing (*Bifidobacteriaceae*, *Porphyromonadacaeae*, *Prevotellaceae*, *Staphylococcaceae*, *Lachnospiraceae*, and *Lactobacillaceae*)	Veiga et al. (2010)
PCR and 454-pyrosequencing	Biopsy of mucosal tissue	Investigation of intestinal microbiota composition and characterization of the probiotic effects (mix VSL#3) in the mucosa of patients with irritable bowel syndrome[a]	*Lactobacillus* (*L. casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*), *Bifidobacterium* (*B. longum*, *B. breve*, *B. infantis*), and *S. salivarius* subsp. *thermophilus*	Firmicutes, Bacteroidetes, Synergistetes, Actinobacteria, and Cyanobacteria	Ng et al. (2013)
qPCR and FISH	Feces and biopsy of duodenal mucosa	Comparison of intestinal microbiota composition of healthy and sick individuals (irritable bowel syndrome)[a]	–[b]	qPCR (*B. adolescentis*, *B. bifidum* and *B. longum*, and *B. catenulatum*); FISH (*Faecalibacterium prausnitzii*, *C. coccoides-E. rectale* group, *Bifidobacterium*, *Lactobacillus-Enterococcus* group, *C. histolyticum* group, *Bacteroides-Prevotella* group, *C. difficile*, *C. lituseburense* group)	Kerckhoffs et al. (2009)

Table 1 (continued)

Method	Source	Application	Probiotic microorganism (s) tested	Detected microbiota	References
FISH	Feces	Investigation of the role of colonic microbiota on the development of lactose intolerance[a]	–[b]	*E. rectale*/*C. coccoides*, *Bacteroides*/*Prevotella*, bifidobacteria, and *Atopobium* group	Zhong et al. (2004)
FISH	Feces	Assessment of how the ingestion of different amounts of a probiotic (*L. johnsonii* La1) affects main bacterial populations in human fecal microbiota[a]	*L. johnsonii* La1	*E. rectale*, *Fusobacterium prausnitzii*, *Bacteroides*, *Atopobium*, *C. histolyticum*, *Bifidobacterium*, and *Lactobacillus*	Garrido et al. (2005)
TGGE and FISH	Feces	Assessment of the impacts of regular yogurt consumption on composition and metabolism of human intestinal microbiota[a]	–[b]	TGGE (*L. bulgaricus*, *L. casei*, and *L. mesenteroides*); FISH (*C. coccoides-Eubacterium rectale* group, *Clostridium leptum* subgroup, *Prevotella and Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Weissella*, *Pediococcus*, *Vagococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus* and *Lactococcus*, *Atopobium* group and enteric group)	Alvaro et al. (2007)
DGGE	Feces	Evaluation of the therapeutical effects of probiotic yogurts in patients with irritable bowel syndrome (change of microbiota and relief of irritable bowel syndrome's symptoms)[a]	*B. lactis* Bb-12, *L. casei* 431, *L. acidophilus* La-5, and *L. fermentum* ME-3	*E. coli*, *C. difficile*, *L. rhamnosus* GG, and *Bifidobacterium*	Lee et al. (2013)
DGGE	Mucosa	Assessment of the effects of probiotic supplementation (*P. acidilactici*) on intestinal microbiota and relationship with growth performance of trout (*Oncorhynchus mykiss*)[e]	*P. acidilactici*	–[f]	Ramos et al. (2013)
DGGE and RISA	Feces	Characterization of intestinal microbiota of children[a]	–[b]	*B. bifidum*, *B. adolescentis*, *B. angulatum*, *B. catenulatum*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. pseudolongum* subsp. *pseudolongum*, *B. breve*, and *B. gallicum*	Roger et al. (2010)
DGGE and RISA	Feces	Identification of bacterial colonization (*Bacteroides fragilis*, *C. leptum*, and *C. coccoides*) in patients with diseases such as cancer[a]	–[b]	*Bacteroides fragilis* group, *Clostridia leptum* subgroup, and *Clostridia coccoides* subgroup	Scanlan et al. (2008)
qPCR	Tissue (ileal mucosa)	Assessment of the impact of probiotic supplementation (*B. longum* AH1206) on health, growth, and development of neonatal pigs[g]	*B. longum* AH1206	Bifidobacteria	Herfel et al. (2013)

Table 1 (continued)

Method	Source	Application	Probiotic microorganism (s) tested	Detected microbiota	References
qPCR and TRFLP	Feces	Assessment of bacterial community profile in fecal microbiota of healthy adult human after ingestion of probiotic yogurt containing *B. lactis* Bb-12 and *L. acidophilus* LA-5[a]	*B. lactis* Bb-12 and *L. acidophilus* LA-5	qPCR (*B. adolescentis/ruminantim*, *B. bifidum*, *B. breve*, *B. catenulatum* group, *B. longum* group, *Bacteroides-Prevotella* group, *Blautia coccoides* group, *C. leptum* group, Enterobacteriaceae, *Enterococcus*, and total bacteria); TRFLP (*Clostridium*, *Lachnospiraceae*, *Faecalibacterium*, *Blautia*, *Subdoligranulum*, *Enterococcus faecalis*, *Desulfovibrio*, *Eubacterium*, *Ruminococcaceae*, *Peptostreptococcaceae*, *Alistipes*, *Bacteroides*, *Parabacteroides*, *Prevotella*, *E. coli*, and *Blautia*)	Filteau et al. (2013)
TRFLP	Feces	Characterization of human intestinal microbiota variability[a]	–[b]	Total bacteria	Li et al. (2007)
qPCR	Feces	Identification and quantification of a probiotic bacterium (*L. rhamnosus* GG) in human fecal samples[a]	*L. rhamnosus* GG	*L. rhamnosus* Lc705, *L. casei* ATCC 334, *L. zeae* ATCC 393, *L. rhamnosus* ATCC 7469, *L. rhamnosus* 1/6, *L. rhamnosus* E-97800, *L. rhamnosus* VS 1020, *L. rhamnosus* VS 1019, and *L. rhamnosus* GG	Ahlroos and Tynkkynen (2009)
Metagenomics	Feces	Comparison of intestinal microbiomas of adults and children[a]	–[b]	*Bacteroides*, *Dorea*, *Enterococcus*, *Citrobacter*, *Eubacterium*, *Streptococcus*, *Propionilbacterium*, *Raoultella*, *Bifidobacterium*, *Parabacteroides*, *Salmonella*, *Ruminococcus*, *Collinsella*, *Enterobacter*, *Clostridium*, *Escherichia*, *Shigella*, and *Klebsiella*	Kurokawa et al. (2007)
FISH and metagenomics	Feces	Investigation of fecal microbiota in patients with Crohn's disease[a]	–[b]	Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria	Manichanh et al. (2006)
Metagenomics	Feces	Analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis[a]	–[b]	*Escherichia*, *Haemophilus*, *Clostridium*, *Klebsiella*, *Serratia*, *Veillonella*, *Enterobacter*, *Enterecoccus*, *Megasphaera*, *Pseudomona*, *Staphylococcus*, *Bacteroides*, *Shigella*, and *Gemella*	Wang et al. (2009)
Metaproteomics	Feces	Identification of microbial proteins in fecal samples to gain information about genes expressed and key microbial functions in human gut[a]	–[b]	–[f]	Verberkmoes et al. (2009)

Table 1 (continued)

Method	Source	Application	Probiotic microorganism (s) tested	Detected microbiota	References
Metatranscriptomics	Feces	Functional analysis of human intestinal microbiota[a]	–[b]	*Lachnospiraceae, Ruminococcaceae, Bacteroidaceae, Prevotellaceae* and *Rickenellaceae*	Gosalbes et al. (2011)
Metabolomics	Blood	Effects of intestinal microbiota on blood metabolites[c]	–[b]	–[f]	Wikoff et al. (2009)
Microarray	Feces	Evaluation of the effect of probiotics-containing biscuit in the intestinal microbiota of the elderly[a]	*B. longum* Bar33 and *L. helveticus* Bar13	*Bacteroides-Prevotella, Clostridium* cluster, *Lactobacillaceae, Bifidobacteriaceae, Bacillaceae, Fusobacterium, Cyanobacteria,* Enterococcales, *Enterobacteriaceae,* and *Campylobacter*	Rampelli et al. (2013)
454-Pyrosequencing	Feces	Assessment of the effect of probiotic preparation in digestive health of cystic fibrosis patients[a]	*L. reuteri* DSM 17938	Fusobacteria, Bacteriodetes, Actinobacteridae, Firmicutes and Proteobacteria	Del Campo et al. (2014)
qPCR and 454-pyrosequencing	Fecal and cecal samples	Evaluation of the effect of host diet on the intestinal persistence and gene expression of *L. plantarum* WCFS1 in healthy and health-compromised[c]	*L. plantarum* WCFS1	*Lactobacillus, Ruminococcus, Lachnospiraceae, Bacteroides,* Bacteroidales, *Desulfovibrio, Desulfovibrionaceae,* and *Anaerotruncus*	Tachon et al. (2014)
qPCR and 454-pyrosequencing	Feces	Assessment of changes in the fecal microbiota of adults consuming probiotic (*L. casei* Zhang)[a]	*L. casei* Zhang	*Phascolarctobacterium, Bacteroides, Roseburia, Faecalibacterium, Lachnospiraceae, Bifidobacterium, Lactobacillus, Coprococcus, Subdoligranulum, Anaerostipes, Prevotella, Dorea,* and *Parasutterella*	Zhang et al. (2014)

Only some of the studies listed in this table were discussed in the manuscript text since it would not be feasible to handle all of them in a minireview; moreover, this was not the only purpose of the work. For further details, please go through the references provided in the table

DGGE denaturing gradient gel electrophoresis method, *FISH* fluorescent in situ hybridization method, *PCR* polymerase chain reaction, *qPCR* quantitative (real-time) polymerase chain reaction, *RISA* ribosomal intergenic spacer analysis, *TGGE* temperature gradient gel electrophoresis method, *TRFLP* terminal restriction fragment length polymorphism analysis

[a] Observed in humans

[b] In this study, the method was used only for the analysis of the intestinal microbiota (without administration of probiotics)

[c] Observed in mouse

[d] Observed in rats

[e] Observed in fish

[f] The intestinal microbiota was not fully described in the study

[g] Observed in pigs

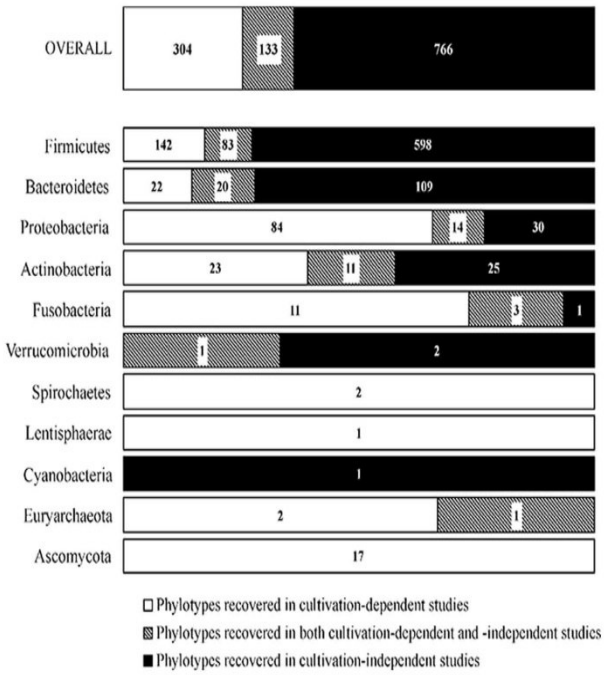

Fig. 3 Distribution of prokaryotic phylotypes in the GIT, shown overall and in terms of phylotypes (eight bacterial, one archaeal, and one eukaryal). The diversity of the gastrointestinal isolates that have been fully characterized, but lack the SSU rRNA gene sequence, was taken into account for construction of this figure (Rajilic-Stojanovic et al. 2007, with permission)

Sanger sequencing

Considering the limitations of culture-dependent methods, molecular techniques based on the analysis of the 16S ribosomal RNA (rRNA) bacterial gene, a marker of genetic diversity, have emerged. This gene was chosen because of its small size (1.5 kb) and due to the fact that it strikes an appropriate balance of conservation and variability, which enables the differentiation of species and strains but also the identification of members belonging to the same phylogenetic group (Peterson et al. 2008).

Sanger sequencing is used to analyze the microbial diversity as 16S rRNA gene sequences are resolved into operational taxonomic units (entities of taxonomic classification of group or individual species) based on their individual percent sequencing identity (%ID). Hence, specific %IDs are recognized as indicators of taxonomic resolutions, which indicate the species, genus, and family (Peterson et al. 2008). The technique has the advantages of presenting good resolution and sensitivity; however, its main limitations are the time required for its performance, low yield, high cost, and the need for an extensive data analysis (Delmont et al. 2012). The method was used by Finegold et al. (2002) to compare the intestinal microbiota in children with regressive autism and healthy ones. The authors observed that the number of clostridial species in the feces in the former group was greater than the one determined in the control group.

Pyrosequencing

Pyrosequencing is one of latest low-cost options that are replacing the need to sequence every 16S rRNA gene in its totality. The technique generates large numbers of 16S rDNA sequences by amplification of the variable regions selected on the inside of the 16S rRNA gene. Pyrosequencing is able to sequence a large amount of bases with good precision and yield (Margulies et al. 2005). In addition, the technique shows optimum sensitivity. Interestingly, pyrosequencing is able to determine the entire phylogenetic spectrum and allows taxonomic characterization and assessment of intestinal microbial populations at different taxonomic levels (Hooda et al. 2012). Several researchers have successfully used the method to evaluate the impact of probiotic consumption on the intestinal microbiota, as demonstrated by the studies published by Del Campo et al. (2014), Tachon et al. (2014), and Zhang et al. (2014).

DNA microarrays

DNA microarray technology is a powerful tool developed specifically for high-throughput screening of microbial communities. This methodology has the potential to provide information about the pathogenesis of several diseases—infectious and noninfectious ones (e.g., cancer) (Paul et al. 2007). Moreover, it exhibits an efficient cost-benefit relationship and good levels of sensitivity and selectivity and requires short time for performance of analysis (Heller 2002). Its main

drawbacks include the low detection limit and the inability to identify new species and strains (Sekirov et al. 2010). This technique was able to detect changes on the intestinal microbiota of elderly people that consumed probiotics-containing biscuits once a daily for a month (Rampelli et al. 2013). According to the authors, in individuals that ingested the probiotic, the age-related increase of the opportunistic pathogens was reverted, in comparison to individuals supplemented with placebo-containing biscuits.

Fluorescence in situ hybridization (FISH) and real-time quantitative polymerase chain reaction (qPCR)

FISH and qPCR are valuable tools to assess the intestinal microbiota. Furthermore, both techniques can be combined to confirm the results observed. Kerckhoffs et al. (2009) used FISH and qPCR techniques to evaluate the intestinal microbiota composition of healthy individuals and those diagnosed with irritable bowel syndrome. The authors observed a decrease in the bifidobacteria populations in the latter, in comparison to the levels determined in healthy subjects.

FISH uses oligonucleotide probes labeled with florescence, conceived to hybridize with unique 16S rRNA sequences present in specific microorganisms. One of the main drawbacks for FISH is the fact that only a few probes can be used per analysis (Zoetendal and Mackie 2005).

The qPCR is a precise and sensitive method for the enumeration of microorganisms in complex ecosystems. In this technique, specific or universal primers can be used and a standard curve is generated with the use of a reference strain, from which results will be derived for microorganisms' enumeration. An important limitation of both methods is seen in cases where no adequate cultivable strains are available for use as a reference strain. Furthermore, they do not allow the identification of new species since the primers used target a specific previously known bacterial taxonomic group (Prakash et al. 2011).

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)

DGGE and TGGE are basically forms of electrophoresis which use either a temperature or a chemical gradient, respectively, to denature samples as they move across an acrylamide gel. In reality, the samples analyzed by these methods correspond to DNA fragments previously extracted directly from the microbial community, without the need of pre-enrichment steps, and then amplified by the PCR method (Valásková and Baldrian 2009). According to their guanine and cytosine contents (GC), the PCR products will migrate in the acrylamide gel and a differential band pattern will be observed, which represents the sample microbial diversity. DGGE and TGGE allow a high number of samples to be screened simultaneously (McCartney 2002). The techniques can be used to analyze the whole community or specific populations or groups within a sample and have been successfully used to study the complexity and dynamics of human fecal microbiota (Alvaro et al. 2007; Lee et al. 2013). However, the main limitation of these methods is the detection of heteroduplex molecules, which can result in an overestimation of the real microbial community and, therefore, lead to inaccurate conclusions (McCartney 2002). Moreover, the PCR products are short and limited taxonomic information can be obtained. Both methods also have limited sensitivity and the reproducibility between gels is difficult (Muyzer 1999).

Terminal restriction fragment length polymorphism (TRFLP)

TRFLP is a powerful tool to evaluate the microbial diversity. TRFLP profiles are generated by the digestion of PCR-amplified 16S rRNA genes with a restriction endonuclease, originating a florescence-labeled terminal restriction fragment of variable length (Osborn et al. 2000). The fragments migrate distinctly in the gel electrophoresis, creating a specific band pattern for each sample. TRFLP is a useful quantitative technique for the evaluation of changes in the microbial populations and comparison of communities (Abdo et al. 2006). TRFLP is a fast, low-cost, and reproducible method (Smith et al. 2005); however, the TRFLP profile characterization is not easy since databases of TRF sizes may be imprecise (Kitts 2001). This technique was used by Filteau et al. (2013) to evaluate the impact of probiotic yogurt consumption on the fecal microbiota composition in healthy adults. According to the authors, no major differences were determined in the microbial profiles determined in fecal samples obtained from individuals fed with yogurt or placebo.

Ribosomal intergenic spacer analysis (RISA)

The RISA is a relatively new technique used for the analysis of the intestinal microbiota, as shown by Scanlan et al. (2008) and Roger et al. (2010). The method involves the PCR amplification of the intergenic space between the 16S and 23S rRNA genes (Feligini et al. 2015). It shows good resolution, detection limit, and reproducibility, besides being fast and inexpensive (Rastogi and Sani 2011); however, the application of the method can be limited by the absence of an available extensive database comprising the RIS taxonomy for intestinal microorganisms (Brown et al. 2005; Prakash et al. 2011). Nevertheless, the use of RIS sequences for the differentiation of microorganisms is known to be more useful than the analysis based on the 16S rRNA gene sequence. As an example, 16S rRNA gene sequences of species belonging to the genus *Bifidobacterium* can show 99 % similarity, whereas

RIS genetic sequences show greater divergence in the sequences of closely related species (Ventura et al. 2001).

Analyses focused on function—the "meta" family

Metagenomics

Metagenomics is a recent tool for the analysis of microbial communities. Indeed, it is a genomic analysis applied to all microorganisms present in a given microbial ecosystem, without prior identification. Metagenomics comprises culture-independent studies of the structures and functions of microbial communities and their interactions with the habitat (Lepage et al. 2013). The process is divided into two areas: (a) individual targets are amplified using the PCR method and the products sequenced—metagenomic study focused on a single gene; and (b) the total DNA is isolated from a sample and sequenced—random metagenomic study of all genes. The method provides a detailed survey of all genes that exist in a specific community (structure, composition, and function) in a single experiment (Gilbert and Dupont 2011). The technique provides information about the sequence of the microbiota genomes and can consequently be used to identify biological contributions and functions in this complex community, comparing healthy and ill individuals (Manichanh et al. 2006; Wang et al. 2009), as well as people of different ages (Kurokawa et al. 2007). The main disadvantage associated with the method includes the fact that the reads (one read is approximately equivalent to one gene size), mapped for reference genomes, are limited by the number of available sequenced genomes. This method depends on extensive bioinformatics analysis, and the cloning procedure is of great importance since it can directly affect the genetic information obtained (Sekirov et al. 2010).

Metatranscriptomics

Metatranscriptomics is another new genomic tool applied to the analysis of microbial communities and is based on the sequencing of nucleic acids extracted from microbial populations, as seen for metagenomics. While metagenomics deals with the evaluation of DNA sequences, metatranscriptomics involves the characterization of messenger RNA (mRNA), directly extracted from microbial populations (Gosalbes et al. 2011). Metatranscriptomics enables researches to understand how changes in the environment induce alterations in gene(s) expression in the whole community. Since RNA degradation is more likely to happen than expected for DNA, the sensitivity of metatranscriptomics depends on the number of read sequences obtained, which is the main disadvantage of the method and may result in relevant data loss (Sekirov et al. 2010). This technique can differentiate between expressed and nonexpressed genes. Thus, it focuses on the metabolically active members of a community (Su et al. 2012). Gosalbes et al. (2011) evaluated the gut microbiome and its functionality in health volunteers using metatranscriptomic analysis. According to the authors, the predominant families detected in the active microbiota included *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceae*, *Prevotellaceae*, and *Rickenellaceae* and were related to important functions such as carbohydrate metabolism, energy production, and synthesis of cellular components.

Metabolomics

Metabolomics provides an overall description of the metabolites present in a biological sample. The assessment of human metabolites can be an excellent indicator of a pathogenic process and also predict the effect of diet on human health (Russell and Duncan 2013). Wikoff et al. (2009) used metabolomics analysis and demonstrated an important relation between the intestinal microbiota and metabolite production, which were determined in plasma samples obtained from germ-free and conventional mice. According to the authors, the production of amino acid metabolites, including the antioxidant indole-3-propionic acid, and also organic acids containing phenyl groups was remarkably upregulated by the presence of gut microbes.

Metabolomics methods are considered to be faster and cheaper than the ones used for metagenomics studies. In addition, the method is very selective and sensitive (Dunn et al. 2005). However, one major limitation of metabolomics is that given the high complexity of the majority of tissues and fluids in the body, an overall view of all the metabolites is not feasible. Thus, not all metabolites are detected in heterogeneous natural environments and the presence of interfering compounds may burden the determination of their exact origin (Sekirov et al. 2010).

Metaproteomics

Although metatranscriptomics provides data about the genetic expression and activity, additional levels of cell localization and regulation occur at the protein level. Thus, the data obtained from the transcriptome and proteome can be substantially different. By the use of metaproteomics, the proteins are extracted from samples of mixed microbial populations, fractionated, separated by the use of the liquid chromatography method, and detected by the use of mass spectroscopy techniques (Langley et al. 2013). Metaproteomics has been used to study the main microbial functions in the intestine, including the diversity and abundance of the proteins in this organ, known as metaproteome. It is less expensive and faster than the metagenomic method(s) (Verberkmoes et al. 2009).

However, it is worth mentioning that the suitable protein fraction is difficult to be extracted and estimated (Su et al. 2012).

Probiotics and their mechanisms of action in the GIT

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014). Throughout the years, foods have been used as vehicles for probiotics delivery. The reasons for the successful application of foods as probiotic carriers include the following: (i) the positive health image of most foods; (ii) the fact that some foods, such as the fermented ones, contain live microorganisms, making the addition of probiotics easily acceptable by consumers; (iii) the ability of several foods to deliver probiotic microorganisms with good technological properties, which means that they are able to remain viable and physiologically active even at the end of their shelf-lives and do not negatively impact the food sensory attributes; (iv) the ability of foods to improve probiotics survival throughout the GIT; and (v) the fact that some foods provide bioactive food compounds, which can synergistically interact with probiotics and improve their benefits on the host health (Heller 2001; Da Cruz et al. 2010; Ranadheera et al. 2010; Staliano et al. 2015).

The main action mechanisms of probiotics are related to the GIT (Howarth and Wang 2013). Probiotics consumption seems to be an interesting and feasible approach to modulate the intestinal microbiota and to maintain or restore human health (FAO/WHO 2002). As previously shown (item 2), a wider knowledge on the intestinal microbiota composition and activity may facilitate the identification of microorganisms associated with different diseases and, hence, guide the development of probiotics able to precisely act in this microecosystem (Gueimonde and Collado 2012). In this sense, perturbations in the intestinal microbiota associated with intestinal disorders could be adequately prevented/treated by the use of selected probiotic strains (Bull-Otterson et al. 2013).

The main probiotic microorganisms include species belonging to the genera *Lactobacillus* and *Bifidobacterium*, although strains within few species of *Lactococcus*, *Streptococcus*, *Enterococcus*, *Saccharomyces*, *Bacillus*, *Brevibacillus*, and *Sporolactobacillus* have also been reported as probiotics (Borchers et al. 2009).

Probiotic microorganisms present certain characteristics related to their origin source, as well as regarding their physiology. These features include isolation from humans (not restricted to), resistance to a certain extent to food processing, and ability to adhere to the epithelial cells and to persist in the GIT. Furthermore, probiotics should exert benefit(s) on consumer health and not have pathogenic/virulence traits or toxic properties (Sanders et al. 2007; Nogueira and Gonçalves 2011). Since the beneficial effects of probiotic microorganisms are known to be strain-specific, they cannot be extrapolated to another strain or mixture of strains. Mixed probiotic cultures have the advantage of showing both the properties of the individual strains and the synergetic effects, thus increasing their overall efficiency. However, a study published by Almeida et al. (2008) suggested an antagonistic effect between the strains *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* in probiotic *açaí* yogurt, in which the growth of the latter was inhibited by an excessive production of hydrogen peroxide by *L. bulgaricus*. Therefore, inappropriate mixtures of probiotic strains may also result in reduced/loss health effects (Christensen et al. 2002; Gerritsen et al. 2011).

The main action mechanisms of probiotics include epithelial barrier function enhancement, improved adhesion to intestinal cells and pathogen inhibition by competition for adhesion sites, production of antimicrobial substances, and modulation of the immune system (Fig. 4) (Rijkers et al. 2010). Together, these mechanisms are able to modulate the composition of the intestinal microbiota and to prevent the growth of pathogenic bacteria as further detailed in the succeeding subsections.

Strengthening of the epithelial barrier

The main functions of the intestinal barrier are the maintenance of the epithelial integrity and protection of the host (Bermudez-Brito et al. 2012). The intestinal barrier defense mechanisms include the mucous layer, antimicrobial peptides, secretion of IgA, and the epithelial tight junctions (Ohland and Macnaughton 2010). Once the epithelial barrier is damaged, pathogenic microorganisms can lead to intestinal disorders, including inflammatory diseases (Hooper et al. 2001, 2003; Sartor 2006). Probiotics are important to maintain the integrity of this barrier and also to repair its damages (Bermudez-Brito et al. 2012). Probiotics can also increase the expression of genes that codify junction proteins and promote the mucous secretion (mucins) to improve the barrier function and to exclude pathogens (Anderson et al. 2010).

Increased adhesion to the intestinal mucosa and competitive exclusion of pathogenic microorganisms

Adhesion to the intestinal mucosa is an important property for the interaction between the probiotic microorganism and the host. Other features are also important, including the ability of probiotic microorganisms to modulate the immune system and to adhere to the intestinal mucosa (Collado et al. 2009).

Probiotics prevent the binding of pathogens to the intestinal cells by competitive exclusion for nutrients and adhesion sites in the mucosa (Collado et al. 2005; Bermudez-Brito et al. 2012; González-Rodríguez et al. 2012) and also by promoting

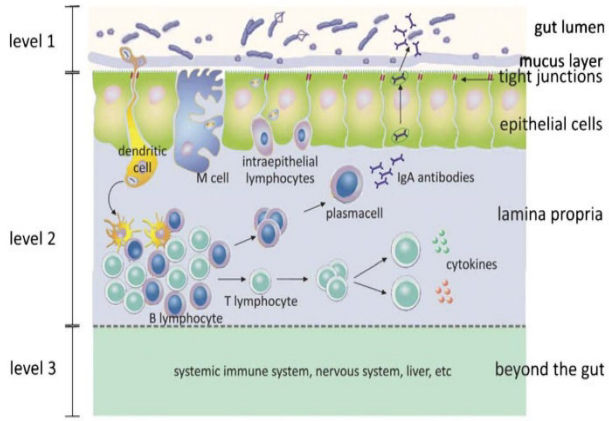

Fig. 4 Main mechanisms of probiotics action. Three levels of known or potential actions are presented. *Level 1*: Interference in the growth or survival of pathogenic microorganisms in the gut lumen; *level 2*: improvement of the mucosal barrier function and mucosal immune system; *level 3*: effect on the systemic immune system, as well as other cell and organ systems, including the liver and brain (Rijkers et al. 2010, with permission)

qualitative alterations in the intestinal mucin (Kim and Ho 2010).

Overall, probiotics are able to suppress the growth of pathogenic or potentially pathogenic microorganisms by the creation of a hostile microenvironment, elimination of available bacterial receptor sites, depletion of essential nutrients, and production/secretion of antimicrobial substances (Rolfe 1991).

Production of antimicrobial substances

Low molecular weight compounds such as organic acids and proteinaceous antimicrobial substances, known as bacteriocins, are produced by some probiotic strains (Bermudez-Brito et al. 2012). Organic acids such as acetic and lactic acid show good inhibitory effects against pathogenic microorganisms (Alakomi et al. 2000; De Keersmaecker et al. 2006; Makras et al. 2006). The bactericidal mechanism mediated by bacteriocins involves the destruction of the target cell by membrane pore formation or by the inhibition of cell wall synthesis (Nielsen et al. 2010; Hassan et al. 2012). Many lactic acid bacteria (LAB) also produce small antimicrobial proteins (AMPs) that can act against foodborne pathogens.

Probiotic bacteria are able to produce conjugated bile acids (bile salt derivatives) that have strong antimicrobial activity, and some probiotic strains produce metabolites that are able to inhibit the growth of fungi and Gram-negative bacteria (Bermudez-Brito et al. 2012).

Probiotics and the immune system

The immune system comprises the innate immune and the adaptive response. The innate system is the first line of defense and acts in a nonspecific way, which includes neutrophils, eosinophils, basophils, monocytes, dendritic cells, natural killer cells, and soluble factors. On the other hand, the adaptive system responds to antigens in a specific way and consists of T and B lymphocytes, humoral factors, and immunoglobulins. The innate and adaptive immunity work in collaboration (Tsai et al. 2012). The immune system activation may occur due to the competition for nutrients and colonization sites, antimicrobials production, and changes in the intestinal pH, among others. However, in most cases, the immune response is initiated by unknown factors (Shah 2007).

The host cells that most interact with probiotics are both the intestinal epithelial cells (IECs) and the dendrite cells (DCs) (Bermudez-Brito et al. 2012). The IECs and DCs can interact and respond to intestinal microorganisms through their pattern recognition receptors, which bind pathogen-associated molecular patterns present in the majority of pathogens, modulating the intestinal immune system (Gómez-Llorente et al. 2010; Lebeer et al. 2010). Furthermore, probiotic bacteria can also exert immune-modulating effects by interaction with monocytes/macrophages and lymphocytes (Bermudez-Brito et al. 2012).

Probiotics, intestinal microbiota, and health

The intestinal microbiota exerts an important role on human health and disease. The manipulation of these microorganisms by probiotics intake is an attractive approach, since they act by modulating the intestinal microbiota and can contribute with health maintenance and restoration (Gerritsen et al. 2011). A study performed by Sun et al. (2011) showed that *Bacillus pumilus* SE5, administered to fish as a dietary supplement, was capable to modulate the intestinal microbiota. It is believed that probiotics can act in three ways: (a) directly within the GIT, (b) interact directly with the mucous layer and the intestinal epithelium, and (c) outside the GIT, by impacting the immune system and other sites (Rijkers et al. 2010).

The mechanisms by which probiotic microorganisms are able to alter the intestinal microbiota include the reduction of the luminal pH, competition for nutrients, secretion of antimicrobial compounds (organic acids, biosurfactants, hydrogen peroxide, and bacteriocins, among others), prevention of both bacterial adhesion and invasion of the epithelial cells, and induction of antimicrobial compound production by the host (Fooks and Gibson 2002; Ng et al. 2009; Gerritsen et al. 2011). Table 2 lists some of the beneficial effects of probiotics in the GIT and at other sites; below, we further characterize these potential uses.

Inflammatory bowel diseases

Inflammatory bowel diseases include ulcerative colitis and Crohn's disease. Crohn's disease is characterized by an unequal inflammation that can affect any part of the GIT, whereas ulcerative colitis is a chronic inflammatory condition which only involves the large intestine (Prakash et al. 2011). Im et al. (2009) showed that *Bacillus polyfermenticus*, administered to rats, reduced the mortality rates, the seriousness of the colitis (according to weight loss, diarrhea, and mucosal damage occurrence), and the expression of inflammatory molecules (e.g., tumor necrosis factor alpha). The authors also reported that by the probiotic administration, apoptosis was overcome both in vivo and in vitro and a proliferation of epithelial cells was observed. Although not fully elucidated, the possible mechanisms involved could include the secretion of components that inhibit NF-κB activation and IL-8 secretion and induction of large amounts of IL-10 and low levels of IL-12 (Miquel et al. 2013).

The irritable bowel syndrome

The irritable bowel syndrome is characterized by abdominal pain, swelling, and alterations in the intestinal habits in the absence of any abnormality of the mucous. The disease is associated with abnormal intestinal communities, but their importance in the pathogenesis of the syndrome is not clear (Prakash et al. 2011). According to Dolin (2009) and Hun (2009), capsules containing *Bacillus coagulans* strains GBI-30 and 6086 were administered to patients and improved the symptoms of the irritable bowel syndrome, such as swelling and abdominal pain. A study published by Nobaek et al. (2000) reported that patients diagnosed with the irritable bowel disease that received, for 4 weeks, a supplement containing *Lactobacillus plantarum* DSM 9843a showed alleviation of pain and less flatulence. Malted milk containing *Bifidobacterium infantis* 35624 also alleviated the symptoms of the irritable bowel syndrome when administered to subjects. This response was associated with a normalization of the levels of anti-inflammatory and proinflammatory cytokines, suggesting an immune-modulating role for the microorganism (O'Mahony et al. 2005). A supplement containing *Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium animalis* ssp. *lactis* Bb-12 was able to stabilize the intestinal microbiota and reduce the symptoms of the irritable bowel syndrome in patients, reducing the distension and abdominal pains (Kajander et al. 2008). Finally, a treatment using probiotic mix VSL#3 (*Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *B. infantis*, and *Streptococcus salivarius* subsp. *thermophilus*) during 4 weeks in patients with irritable bowel syndrome improved the symptoms of the disease. The authors associated the positive outcome with the modulation of the gut microbiota, as seen by the reduction of microorganisms' population belonging to the genus *Bacteroides* (Ng et al. 2013).

Lactose intolerance

Lactose is a sugar found in milk, which can be broken into glucose and galactose by the action of the lactase enzyme, mainly produced by children and some adults. However, some humans stop producing the enzyme in infancy, and if these individuals consume dairy products containing lactose, they can develop gastrointestinal symptoms such as abdominal distension, pain, flatulence, and diarrhea (Sanders et al. 2007). The use of probiotics to alleviate the symptoms of lactose intolerance is common due to the improvement in lactose digestion associated with the microorganisms (Maathuis et al. 2010). A study performed by He et al. (2008) suggested that the consumption of yogurt enriched with *B. animalis* for 2 weeks modified the composition of the intestinal microbiota, which could had been responsible, at some extent, for the alleviation of lactose intolerance symptoms in intolerant subjects. The consumption of a probiotic product containing *L. casei* Shirota and *B. breve* Yakult improved the lactose digestibility in patients with lactose intolerance. This positive observation could be related to an increase in the microbiota β-galactosidase activity and changes in the gut microbiota composition (Almeida et al. 2012).

Metabolic diseases

Obesity is a complex syndrome that develops from a prolonged disequilibrium in the energetic balance between caloric ingestion and expenditure (Guinane and Cotter 2013). Environmental and genetic factors influence obesity, and intestinal dysbiosis can also contribute to its development, since bacteria present in the intestinal microbiota influence nutrient uptake and energy regulation (Tilg et al. 2009; Prakash et al. 2011). Lines of evidence have shown that changes in the balance of the intestinal microbiota are associated with the development of metabolic diseases, including obesity, diabetes, hypercholesterolemia, and high blood pressure (Ebel et al. 2014).

Table 2 Selected beneficial effects of probiotics

Microorganism/product	Observed effect	References
B. polyfermenticus	Improvement of colon inflammation (mechanism of suppression of apoptosis and proliferation and migration in epithelial cells)	Im et al. (2009)
B. angulatum DSM 20098	Decrease in cholesterol levels	Al-Saleh et al. (2006)
B. infantis DSM 20088	Decrease in cholesterol levels	Al-Saleh et al. (2006)
Fermented cereal containing *L. casei* NCDC-19	Decrease in total cholesterol levels	Sindhu and Khetarpaul (2003)
Ganeden BC^{30} (*B. coagulans* GBI-30, 6086)	Reduction of evacuation in patients with irritable bowel syndrome	Dolin (2009)
		Hun (2009)
	Improvement of abdominal pain and swelling (symptoms of irritable bowel syndrome)	Maathuis et al. (2010)
	Relief of lactose intolerance symptoms	Kimmel et al. (2010)
	Improvement of immune system	Jensen et al. (2010)
	Anti-inflammatory effects and immune modulation	
Yogurt containing *B. animalis* DN-173010	Relief of lactose intolerance symptoms	He et al. (2008)
Probiotic product (*L. casei* Shirota and *B. breve* Yakult)	Improvement of lactose digestibility in patients with lactose intolerance	Almeida et al. (2012)
Yogurt containing *L. acidophilus* and *B. lactis*	Decrease in cholesterol levels	Ataie-Jafari et al. (2009)
L. reuteri	Reduction of gingivitis and dental plaque	Krasse et al. (2005)
L. rhamnosus JB-1	Reduction of symptoms of anxiety and depression	Bravo et al. (2011)
Fermented milk containing *L. casei* and *L. plantarum*	Reduction of symptoms and severity of infection caused by *E. coli*	Mirzaei et al. (2012)
Fermented milk containing *L. helveticus* LBK 16H	Reduction of blood pressure	Seppo et al. (2003)
Fermented milk containing *L. helveticus*		Jauhiainen et al. (2005)
Fermented milk containing *L. helveticus* IDCC3801	Prevention and relief of Alzheimer disease and other memory dysfunctions	Yeon et al.(2010)
Fermented milk containing *L. paracasei*-33	Improvement of allergic rhinitis symptoms	Wang et al. (2004)
Malted milk containing *B. infantis* 35624	Relief in symptoms of irritable bowel syndrome and immunomodulatory effects	O'Mahony et al. (2005)
L. reuteri	Improvement of periodontitis symptoms	Teughels et al. (2013)
Malt containing *L. mesenteroides* and *B. subtilis natto* RG4365	Improvement of *V. cholerae* inhibition by combination of two probiotic strains	Vidyalaxme et al. (2012)
Cheese containing *B. bifidum* A12, *L. acidophilus* A9, and *L. paracasei* A13	Immunomodulatory effects in intestine	Medici et al. (2004)
L. rhamnosus GG	Influence in the innate immunity and reduction in the proinflammatory cytokines expression, tumor necrosis factor-α and IL-6, consequently reduction in the inflammatory response	Amit-Romach et al. (2010)
B. breve M-16 V	Attenuation of allergic symptoms in allergic asthma-induced mice	Hougee et al. (2010)
L. plantarum 06CC2	Relief of influenza symptoms in mice	Takeda et al. (2011)
L. brevis CD2	Inhibition of periodental inflammation	Maekawa and Hajishengallis (2014)
S. thermophilus DSM 20617	Decrease in cholesterol levels	Al-Saleh et al. (2006)
Supplement containing *B. lactis*, *L. acidophilus*, and *L. rhamnosus*	Reduction of glucose levels in blood	Al-Salami et al. (2008)
Supplement containing *E. faecium* RM11 and *L. fermentum* RM28	Reduction in risk of colorectal cancer	Thirabunyanon et al. (2009)
Lacidofil probiotic (*L. rhamnosus* R0011 and *L. acidophilus* R0052)	Improvement of the irritable bowel symptoms, mental health, and cancer-related fatigue in colorectal cancer patients	Lee et al. (2013)
B. adolescentis SPM0212	Inhibition of the proliferation of human colon cancer cell and fecal enzymes	Kim et al. (2008)
Supplement containing *L. acidophilus* 4356	Decrease in cholesterol levels	Huang et al. (2010)
Supplement containing *L. casei* Shirota	Reduction of anxiety symptoms	Rao et al. (2009)
Supplement containing *L. rhamnosus GG*	Antidiabetic effect	Tabuchi et al. (2003)

Table 2 (continued)

Microorganism/product	Observed effect	References
Supplement containing *L. helveticus* R0052 and *B. longum* R0175	Mental well-being (decrease in levels of stress)	Messaoudi et al. (2011)
Supplement containing *L. plantarum* DSM 9843	Pain relief and reduction in flatulence (irritable bowel syndrome)	Nobaek et al. (2000)
Supplement containing *L. rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *B. animalis* subsp. *lactis* Bb-12	Stabilization of intestinal microbiota and reduction in symptoms of irritable bowel syndrome	Kajander et al. (2008)
Supplement containing *B. polyfermenticus* SCD	Modulation of physiological functions (lipid and antioxidant profiles in hypercholesterolemia)	Paik et al. (2005)
Supplement containing *B. pumilus* SE5	Modulation of intestinal microbiota	Sun et al. (2011)
Probiotic mix VSL#3 (*L. casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *B. longum*, *B. breve*, *B. infantis*, and *S. salivarius* subsp. *thermophilus*)	Modulation of intestinal microbiota in patients with irritable bowel syndrome	Ng et al. (2013)

The consumption of probiotics has shown positive effects on different disorders such as diabetes, hypercholesterolemia, and high blood pressure. A study published by Al-Salami et al. (2008) showed that the administration of probiotics (*L. acidophilus*, *B. lactis*, and *L. rhamnosus*) reduced the glucose levels in the blood of diabetic rats, thus exerting a hypoglycemic effect. According to Tabuchi et al. (2003), a supplement containing *L. rhamnosus* GG was administered to rats and also presented an antidiabetic effect, which could be attributed to the prevention of the decrease in insulin secretion. When used as a supplement, *B. polyfermenticus* SCD showed significant health benefits by modulating the physiological functions, including the lipid and antioxidant profiles in hypercholesterolemic rats (Paik et al. 2005). Ataie-Jafari et al. (2009) demonstrated that the ingestion of two strains of probiotic bacteria (*L. acidophilus* and *B. lactis*) for 6 weeks was associated with a cholesterol-reducing effect in hypercholesterolemic individuals due to the inhibition of cholesterol absorption or its assimilation/capture by the bacteria cell membrane. Furthermore, the administration of a supplement containing *L. acidophilus* 4356 to rats for 4 weeks reduced the cholesterol levels (Huang et al. 2010). Sindhu and Khetarpaul (2003) reported that the consumption of a fermented cereal added with probiotics (*L. casei* NCDC-19 and *Saccharomyces boulardii*) for 42 days was capable of reducing the total cholesterol levels in mice. Finally, the ingestion of fermented milk containing probiotic *Lactobacillus helveticus* was shown to reduce the blood pressure of both hypertensive rats (Jauhiainen et al. 2005) and individuals (Seppo et al. 2003) and, therefore, could be useful in the treatment of the condition.

Allergic diseases

Atopic diseases are caused by exaggerated or nonequilibrated immunological responses to environmental and inoffensive antigens (allergens) (Sanders et al. 2007). Allergic diseases can be initiated and maintained by environmental factors associated with a change in the intestinal microbiota (Prakash et al. 2011). A study carried out by Wang et al. (2004) showed that the symptoms of patients suffering from allergic rhinitis were alleviated by the consumption of a fermented milk added with *Lactobacillus paracasei*-33, through changes in the composition of the gut microbiota. According to Kirjavainen et al. (2003), a supplement containing *L. rhamnosus* GG showed potential for the treatment of atopic eczema and allergy to cow's milk in children.

Mental diseases

Probiotic bacteria have an important role in bidirectional communication of the intestine-brain axis and can be used as therapeutic adjuncts in stress-related disorders, such as anxiety and depression. When administered to mice, *L. rhamnosus* JB-1 reduced anxiety and depression symptoms via modulation of the intestinal microbiota due to the bidirectional communication between the brain and the gut (Bravo et al. 2011). Similarly, Rao et al. (2009) reported a reduction of anxiety symptoms in patients who used for 2 months a supplement containing *L. casei* Shirota. Moreover, fermented milk added with *L. helveticus* IDCC3801 prevented and alleviated Alzheimer's disease and other memory dysfunctions in mice via reduction of accumulation of neurotoxic peptides, which are involved in the development of the disease (Yeon et al. 2010). Moreover, the administration of a supplement containing *L. helveticus* R0052 and *B. longum* R0175 promoted mental well-being to patients, including a decrease in stress levels, which was associated with the stress state of the individual (Messaoudi et al. 2011).

Periodontal diseases

Gingivitis is an inflammatory reaction caused by the accumulation of bacteria in the gingival gums. Once inflammation and degradation of the collagen increase, the process can lead to periodontitis (Yanine et al. 2013). Some studies have demonstrated the effectiveness of probiotics in the improvement of oral health. *Lactobacillus reuteri* was shown to be able to reduce gingivitis and bacterial plaque in subjects with moderate and severe gingivitis (Krasse et al. 2005). Probiotics lozenges containing *L. reuteri* were shown to be useful in the treatment of patients diagnosed with periodontitis (Teughels et al. 2013). According to Maekawa and Hajishengallis (2014), the topical treatment with *L. brevis* CD2 inhibited the periodontal inflammation in mice through modulatory effects on the periodontal microbiota. Bhardwaj and Bhardwaj (2012) hypothesized that probiotics may improve the signals/symptoms of the disease by controlling the growth of periodontal pathogens.

Bacterial infections

The susceptibility to enteric infections increases with the disrupture of the commensal microbiota equilibrium (Prakash et al. 2011). The consumption of a fermented milk containing *L. plantarum* and *L. casei* led to a decrease in both stool recovery and intestinal colonization rates of *Escherichia coli* O157:H7 in rats and, consequently, minimized the duration and severity of the infection (Mirzaei et al. 2012). According to VidyaLaxme et al. (2012), a food product containing ragi malt and probiotics *Bacillus subtilis* natto RG4365 and *Leuconostoc mesenteroides* inhibited the planktonic growth of *Vibrio cholerae* and affected its ability of biofilm formation and adherence to extracellular matrix proteins. The authors also observed increased amounts of beneficial fatty acids such as linoleic and linolenic acids and higher mineral contents (iron and zinc) when both microorganisms were added to the functional food product, in comparison to the amounts determined when *B. subtilis* natto RG4365 and *L. mesenteroides* were tested alone.

Colorectal cancer

Colorectal cancer is mainly a disease of developed countries with a Western culture, and adenocarcinoma is considered the most common type of the disease (Prakash et al. 2011). Studies have shown an association among the intestinal microbiota, colorectal cancer, and the administration of probiotics (Kim et al. 2008; Levy et al. 2014). *B. polyfermenticus* SCD showed anticarcinogenic effect in rats, which was explained by its strong adherent properties in the intestinal mucosa and also by the inhibition of the growth of human colon cancer cells (Lee et al. 2007). According to Thirabunyanon et al. (2009), in vitro tests demonstrated that *Enterococcus faecium* RM11 and *L. fermentum* RM28 were able to reduce the proliferation of colon cancer cells through the reduction of the viability and induction of the apoptosis of colon cancer cells due to an increase of the adherence of the probiotics to the intestinal epithelial cells. The administration of probiotic Lacidofil containing *L. rhamnosus* R0011 and *L. acidophilus* R0052, for 12 weeks, improved cancer-related fatigue in colorectal cancer patients (Lee et al. 2014). The authors correlated the positive outcome with the probiotics action mechanisms, which could have included the restoration of the gut flora and their anti-inflammatory properties.

Modulation of the immune system

Autoimmune diseases occur when the body's immune system attacks and destroys healthy tissues, as in the case of type 1 diabetes (Boerner and Sarvetnick 2011), celiac disease (de Sousa Moraes et al. 2014), inflammatory bowel diseases (Kostic et al. 2014), and allergic asthma (Bach 2002). Several studies have shown that probiotics can act by modulating the immune system. Capsules containing *B. coagulans* strains GBI-30 and 6086, administered to patients, led to an increase in the levels of immunological markers including cytokines (IL-6, IL-8, and IFN-γ) and CD3CD69 cells (Kimmel et al. 2010). According to Amit-Romach et al. (2010), the administration of *L. rhamnosus* GG affected the innate immunity in the intestine of colitis-induced rats through the increase of the mucin expression, which is important in the inhibition of the adherence of pathogenic bacteria in the gut. Furthermore, the probiotic treatment reduced the expression of proinflammatory cytokines, tumor necrosis factor-α, and IL-6 and, consequently, reduced the inflammatory response. According to Hougee et al. (2010), the treatment using *B. breve* M-16 V in allergic asthma-induced mice attenuated the allergic symptoms due to the reduction of the lung inflammation and the IgE and IgI levels. Moreover, a study carried out by Takeda et al. (2011) showed that heat-killed *L. plantarum* 06CC2 alleviated influenza symptoms in mice by an increase of natural killer cell activity associated with the enhancement of interferon-α and Th1 cytokine production. Furthermore, Medici et al. (2004) demonstrated that the consumption of cheese containing *B. bifidum*, *L. acidophilus*, and *L. paracasei* exerted an immune-modulating effect on mice intestines via interaction of the probiotics with the immune cells of the gut. Finally, Martinez et al. (2009) demonstrated that cell-free supernatants from probiotic strains *L. reuteri* RC-14 and *L. rhamnosus* GR-1 were able to upregulate the secretion of cytokines (IL-8 and IP-10) by vaginal epithelial cells infected with *Candida albicans*. The authors concluded that this mechanism could possibly play an important role to help clear out vulvovaginal candidiasis in vivo.

Conclusions

Until now, although traditional and molecular methods are available for the characterization of the intestinal microbiota, a full understanding of its composition and diversity and how changes in this microecosystem cause or are associated with the development of diseases seems to be beyond our grasp. Thus, the performance of more in vitro and in vivo studies that analyze the diversity, function, and action mechanisms of GIT microorganisms and also elucidate how probiotics can positively affect/interact with the intestinal microbiota is essential for the development of new strategies to prevent/manage several relevant pathologic conditions.

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Resistance characterization of eight probiotic *Bacillus* strains in different food matrices to eight unit operations applied in food industry

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Abstract

This study aims to quantify the resistance of several strains of probiotic *Bacillus* (PB) (*B. flexus* Hk1, *B. subtilis* Bn1, *B. licheniformis* Me1, *B. mojavensis* KJS3, *B. subtilis* PXN21, *B. subtilis* PB6, *B. coagulans* lactospore and *B. coagulans* GBI-30, 6086) inoculated in different food matrices to common unit operations used in the food industry. The unit operations/food matrices studied were pasteurization (milk and fruit juice), cooking (meat product), baking (bread), drying (fruit), acidification/fermentation (yogurt), supercritical carbon dioxide (fruit juice), irradiation (powder pepper) and extrusion (pasta and snacks). The survival/resistence of PB in each food matrix was determined through enumeration using selective culture media. The PB strains that survived to various unit operations were subjected to proteomic analysis to compare how they respond, at protein level, to the environment stresses. According to the results, *B. coagulans* BC30 was able to survive different unit operations studied. (pasteurization-milk, cooking, baking, drying, acidification/fermentation and supercritical dioxide carbon), with decimal reductions of up to 1 $\log_{10}$. On the other hand, *B. subtilis* PXN21 and *B. subtilis* PB6 were the most negatively affected. For these strains, more than 3 $\log_{10}$ decimal reductions were observed when submitted to the pasteurization (juice) or baking processes. As for irradiation, the effect was dose-dependent, ranging from strain to strain, while *B. flexus* Hk1, *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. subtilis* PB6 and *B. subtilis*

PXN21 were the less affected PB strains in powder pepper. Even though PB strains showed resistance to all unit operations studied in different food matrices, the behavior was found to be strain- unit operation-dependent.
Keywords: *Bacillus*; probiotic food; resistence; spore; technological process.

1. Introduction

The probiotics have been definided as "*live microorganisms that when administered in sufficient amounts, confer a health benefit on the host*" (Hill et al., 2014). *Lactobacillus* and *Bifidobacterium* are the main genera used as probiotics (Boyle and Tang, 2006); however, some species of spore forming bacteria have also been used. For instance, spore forming bacteria strains with probiotic properties such as *Bacillus coagulans*, *Bacillus subtilis, Bacillus licheniformis*, *Bacillus flexus*, *Bacillus mojavensis*, *Bacillus polyfermenticus* and *Bacillus pumilis* have been reported in the literature (Cutting, 2011; Hung et al., 2012; Kim et al., 2011; Nithya and Halami, 2013; Paik et al., 2005). Probiotic *Bacillus* (PB) strains are known to survive better to gastrointestinal conditions, as well as present greater resistance to technological food processes, than *Lactobacillus* and *Bifidobacterium* due to their ability to form spores (Hong et al., 2005; Nithya and Halami, 2013).

The probiotic microorganisms have been added to various foods and beverages such as fermented milk (Desrouillères et al., 2015), yogurt (Ranadheera et al., 2012), cheese (Abadia-Garcia et al., 2013), ice cream (Cruz et al., 2009), juices (Céspedes et al., 2013), cereals (Sindhu and Khetarpaul, 2003) and chocolate (Foong et al., 2013) among others. However, the importance of the food matrix and of the probiotic strain on the biological effects observed must considered when selecting a probiotic strain for use (Champagne et al., 2005; Ranadheera et al., 2010). For instance, He et al. (2008) showed that the ingestion of yogurt containing *Bifidobacterium animalis* DN-173010 modified the composition of the intestinal microbiota and relieved lactose intolerance symptoms (He et al., 2008). Juice added of probiotics (*Lactobacillus acidophilus* LA5 or *Lactobacillus casei* 01) modulated the intestinal microbiota, increasing bifidobacteria and decreasing pathogenic bacteria (*in vitro*) (Chaikham et al., 2012). The cholesterol level was reduced with the consumption of probiotic yogurt (*Lactobacillus acidophilus* e *Bifidobacterium lactis*) (Ataie-Jafari et al., 2009), while the severity of pathogen infection minimized with the ingestion of probiotic fermented milk

containing *Lactobacillus casei* and *Lactobacillus plantarum* (Mirzaei et al., 2012). Fermented milk containing the probiotic strains *Lactobacillus paracasei*-33 and *Lactobacillus helveticus* IDCC3801 prevented and relieved Alzheimer disease and other memory dysfunctions (Yeon et al., 2010). The ingestion of probiotic cheese containing *Bifidobacterium bifidum* A12, *Lactobacillus acidophilus* A9, and *Lactobacillus paracasei* A13 led to immunomodulatory effects in intestine (Medici et al., 2004) and attenuation in the development of hypertension was related with the administration of probiotic cheese (*Lactobacillus acidophilus* LA 14 and *Bifidobacterium longum* BL 05) (Lollo et al., 2015).

In order to result in health benefits to the hosts, a food substrate must transport, deliver and regulate colonization of microorganisms in the gastrointestinal tract (Kailasapathy and Chin, 2000; Ranadheera et al., 2010). Nonetheless, for delivery of probiotic in the gastrointestinal tract, the physico-chemical and nutritional properties and interactions between probiotics and components of food carriers should be take into account as they comprise fundamental factors for the survival and efficacy of probiotics (Kailasapathy and Chin, 2000; Ranadheera et al., 2010).

For the successful application of probiotic microorganisms in a food matrix, the viability and functionality of that microorganism must be maintained at all steps of food processing and during gastric transit (Sanz, 2007). Even though are several studies report on the effects of unitary operations on probiotic lactic acid bacteria (Ergin et al., 2016; Leone et al., 2017; Sidira et al., 2014; Tsevdou and Taoukis, 2011; Wirunpan et al., 2016), the fate of spore forming bacteria to unit operations applied in food processing has been scantly documented (Fares et al., 2015; Jao et al., 2011). Therefore, this study aimed to quantify the impact of different unit operations on eight PB strains inoculated in probiotic food matrices. The probiotic spore forming bacteria studied belongs to *Bacillus* genus (*B. flexus* Hk1, *B. subtilis* Bn1, *B. licheniformis* Me1, *B. mojavensis* KJS3, *B. subtilis* PXN21, *B. subtilis*PB6, *B. coagulans* lactospore and *B. coagulans* GBI-30, 6086), while unit operations and food matrixes chosen were pasteurization (milk and fruit juice), cooking (meat product), baking (bread), drying (fruit), acidification/fermentation (yogurt), supercritical carbon dioxide (fruit juice), irradiation (powder pepper) and extrusion (pasta and snacks). Furthermore, the PB strains that survived to various unit operations were selected for proteomic analysis to compare how they respond, at protein level, to the environment stresses.

2. Materials and Methods

2.1. PB Strains

Bacillus subtilis PXN21, *Bacillus subtilis* PB6, *Bacillus coagulans* lactospore, *Bacillus coagulans* GBI-30, 6086, *Bacillus mojavensis* KJS3, *Bacillus licheniformis* Me1, *Bacillus flexus* Hk1 and *Bacillus subtilis* Bn1 were the PB strains used in this study. *Bacillus subtilis* PB6 (Kemin, Sao Paulo, Brazil), *Bacillus subtilis* PXN21 (Probiotics International Ltd, Lopen Head, UK), *Bacillus coagulans* lactospore (Sabinsa, East Windsor, New Jersey, USA) and *Bacillus coagulans* GBI-30, 6086 or *Bacillus coagulans* BC30 (Ganeden Biotech Inc, Mayfield Heights, Ohio, USA) were obtained as powder spores. On the other hand *Bacillus mojavensis* KJS3 (Department of Pharmacy, Kyungsung University, Korea), *Bacillus licheniformis* Me1, *B. flexus* Hk1 and *B. subtilis* Bn1 (CSIR-Central Food Technological Research Institute, India) were donated by researchers as slants and spores suspensions were prepared for use in this study (section 2.2).

2.2. Preparation of spore suspensions

The suspensions of spores were prepared following methodoly of Pflug (1999). *B. licheniformis* Me1, *B. flexus* Hk1 and *B. subtilis* Bn1 strains were stored at −18°C in Luria–Bertani (LB) broth medium containing 20% (v/v) glycerol and *B. mojavensis* KJS-3 strain was stored in tryptic soy broth medium containing 20% (v/v) glycerol. The *B. licheniformis* Me1, *B. flexus* Hk1 and *B. subtilis* Bn1 strains were initially cultivated in Luria–Bertani (LB) broth (g/L: tryptone (10g), yeast extract powder (5g) and NaCl (10g)) and *B. mojavensis* KJS-3 strain in tryptone soya broth (TSB) for 24 h in 150 rpm/min at 37°C incubator shaker Series 25 (Edison New Jersey, USA). Then the grown cultures were separately transferred to uninoculated broths, following overnight incubation a shaker at 37°C and 150 rpm/min. This procedure was repeated twice to increase the concentration of cells and to allow cell's syncronization. Sporulation of *B. licheniformis* Me1, *B. flexus* Hk1 and *B. subtilis* Bn1 were performed in Difco sporulation media (DSM) (g/L: bacto nutrient broth (Difco) (g/L: beef extract (3g) and peptone (5g); KCl 10% (10mL); $MgSO_4.7H_2O$ 1.2% (10mL); NaOH 1M (1,5mL); $Ca(NO_3)_2$ 1M (1mL); $MnCl_2$ 0.01M (1mL); $FeSO_4$ 1mM (1mL) and agar (15g). The sporulation of *B. mojavensis* KJS3 was performed in spore medium (g/L: bacto nutrient broth (Difco) (g/L: beef extract (3g) and peptone (5g); $MgSO_4.7H_2O$ (0.51g); KCl (0.97g);

$CaCl_2.2H_2O$ (0.2g); $MnSO_4.H_2O$ (0.003g); $FeSO_4.7H_2O$ (0.00055g); skim milk 2% (20g) and agar (15g)). Sporulation in solid media were performed in Roux bottles containing 200 ml of medium inoculated with 2 ml of culture especific. Roux bottles were incubated in kiln Fanem, model 315 SE (Sao Paulo,Brazil) at 37°C and the production of spores by each strain was checked frequently. When more than 90% of spores were detected (incubation time of 30 days for *B. licheniformis* Me1, *B. flexus* Hk1 and *B. subtilis* Bn1 and 15 days for *B. mojavensis* KJS3) the cells were harvested as previously described (Peña et al., 2014; Spinelli et al., 2009). Further, centrifugation at 3500 x g for 20 min at 4°C (*B. licheniformis* Me1, *B. flexus* Hk1 and *B. subtilis* Bn1) or 1500 x g for 20 min at 4°C (*B. mojavensis* KJS3) was done in a refrigerated centrifuge Sorvall Instruments Du Pont, model RC5C (Newtown, CO, USA). The pellets obtained were washed three times with sterile distilled water. The washing and centrifugation processes were repeated twice and then, a 2% lysozyme solution was added to the spore suspensions to eliminate vegetative cells (*B. mojavensis* KJS-3, *B. licheniformis* Me1, *B. flexus* Hk1 and *B. subtilis* Bn1). Then, the suspensions of spores were washed and centrifugated twice (5555 x g for 10 min at 4°C and a centrifugation at 7150 x g for 10 min at 4°C, respectively) (Kim and Naylor, 1966).

Once pellets of the strains were obtained, drying was done as described by Weifen, et al., (2012) with modifications. The pellet with spores of the three PB strains were collected and mixed with maltodextrin (1w:1w), following drying at 105°C until water activity values of 0.3-0.4 were obtained. This range was used considering the water activity values of the commercial PB preparations (section 2.1).The spore counts in the suspensions were 5.25×10^{10} CFU/g (*B. mojavensis* KJS3), 2.84×10^{9} CFU/g (*B. licheniformis* Me1), 1.63×10^{10} CFU/g (*B. flexus* Hk1) and 3.57×10^{11} CFU/g (*B. subtilis* Bn1). The commercial PB strains were provided with counts of 8×10^{10} CFU/g (*Bacillus subtilis* PB6), 1.5×10^{11} CFU/g (*Bacillus subtilis* PXN21), 6×10^{9} CFU/g (*Bacillus coagulans* lactospore) and 1×10^{9} CFU/g (*Bacillus coagulans*BC30). All reagents of this stage were from Dinamica quimica (Diadema, SP, Brazil), with the exception of tryptone, yeast extract powder, tryptone soya broth, beef extract, peptone and agar that were from kasvi (Curitiba, PR, Brazil), lysozyme (Sigma-Aldrich, Sao Paulo, Brazil) and skim milk (Nestle, Brazil).

2.3. Resistance/Survival of spores of PB strains to different unit operations

The spores of each of the eight PB strains were challenged in different matrices to specific unit operations: pasteurization (milk and fruit juice), cooking (meat product), baking

(bread), drying (fruit), acidification/fermentation (yogurt), supercritical carbon dioxide (fruit juice), irradiation (powder pepper) and extrusion (pasta and snacks). The processing conditions and formulations followed procedures previously described in the literature. The initial inoculum level was standardized in 10^9 per portion of food. Two independent experiments were made for each process and control foods (absence of probiotic strain) were done in all the processes. Samples (1mL or 1g) were collected before and after of process and the PB strains were enumerated (plate counting) using appropriate culture media and thermal shock, as indicated by suppliers of the strains (section 2.4).

2.3.1. Pasteurization

Cow milk and orange juice (11°brix, prepared from commercial concentrated pulp) added of probiotics *Bacillus* (*B. subtilis* PXN21, *B. subtilis* PB6, *B. coagulans* lactospore, *B. coagulans* BC30, *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. flexus* Hk1 and *B.subtilis* Bn1) (portion of 200 mL) were pasteurized in water bath Quimis, model 0334M-28 (Diadema, SP, Brazil). The pasteurization conditions for cow milk and orange juice were 65°C per 30 minutes and 95°C per 30 seconds, respectively and subsequent cooling to room temperature (Gunasekera et al., 2002; Yeom et al., 2000). The temperature was monitored through the datalog (with thermocouple) Fluke, model Hydra series II (EUA). The initial inoculum level was standardized in 10^9 per 200 mL portion of milk or juice.

2.3.2 Cooking

Meat product (meatballs) added of probiotics *Bacillus* were produced. The process consisted of mixture of bovine meat (100%) with salt (1.5%), pepper (0.1%), corn starch (4%) and ice water (10%). All the ingredients were mixed in container, followed by the addition of each PB strain separately. Then, the meatballs were hand-modeled (portion of 25g), following cooking in cooker Tron (Sao Paulo, Brazil) (72°C at the cold spot of the the product) (Modzelewska-Kapituła, 2012). The temperature was monitored through the datalog (with

thermocouple) Fluke, model Hydra series II (EUA). The initial inoculum level was standardized in 10^9 per 25g portion of meatballs.

2.3.3 Baking

Breads added of probiotics *Bacillus* were prepared according to Schmiele et al., (2012), with modifications. The formulation consisted of wheat flour (100%), biological yeast (6%), water (62%), sugar (6%), gluten (1%), salt (2%), fat (4%) and sodium propionate (0.5%). The process stages were: sponge preparation (mixture of sugar, biological yeast and water), fermentation in kiln Marconi, model MA 032 (Piracicaba, SP, Brazil) at 37°C for 1 hour, mixture of other ingredients (wheat flour, gluten, salt and sodium propionate) and PB strains, adding of sponge in the probiotic mixed pasta, rounding (portion of 25g), fermentation in kiln Marconi, model MA 032 (Piracicaba, SP, Brazil) at 42°C, 1 hour and baking in oven Tron (Sao Paulo, Brazil) (180°C, 20 minutes). The temperature was monitored through the datalog (with thermocouple) Fluke, model Hydra series II (EUA). The initial inoculum level was standardized in 10^9 per 25g portion of bread.

2.3.4 Drying

The resistance of PB to drying was studied through production of crystallized pineapples. The process involved osmotic pre-treatment and drying (Nicoleti et al., 2007). The stages were pineapple slicing (portion of 25g), bleaching (100°C, 5 minutes), osmotic dehydration (42°C, 2 hours, 1:4, pineapple: sugar syrup 45° brix), sieving, crystallization (adding of probiotics *Bacillus* spores and drying in kiln Fanem, model 315 SE (Sao Paulo,Brazil) at 65°C until to reach water activity around 0.65-0.75). The water activity was monitored through the activity analyzer, Aqualab digitaL 4TEV (Decagon, Pullman, USA). The initial inoculum level was standardized in 10^9 per 25g portion of crystallized pineapples.

2.3.5. Acidification/fermentation

The resistance of PB to acidification/fermentation was studied through yogurt production (Tamime and Robinsons, 1999). The commercial sterilized milk was standardized with skim powdered milk (total solids 13%), heat-treated (90°C/5 minutes) in water bath Quimis, model 0334M-28 (Diadema, SP, Brazil), cooled to 42°C, added 2.5% (v/v) traditional lactic culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus,*CHR-Hansen, Brazil) and 1% (v/v) PB strain. Then, the product (portion of 180g) was fermentated at 45°C in kiln Marconi, model MA 032 (Piracicaba, SP, Brazil) until pH 4.6 (5h) and cooled down to 10°C. The pH was monitored through the potentiometers (pH electrode), Akso AK103 model pHmetrer (Rio Grande do Sul, Brazil). The initial inoculum level was standardized in 10^9 per 180g portion of yogurt.

2.3.6. Supercritical carbon dioxide

The orange juices (11°Brix, pH=3.6) were prepared from commercial concentrated pulp (portion of 20 mL), added of probiotics *Bacillus* and subjected to the supercritical CO_2 process (10 MPa, 36°C, 10 minutes) in experimental homemade apparatus (FEA/Unicamp, Campinas, SP, Brazil) (Gasperi et al., 2009; Santos et al., 2016). The initial inoculum level was standardized in 10^9 per 200 mL portion of juice.

2.3.7. Irradiation

Powder peppers were mixed with each PB strain (portion of 5g) and packaged in stomacher bags were irradied in different conditions (3.0, 5.0 and 7.0 KGy) (Oh et al., 2003). The irradiation process was realized in a multi-purpose cobalt-60 irradiator (CTR/IPEN, São Paulo, Brazil).

2.3.8. Extrusion

Snack and pasta were produced to estimate the resistance of each PB strain to thermoplastic and conventional extrusion, respectively. The thermoplastic extrusion was realized at different temperature conditions (100, 115, 130 and 150°C) at constant humidity (16%) (Ding et al., 2006). The process consisted in the mixture of corn starch and water in recipient, followed by addition of PB, extrusion (100, 115, 130 or 150°C) in single screw extruder Brabender, model 20D/N-GNF1014/2 (Duisburg, Germany) and drying in kiln Tecnal, model TE-394/2 (Piracicaba, Brazil) (80°C, 1 hour). The conventional extrusion was done following methodology by Jaekel et al. (2015) with modifications. The flour and water were mixed at pastaia 2 model (Tatui, Brazil), but only one PB strain was tested (*B. coagulans* GBI-30, 6086). Then, the dough was allow to rest for 5 min at pastaia 2 model (Tatui, Brazil). Extrusion was one using a pastaia 2 model (Tatui, Brazil), spaghetti type format using a matrix with 32 openings of 1.7 mm diameter, following drying in Kiln Tecnal, model TE-394/2 (Piracicaba, Brazil) at 60°C for 2 hours and 70°C for 1 hour until humidity around 10-13% was reached. The humidity was monitored through the humidity meter Marte, model ID200 (Sao Paulo, Brazil). The initial inoculum level was standardized in 10^9 per 30 g portion of snack or 80 g portion of spaghetti. Samples (1g) were collected before, after and during all stages of each process. PB were enumerated using appropriate culture media and thermal shock, as indicated by supplier of the strain (section 2.4).

2.4. Determination of bacterial survival

The culture media and incubation conditions for each BP strain were provided by the suppliers of the strains (table 1). All reagents to prepare the medium were from Dinamica quimica (Diadema, SP, Brazil), with the exception of tryptone, peptone, yeast extract powder, tryptone soya broth, tryptic soy agar, agar that were from kasvi (Curitiba, PR, Brazil) and MYP agar (Merck, Darmstadt, Germany).

Table 1

Culture media and incubation conditions for each BP strain.

PB	Culture media	Incubation conditions
B. flexus Hk1, *B.subtilis* Bn1 and *B. licheniformis* Me1	LB agar media (g/L: tryptone (10g), yeast extract (5g), NaCl (10g) and agar (15g)) (Nithya and Halami, 2013)	37°C/24h
B. mojavensis KJS3	Tryptic soy agar (TSA) (g/L: 40g) (Kim et al., 2011)	37°C/24h
B. subtilis PB6	TSAYE media (g/L: tryptic soy ag ar (40g) and yeast extract (6g)) (Teo and Tan, 2005)	30°C/16h
B. subtilis PXN21	MYP agar media (g/L: MYP agar (47.75g), egg emulsion (55.5 mL) and B polymyxin (1.2 mL)) (Mossel et al., 1967)	37°C/24h and 20°C/24h
B. coagulans lactospore	GYEA agar media (g/L: yeast extract powder (5g), peptone (5g), glucose (5g), potassium phosphate dibasic (0.5g), potassium phosphate monobasic (0.5g), magnesium sulfate (0.3g), manganese sulphate (0.01g), zinc sulfate (1 mL), copper sulphate penta (1 mL), cobalt sulfate hepta (1 mL), NaCl (0.01g) and agar (15g)) (Majeed et al., 2016)	37°C/72h
B. coagulans BC30	BC agar media (g/L: yeast extract powder (5g), peptone (5g), glucose (5g), potassium phosphate dibasic (0.5g), potassium phosphate monobasic	42°C/48h

(0.5g), magnesium sulfate
(0.3g), trace mineral
solution (1mL))

The selective enumeration of food matrix probiotic were realized without and with thermal shock (thermal process at 80°C/10 min and cooling). Samples were collected before and after of each process and during all stages. Probiotics *Bacillus* were enumerated using appropriate culture media and incubation conditions, as indicated by suppliers of the strains.The counts were reported as log10 colony forming units (CFU)/ml or g of sample. The number of survivors for each unit operation and food matrix was calculated based on the difference between the initial inoculum (N_0) and final count (N_f) after each experiment.

2.5. Proteomic analysis

2.5.1. Extraction and purification of total protein

The PB strains (*B. subtilis* PXN21, *B. subtilis* PB6, *B. coagulans* lactospore and *B. coagulans* GBI-30, 6086) were mixed with extraction solution ((urea 7M, thiourea 2M, CHAPS 4% (All from GE Healthcare, Uppsala, Sweden) and protease inhibitor (Complete ™ Protease InhibitorCocktail, Roche, Mannheim, Germany)), lysed (5 cycles of 1 minute in vortex) and centrifuged (1000 × g, 4°C, 5 minutes) in centrifuge (Eppendorf 5810 R, Hamburg, Germany). The supernatants (extracted proteins) were collected and purified (Wessel and Flugge, 1984). The protein samples were added methanol (Synth, Diadema, Brazil), vortexed and centrifuged (9000 × g, 10 seconds). Then, chloroform (Synth, Diadema, Brazil) was mixed to samples, vortexed and centrifuged again (9000 × g, 10 seconds). For separation, water was added to samples, vortexed and centrifuged (9000 × g, 1 minute). The upper phase was discarded. Then, further methanol was added to samples, vortexed and centrifuged again (9000 × g, 2 minutes). The supernatants were removed and the protein pellets were dried at room temperature. The purified proteins were solubilized in extraction solution described above and quantified using the 2-D Quant Kit (GE Healthcare, Uppsala, Sweden). Then, the protein solutions were stored in a freezer at -70°C until use.

2.5.1.1.Two-dimensional SDS-Page

For two-dimensional SDS-Page uses a methodology described by Barros et al. (2010). As for isoelectric focusing, the protein concentration was adjusted in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, IPG buffer 3-10 NL (GE Healthcare, Uppsala, Sweden) 0.5% (v/v), 40 mM dithiothreitol (DTT, Sigma, St. Louis, USA), 0.002% bromophenol blue (Sigma, St. Louis, USA) and protease inhibitor (Roche, Mannheim, Germany)), applied to 13 cm strips (3-11 NL, GE Healthcare, Uppsala, Sweden) and rehydrated for 16-18 h. Then, the focusing was performed on IPGphor 3 (GE Healthcare, Uppsala, Sweden) equipment, according to the following protocol: 500 V (stepandhold) up to 0.5 KVh, 1000 V (gradient) to 0.8 KVh, 8000 V (gradient) to 11, 3 KVh and 8000 V (stepand hold) until a total of 25 KVh was reached.After focusing, the strips were equilibrated in solution containing Tris-HCl pH (8.875 mM), 6 M urea, 30% glycerol, 2% SDS and 65 mM DTT, for 15 minutes. The same step was repeated again, however by replacing DTT with 135 mM iodoacetamide (Sigma, St. Louis, USA). Polyacrylamide gel electrophoresis (SDS-PAGE) was performed on SE 600 Ruby (GE Healthcare, Uppsala, Sweden) equipment, using gels in 12.5% (GE Healthcare, Uppsala, Sweden) Tris-glycine-SDS buffer (25 mM Tris, 192 mM glycine and 1% SDS) (Sigma, St. Louis, USA). Initially, the proteins were fixed in 50% (v/v) methanol solution and 10% acetic acid (Sigma, St. Louis, USA), for 1 hour. The solution was decanted and replaced with 5% (v/v) methanol and the gels were incubated for 15 min. Again, this solution was decanted and the gels were washed with deionized water three times, 5 min each wash. Then, the gels were exposed to 0.02% (w/v) sodium thiosulfate solution (Sigma, St. Louis, USA) for 3 min. Again, the gels were washed with deionized water, but only for 30 seconds per wash. The gels were then incubated in 0.2% (w/v) silver nitrate solution (Merck, Darmstadt, Germany) for 23 min. The gels were again washed with deionized water for 30 seconds per wash. Finally, the development was done in a solution containing 3% (w/v) sodium carbonate (Sigma, St. Louis, USA)), 2% (v/v) of 0.02% (w/v) sodium thiosulfate solution and 0.05% (v/v) of 37% formaldehyde (Sigma, St. Louis, USA). The developing reaction was stopped by adding 1.4% (w/v) EDTA for 10 min. The gels were washed one last time with deionized water to remove EDTA. The procedure of staining the gels with silver nitrate was based on the protocol "Short silver nitrate staining" described in Chevallet et al. (2006). Then, the gels were scanned using the ImageScanner III (GE Healthcare, Uppsala, Sweden) and Labscan 6.0 (GE Healthcare, Uppsala, Sweden) software.

2.6. Statical analysis

The results were expressed as means ± standard deviation and analyzed by ANOVA, followed by the t test and Scott-Knott using the Sisvar software 5.6 (Lavras, MG, Brazil) and differences were considered statistically significant when $p < 0.05$. Two replicates were made for each process.

3. Results and discussion

Currently, the pursuit of health, wellness and healthy eating has led the food industry and researchers to study and develop functional foods such as probiotics (Martins, 2013). Researchs that addresses the survival of probiotic microorganisms in foods is of fundamental importance for the development of new probiotic food (Kailasapathy, 2006). In this way, this study evaluated the survival of PB in foods to various unit operations applied in food processing (Tables 2 to 4).

3.1. Pasteurization

The survival of PB in the pasteurization process was analyzed in milk and orange juice (Table 2). The eight probiotic PB strains in milk and juice showed decimal reductions (gamma) ranging from 0.00 to 0.51 log CFU/g and 1.19 to 3.02 log CFU/g without thermal shock, respectively. While, with thermal shock (CH) ranged from 0.03 to 0.64 log CFU/g and 0.43 to 2.50 log CFU/g, respectively, there was no significant difference ($p<0.05$) in survival both in milk and juice when compared gamma without thermal shock and with thermal shock (CH) in all strains, respectively, individually. When comparing the gamma of strains in milk and juice between to each other, both without thermal shock and with thermal shock (CH) there was no significant difference ($p<0.05$) between them. The resistence of nine strains of *Lactobacillus paracasei* (4R4, 61H4, 61R3, 7R1, 8I2, 8R2, 162M6, 171R4 and 171R7) in buffer and UHT whole milk at the pasteurization process (73°C, 15 s) was reported. None of the nine *L. paracasei* strains survived after pasteurization in buffer. When grown in milk,

seven of the nine *L. paracasei* strains (61H4, 61R3, 7R1, 8I2, 8R2, 171R4 and 171R7) survived, but at low numbers. The milk components may protect the bacterial cells from the heat injury (Christiansen et al., 2006). Study of Zheng et al. (2014) showed that heat treatment (95°C/1 min) in litchi juice took the lactic acid bacteria to levels not detectable. However, different of the lactic acid bacteria, all eight *Bacillus* strains showed considerable resistance to both the neutral pH of the milk (~6.6-6.8) and the acid pH of the juice (~3.6) in our study. There was no significant difference in the decimal reductions (gamma) when compared to each other.

3.2. Cooking

As for the cooking process, the eight probiotic PB strains in meatballs showed decimal reductions (gamma) ranging from 0.33 to 1.15 log CFU/g without thermal shock and with thermal shock (CH) ranged from 0.36 to 2.00 log CFU/g, there was no significant difference ($p<0.05$) in survival when compared gamma without thermal shock and with thermal shock (CH) in all strains, respectively, individually. When comparing the gamma of strains in meatballs between to each other, both without thermal shock and with thermal shock (CH) there was no significant difference ($p<0.05$) between them (Table 2). Pérez-Chabela et al. (2013) observed that lactic acid bacteria strains (*Aerococcus viridans* UAM21, *Enterococcus faecium* UAM10a, *Lactobacillus plantarum* UAM17, and *Pediococcus pentosaceus* UAM11) encapsulated with acacia gum and inoculated in cooked meat batters were able of survival the cooking process (72°C, 20 min) with reduction of 1 log (values about 9 log CFU/g). Therefore, in general, the *Bacillus* strains showed good resistence at the cooking process in our study, without the need for microencapsulation.

3.3. Baking

The resistence of *Bacillus* to the baking process was analyzed in bread (Table 2). The eight probiotic PB strains in bread showed decimal reductions (gamma) ranging from 0.65 to

1.78 log CFU/g without thermal shock and with thermal shock (CH) ranged from 0.00 to 3.23 log CFU/g. The resistence of strains (gamma) did not differ significantly from one another rafter the process with thermal shock (CH). As for gamma without thermal shock, there are two groups in descending order of higher gamma (lower resistance) with significant difference ($p<0.05$): (*B. subtilis* PB6, *B. subtilis* PXN21, *B. coagulans* lactospore and *B. flexus* Hk1) > (*B. subtilis* Bn1, *B. coagulans* BC30, *B. licheniformis* Me1 and *B. mojavensis*KJS3). When compared gamma without thermal shock and with thermal shock (CH) there was a significant difference ($p<0.05$) in the strains of *B. subtilis* Bn1, *B. subtilis* PB6 and *B. coagulans* lactospore, respectively. The others strains did not differ significantly when compared gamma without thermal shock and with thermal shock (CH). Zhang et al., 2014 reported the survival of *Bifidobacterium lactis* BB12 to the baking process at various temperatures (165, 185, 205°C) for 12 minutes. Viable counts of *Bif. lactis* BB12 declined significantly during baking. The initial count was 10^6 CFU/g, in the first 6 minutes it has declined to 10^3 CFU/g. At the end of 12 minutes still had survival, but very low. Additionaly, the survival rate of bacteria was affected by heating temperature and heating time. Thus, in general, in our study the most *Bacillus* strains showed good resistance to the baking process. The viability of *B. coagulans* GBI-30, 6086 (*B. coagulans* BC30) in baking process of eight different baking products (chrysanthemum cookies, egg pastry cakes, mooncakes, muffins, polo breads, soda cookies, sponge cakes, and toasts) and the viability during the storage at 4 or 25°C for 15 and 6 days, respectively, were reported (Jao et al., 2011).The values of strain counts in the eight baking products were less than their raw doughafter of baking process and storage. The results showed that the strain viability of baking products decreased with storage days, both 4 and 25°C. However, the probiotic strain survived in significant amounts to the baking process and storage.

3.4. Drying

The table 2 represents the survival of *Bacillus* in crystallized pineapple to the drying process. The resistence of strains did not differ significantly when compared gamma without thermal shock and with thermal shock (CH). The survival of all strains did not differ significantly from one another when compared gamma without thermal shock. However, the gamma with thermal shock (CH) of *B. subtilis* PB6 differed significantly ($p<0.05$) from the

other strains with thermal shock (CH), when compared to each other. The eight probiotic PB strains in crystallized pineapple showed decimal reductions (gamma) ranging from 0.15 to 0.70 log CFU/g without thermal shock and with thermal shock (CH) ranged from 0.10 to 1.84 log CFU/g. Barbosa et al. (2015) showed the survival of *Lactobacillus plantarum* 299v, a commercial probiotic (Probis Probiotika) and *Pediococcus acidilactici* HA-6111-2 in orange powders obtained by at drying process (spray, freeze and convective hot air drying - 40°C/48h) and subsequent storage. There was no decrease in the cell number in the spray and freeze, but a reduction of ~2 log cycles in convective hot air drying during storage at ambient temperature and no significant differences during storage at 4 °C. Wirunpan et al. (2016) reported that it is possible to prepare shrimp feed pellet with high viable cell numbers of probiotic bacteria. *Lactobacillus lactis* 1464 strain incorporated in shrimp feed pellets were dried using at 50, 60, 70 and 80°C with addition of protectants (milk powder and monosodium glutamate). The viability of the strain during drying varied depending on the drying temperature, culture pH and type of protectants. The resistance of *Lactobacillus casei* NRRL B-442 to the drying process by spray drying in orange juice with maltodextrin or gum arabic as drying agents was observed. The strain was able to survive the process in significant counts (Alves et al., 2016). Therefore, the *Bacillus* strains in our study presented good resistence the drying process, without the need for microencapsulation or addition of protective agents.

3.5. Acidification/fermentation

As for the acidification/fermentation process, the resistence of *Bacillus* in yogurt was demonstrated in the table 2. The survival of strains did not differ significantly when compared gamma without thermal shock and with thermal shock (CH), respectively. The resistence of strains did not differ significantly when compared the gamma the strains each other on the process of acidification/fermentation without thermal shock. However, gamma differed significantly from one another on the process of acidification/fermentation with thermal shock (CH), respectively. In descending order of higher gamma (lower resistance), there are two groups with significant difference ($p<0.05$): (*B. subtilis* PXN21 and *B. coagulans* BC30) > (*B. coagulans* lactospore, *B. mojavensis* KJS3, *B. subtilis*Bn1, *B. licheniformis*Me1, *B. subtilis* PB6 and *B. flexus* Hk1). Corroboring with our study, Rutella et al. (2016) tested the

survivability in yogurt fermentation and cold storage of *L. casei* PRA205 and *Lactobacillus rhamnosus* PRA331 strains. Both the strains survived the fermentation and cold storage (viable counts $>10^8$ CFU/g), higher than the minimum therapeutic threshold (10^6 CFU/g). However, the *L. casei* PRA205 strain showed higher survivability during refrigerated storage for 28 days. Kailasapathy (2006) reported that *L. acidophilus* and *Bif. lactis* in yogurt was able to survival at fermentation both free and calcium-induced alginate–starch encapsulated. The survival was increased of 2 and 1 log cell numbers of *L. acidophilus* and *Bif. lactis*, respectively due to protection of cells by microencapsulation. In this way, all analyzed *Bacillus* strains showed good survival to the acidification/fermentation process in our study showing decimal reductions (gamma) ranging from 0.04 to 1.23 log CFU/g without thermal shock and with thermal shock (CH) ranged from 0.09 to 1.08 log CFU/g.

3.6. Supercritical carbon dioxide

The resistence of *Bacillus* to the supercritical CO_2 process also was analyzed in juice (Table 2). The eight probiotic PB strains in juice showed decimal reductions (gamma) ranging from 0.14 to 0.92 log CFU/g without thermal shock and with thermal shock (CH) ranged from 0.01 to 0.67 log CFU/g. The resistence of all strains did not differ significantly when compared gamma without thermal shock and with thermal shock (CH), respectively. The survival of all strains did not differ significantly from one another on the process of supercritical CO_2 without thermal shock. However, on the process of supercritical CO_2 with thermal shock (CH), the gamma of strain *B. licheniformis* Me1 differed significantly ($p<0.05$) from the other strains on process of supercritical CO_2 with thermal shock (CH). The others strains did not differ significantly when compared to each other on process supercritical carbon dioxide with thermal shock (CH), respectively. Spilimbergo and Ciola (2010) checked that the total inativation of *Saccharomyces cerevisiae* strain (10^5 CFU/mL) was obtained after 15 min of treatment, 10 MPa and 35°C, for both peach and kiwi juices. A range of Gram-negative and Gram-positive bacteria, yeasts and spores were treated with pressurized CO_2 at 10.5 MPa and 35 °C during 20 min. The bacterial susceptibility at the treatment followed the sequence Gram-negative≈Gram-positive>yeasts>spores (Garcia-Gonzalez et al., 2009). Bae et al. (2009) reported lethal effect of supercritical carbon dioxide (temperature: 65, 70°C, pressure: 80, 100, 120 bar, time: 10–40 min) on *Alicyclobacillus acidoterrestris* spores (10^6–

10^7 spores/ml) suspended in apple juice. *A. acidoterrestris* spores were completely inactivated by supercritical carbon dioxide process to undetectable levels in above 65°C, 100 bar for 40 min and 70°C, 80 bar for 30 min. In this way, spores are highly resistent at the supercritical CO_2 process. Thus, corroboring with our study that showed high resistance of *Bacillus* strains at the supercritical CO_2 process.

Table 2

Survival of *Bacillus* in foods and drinks to the technological processes.

Samples	**Without thermal shock No (log CFU/mL ou g)**	**With thermal shock (CH) No (log CFU/mL ou g)**	**Without thermal shock γ (log CFU/mL ou g)**	**With thermal shock (CH) γ (log CFU/mL ou g)**
Pasteurization (Milk)				
B. flexus Hk1	6.77	6.65	0.12±0.03 ^{aA}	0.15±0.03 ^{aA}
B. subtilis Bn1	6.57	6.37	0.20±0.10 ^{aA}	0.03±0.00 ^{aA}
B. mojavensis KJS3	6.94	6.85	0.08±0.00 ^{aA}	0.14±0.07 ^{aA}
B. licheniformis Me1	6.48	6.24	0.25±0.09 ^{aA}	0.05±0.00 ^{aA}
B. subtilis PB6	7.06	6.90	0.32±0.02 ^{aA}	0.50±0.08 ^{aA}
B. subtilis PXN21	6.62	6.63	0.00±0.00 ^{aA}	0.21.±0.07^{aA}
B. coagulans lactospore	6.94	6.84	0.10±0.00 ^{aA}	0.33±0.16 ^{aA}

B. coagulans BC30	7.25	6.75	0.51±0.12 aA	0.64±0.15 aA
Pasteurization (Juice)				
B. flexus Hk1	6.60	5.20	1.40±0.00 aA	1.04±0.39 aA
B. subtilis Bn1	6.38	4.45	1.94±0.40 aA	0.48±0.06 aA
B. mojavensis KJS3	6.84	4.46	2.38±0.21 aA	0.83±0.06 aA
B. licheniformis Me1	6.26	4.74	1.52±0.44 aA	0.43±0.40 aA
B. subtilis PB6	6.69	3.67	3.02±0.13 aA	1.09±0.46 bA
B. subtilis PXN21	6.53	4.40	2.13±0.11 aA	1.48±0.11 aA
B. coagulans lactospore	6.75	5.56	1.19±0.27 aA	1.64±0.40 aA
B. coagulans BC30	6.75	4.45	2.30±0.08 aA	2.50±0.41 aA
Cooking				
B. flexus Hk1	7.41	6.52	0.89±0.02 aA	1.46±0.49 aA
B. subtilis Bn1	7.09	6.30	0.79±0.17 aA	1.92±0.09 aA
B. mojavensis KJS3	7.34	6.57	0.78±0.26 aA	0.94±0.21 aA
B. licheniformis Me1	7.63	6.48	1.15±0.41 aA	1.61±0.59 aA
B. subtilis PB6	7.54	6.57	0.96±0.03	1.28±0.04 aA

			aA	
B. subtilis PXN21	7.34	6.37	0.97±0.14 aA	2.00.±0.32 aA
B. coagulans lactospore	7.69	7.25	0.44±0.18 aA	1.23±0.49 aA
B. coagulans BC30	7.63	7.30	0.33±0.17 aA	0.36±0.11 aA
Baking				
B. flexus Hk1	7.10	5.68	1.42±0.23 aB	1.43±0.34 aA
B. subtilis Bn1	7.17	6.13	1.04±0.08 aA	0.00±0.29 bA
B. mojavensis KJS3	7.44	6.79	0.65±0.15 aA	1.51±0.08 aA
B. licheniformis Me1	7.45	6.79	0.67±0.03 aA	1.51±0.39 aA
B. subtilis PB6	7.32	5.55	1.78±0.02 aB	3.23±0.37 bA
B. subtilis PXN21	7.27	5.52	1.76±0.31 aB	2.17±0.57 aA
B. coagulans lactospore	8.13	6.49	1.64±0.18 aB	0.69±0.13 bA
B. coagulans BC30	7.38	6.36	1.02±0.38 aA	1.06±0.15 aA
Drying				
B. flexus Hk1	7.10	6.51	0.15±0.06 aA	0.12±0.22 aA
B. subtilis Bn1	7.14	6.72	0.42±0.13 aA	0.66±0.03 aA

B. mojavensis KJS3	7.12	7.09	0.09±0.00 ^{aA}	0.33±0.07 ^{aA}
B. licheniformis Me1	7.24	7.16	0.31±0.02 ^{aA}	0.23±0.00 ^{aA}
B. subtilis PB6	7.16	6.46	0.70±0.16 ^{aA}	1.84±0.17 ^{aB}
B. subtilis PXN21	7.08	6.78	0.30±0.02 ^{aA}	0.10±0.02 ^{aA}
B. coagulans lactospore	7.48	6.36	1.12±0.16 ^{aA}	2.26±0.30 ^{aB}
B. coagulans BC30	7.34	7.33	0.00±0.00 ^{aA}	0.51±0.09 ^{aA}
Acidification/Fermentation				
B. flexus Hk1	6.64	6.54	0.10±0.03 ^{aA}	0.09±0.00 ^{aA}
B. subtilis Bn1	6.47	5.95	0.52±0.18 ^{aA}	0.27±0.02 ^{aA}
B. mojavensis KJS3	6.86	6.57	0.30±0.06 ^{aA}	0.45±0.00 ^{aA}
B. licheniformis Me1	6.85	6.44	0.41±0.13 ^{aA}	0.19±0.04 ^{aA}
B. subtilis PB6	6.86	6.70	0.16±0.02 ^{aA}	0.19±0.02 ^{aA}
B. subtilis PXN21	6.74	5.51	1.23±0.25 ^{aA}	1.08±0.51 ^{aB}
B. coagulans lactospore	7.01	6.24	0.77±0.32 ^{aA}	0.59±0.29 ^{aA}
B. coagulans BC30	6.98	6.94	0.04±0.00 ^{aA}	0.97±0.02 ^{bB}
Supercritical carbon				

dioxide				
B. flexus Hk1	6.82	6.68	0.14±0.06 aA	0.12±0.04 aA
B. subtilis Bn1	6.02	5.10	0.92±0.04 aA	0.33±0.07 aA
B. mojavensis KJS3	6.89	6.81	0.16±0.00 aA	0.26±0.00 aA
B. licheniformis Me1	6.91	6.53	0.39±0.37 aA	0.67±0.22 aB
B. subtilis PB6	6.53	6.58	0.25±0.07 aA	0.29±0.03 aA
B. subtilis PXN21	6.49	6.20	0.29±0.09 aA	0.09±0.00 aA
B. coagulans lactospore	6.60	6.56	0.22±0.06 aA	0.01±0.00 aA
B. coagulans BC30	6.89	6.49	0.41±0.37 aA	0.07±0.00 aA

– (CH) Samples that were subjected to thermal shock; (No) Initial count; (Nf) Final count; (γ=No-Nf); Means ± standard deviation of two replicates; Means with different superscript lowercase letters in the same line indicate significant difference between samples without thermal shock and samples with thermal shock (CH), respectively, by Test-t ($p<0.05$). Means with different superscript capital letters on the same column indicate significant difference between strains, by Scott-Knott ($p<0.05$).

3.7. Irradiation

The survival of *Bacillus* in powder pepper to the irradiation process ranged from strain to strain (Table 3). The resistance of the *Bacillus* strains was decreased according to the increase of irradiation dose. Thus, the response of the strain to the irradiation process was dose dependent. The eight probiotic PB strains in powder pepper showed decimal reductions (gamma) ranging from 0.83 to 5.23 log CFU/g without thermal shock and with thermal shock (CH) ranged from 0.57 to 4.93 log CFU/g. The resistence of all strains did not differ significantly on the irradiation process without thermal shock in doses of 3, 5 and 7 KGy, when compared the gamma of different doses (3, 5 and 7 KGy) of the same strain,

respectively. As for the gamma on irradiation process with thermal shock (CH), only the survival of strains *B. subtilis* Bn1 and *B. coagulans* BC30 reduced significantly ($p<0.05$) in doses of 3, 5 and 7 KGy. The survival of others strains did not differ significantly on the irradiation process with thermal shock (CH), when compared different doses of the same strain. However, there are significance difference ($p<0.05$) on the gamma of the strains of *Bacillus* on the irradiation process without thermal shock and with thermal shock (CH) between samples of different strains in the same dose. In descending order of higher gamma (lower resistance) the doses without thermal shock: 3 KGy (*B. coagulans* BC30 > *B. coagulans* lactospore and *B. subtilis* Bn1 > *B. subtilis* PB6 > *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. flexus* Hk1 and *B. subtilis* PXN21); 5 KGy (*B. coagulans* BC30 > *B. subtilis* Bn1 > *B. coagulans* lactospore > *B. licheniformis* Me1, *B. subtilis* PB6 and *B. flexus* Hk1 > *B. mojavensis* KJS3 and *B. subtilis* PXN21); 7 KGy (*B. coagulans* BC30 and *B. subtilis* Bn1 > *B. coagulans* lactospore > *B. licheniformis* Me1, *B. subtilis* PXN21, *B. flexus* Hk1, *B. subtilis* PB6 and *B. mojavensis* KJS3). With thermal shock (CH): 3 KGy (*B. coagulans* BC30, *B. subtilis* Bn1 and *B. coagulans* lactospore > *B. licheniformis* Me1, *B. mojavensis* KJS3, *B. subtilis* PB6, *B. flexus* Hk1 and *B. subtilis* PXN21); 5 KGy (*B. coagulans* BC30, *B. coagulans* lactospore and *B. subtilis* Bn1 > *B. licheniformis* Me1, *B. mojavensis* KJS3, *B. subtilis* PB6, *B. flexus* Hk1 and *B. subtilis* PXN21); 7 KGy (*B. coagulans* BC30 > *B. coagulans* lactospore and *B. subtilis* Bn1 > *B. licheniformis* Me1, *B. subtilis* PXN21, *B. subtilis* PB6, *B. mojavensis* KJS3 and *B. flexus* Hk1). The resistence of all strains did not differ significantly when compared gamma without thermal shock and gamma with thermal shock (CH), respectively. In this way, the strains *B. Flexus* Hk1, *B. mojavensis*, *B. licheniformis* Me1, *B. subtilis* PB6 and *B. subtilis* PXN21 were the less affected PB strain in powder pepper and *B. subtilis* Bn1 and *B. coagulans* BC30 were the most affected. Oh et al. (2003) showed that the irradiation was able of decrease the initial viable microorganisms (10^6-10^5) in various spices (pepper powder, garlic powder, onion powder and ginger powder) to the level of 10^3 (reduction of 2-3 log cycles), doses of 3 KGy or more were effective.

Table 3

Survival of *Bacillus* in powder pepper to the irradiation process.

Samples	Dose (KGy)	Without thermal shock γ (log CFU/g)	With thermal shock (CH) γ (log CFU/g)
B. flexus Hk1	3	0.93± 0.09 ^{aA*}	0.87±0.24 ^{aA*}
	5	1.69± 0.09 ^{aB*}	1.73±0.22 ^{aA*}
	7	1.98± 0.04 ^{aA*}	1.92±0.25 ^{aA*}
B. subtilis Bn1	3	2.43±0.02 ^{aC*}	3.41±0.14 ^{aB*}
	5	4.00± 0.01 ^{aD*}	3.90±0.00 ^{bB*}
	7	5.01± 0.03 ^{aC*}	4.01±0.09 ^{bB*}
B. mojavensis KJS3	3	1.18± 0.01 ^{aA*}	1.46±0.10 ^{aA*}
	5	1.38± 0.10 ^{aA*}	2.18±0.46 ^{aA*}
	7	1.96± 0.28 ^{aA*}	2.48±0.29 ^{aA*}
B. licheniformis Me1	3	1.13±0.34 ^{aA*}	2.04±0.70 ^{aA*}
	5	2.03±0.38 ^{aB*}	2.82±0.56 ^{aA*}
	7	2.51±0.18 ^{aA*}	3.16±0.15 ^{aA*}
B. subtilis PB6	3	1.63±0.22 ^{aB*}	1.11±0.12 ^{aA*}
	5	1.86±0.32 ^{aB*}	1.97±0.51 ^{aA*}
	7	1.98±0.35 ^{aA*}	2.61±0.39 ^{aA*}
B. subtilis PXN21	3	0.83±0.05 ^{aA*}	0.57±0.24 ^{aA*}
	5	1.13±0.20 ^{aA*}	0.93±0.34 ^{aA*}
	7	2.23±0.08 ^{aA*}	2.94±0.09 ^{aA*}
B. coagulans lactospore	3	2.60±0.04 ^{bC*}	2.72±0.04 ^{aB*}
	5	3.01±0.10 ^{aC*}	3.98±0.14 ^{aB*}
	7	3.24±0.09 ^{aB*}	4.08±0.29 ^{aB*}
B. coagulans BC30	3	3.26±0.03 ^{aD*}	3.46±0.32 ^{aB*}
	5	4.71±0.13 ^{aE*}	4.34±0.39 ^{bB*}
	7	5.23±0.04 ^{aC*}	4.93±0.46 ^{bC*}

– (CH) Samples that were subjected to thermal shock; (γ=No-Nf); Nf (Final count); No (Initial count): *B. flexus* Hk1: 8.57 log CFU/g; *B. subtilis* Bn1: 8.36 log CFU/g; *B. mojavensis* KJS3: 8.42 log CFU/g; *B. licheniformis* Me1: 8.70 log CFU/g; *B. subtilis* PB6: 8.31 log CFU/g; *B. subtilis* PXN21: 8.60 log CFU/g; *B. coagulans*

lactospore: 8.48 log CFU/g; *B. coagulans* BC30: 8.96 log CFU/g; *B. flexus* Hk1(CH): 8.26 log CFU/g; *B. subtilis* Bn1(CH): 6.69 log CFU/g; *B. mojavensis* KJS3(CH): 6.59 log CFU/g; *B. licheniformis* Me1(CH): 6.48 log CFU/g; *B. subtilis* PB6(CH): 6.66 log CFU/g; *B. subtilis* PXN21(CH): 7.24 log CFU/g; *B. coagulans* lactospore(CH): 8.41 log CFU/g; *B. coagulans* BC30(CH): 8.58 log CFU/g. Means ± standard deviation of two replicates; Means with different superscript lowercase letters on the same colunn indicate significant difference between samples of different doses of the same strain, by Scott-Knott ($p<0.05$). Means with different superscript capital letters on the same colunn indicate significant difference between samples of different strains in the same dose, by Scott-Knott ($p<0.05$). Means with different superscript symbols on the same line indicate significant difference between samples without thermal shock and with thermal shock (CH), by Test-t ($p<0.05$).

3.8. Extrusion

As for the process of extrusion, the survival of *B. coagulans* BC30 did not differ significantly in both in snack to the thermoplastic extrusion process and in spagghetti to the conventional extrusion process. There was no significant difference between samples of different steps at the same temperature and between samples of different temperatures in the same step in the survival of *B. coagulans* BC30 in snack (Table 4). In spagghetti, there was no significant difference between samples of steps in the survival of *B. coagulans* BC30. Additionaly, the survival did not differ significantly when compared gamma without thermal shock and gamma with thermal shock (CH), respectively. In this way, *B. coagulans* BC30 showed excellent resistence the extrusion process both thermoplastic and conventional, showing not significant decimal reductions (gamma) ranging from 0.19 to 0.61 log CFU/g without thermal shock and with thermal shock (CH) ranged from 0.22 to 0.72 log CFU/g in snack to the thermoplastic extrusion process. As for the conventional extrusion process, the decimal reductions (gamma) varied from 0.12 to 2.59 log CFU/g without thermal shock and with thermal shock (CH) ranged from 0.14 to 2.63 log CFU/g in spaghetti (Table 4). Corroboring with our study, Fares et al. (2015) evaluated the resistance of *B. coagulans* GBI-30, 6086 (*B. coagulans* BC30) in pasta to the extrusion and cooking process. The strain was able to survive the process significantly, only dropping 1 log.

Table 4

Survival of *Bacillus* in snack to the thermoplastic extrusion process and in spagghetti to the conventional extrusion process.

Temperature (°C)	Samples	Without thermal shock γ (log CFU/g)	With thermal shock (CH) γ (log CFU/g)
Thermoplastic extrusion			
100	Extrusion	0.19±0.12 ^{aA*}	0.54±0.09 ^{aA*}
	Drying	0.61±0.11 ^{aA*}	0.47±0.19 ^{aA*}
115	Extrusion	0.36±0.00 ^{aA*}	0.72±0.00 ^{aA*}
	Drying	0.56±0.33 ^{aA*}	0.65±0.04 ^{aA*}
130	Extrusion	0.43±0.03 ^{aA*}	0.41±0.07 ^{aA*}
	Drying	0.49±0.04 ^{aA*}	0.68±0.09 ^{aA*}
150	Extrusion	0.33±0.05 ^{aA*}	0.22±0.07 ^{aA*}
	Drying	0.60±0.07 ^{aA*}	0.34±0.02 ^{aA*}
Conventional extrusion			
Cold	Extrusion	0.12 ±0.00 ^{a*}	0.14±0.08 ^{a*}
	Drying	0.77±0.35 ^{a*}	0.84±0.17 ^{a*}
	Cooking	2.59±0.42 ^{a*}	2.63±0.35 ^{a*}

– (CH) Samples that were subjected to thermal shock; (γ=No-Nf); Nf (Final count); No (Initial count): 100: 7.74 log CFU/g; 115: 7.80 log CFU/g; 130: 7.85 log CFU/g; 150: 7.69 log CFU/g; cold: 7.38 log CFU/g; 100(CH): 7.74 log CFU/g; 115(CH): 7.86 log CFU/g; 130(CH): 7.85 log CFU/g; 150(CH): 7.65 log CFU/g. Means ± standard deviation of two replicates; Means with different superscript lowercase letters on the same colunn indicate significant difference between samples of different steps at the same temperature, by Scott-Knott ($p<0.05$). Means with different superscript capital letters on the same colunn indicate significant difference between samples of different temperatures in the same step, by Scott-Knott ($p<0.05$). Means with different superscript symbols on the same line indicate significant difference between samples without thermal shock and with thermal shock (CH), by Test-t ($p<0.05$).

3.9. Correlation between strains and technological processes

According to the results, the resistance of the strains varied from process to process. As for the processes of pasteurization (milk), pasteurization (juice) and cooking (meatballs) all eight strains behaved similarly both without thermal shock and with thermal shock (CH). The other processes the resistence of strains varied according to strain and application of thermal shock (CH). Baking without thermal shock, *B. subtilis* Bn1, *B.mojavensis* KJS3, *B. licheniformis* Me1 and *B. coagulans* BC30 positively stood out. However, with thermal shock (CH) all eight strains behaved similarly. All eight strains behaved similarly on the drying without thermal shock. With thermal shock (CH), the strains of *B. flexus* Hk1, *B. subtilis* Bn1, *B.mojavensis* KJS3, *B. licheniformis* Me1, *B. subtilis* PXN21 and *B. coagulans* BC30 positively stood out. As for acidification/fermentation all eight strains behaved similarly without thermal shock. With thermal shock (CH), *B. flexus* Hk1, *B. subtilis* Bn1, *B.mojavensis* KJS3, *B. licheniformis* Me1, *B. subtilis* PB6 and *B. coagulans* lactospore positively stood out. All eight strains behaved similarly on the supercritical carbon dioxide without thermal shock. With thermal shock (CH), *B. flexus* Hk1, *B. subtilis* Bn1, *B.mojavensis* KJS3, *B. subtilis* PB6, *B. subtilis* PXN21, *B. coagulans* lactospore and *B. coagulans* BC30 positively stood out. As for irradiation, *B. flexus* Hk1, *B.mojavensis* KJS3, *B. licheniformis* Me1, *B. subtilis* PB6, *B. subtilis* PXN21 behaved similarly without thermal shock and with thermal shock (CH) (Table 5). The strains without thermal shock that most stood out negatively were *flexus* Hk1, *B. subtilis* PB6, *B. subtilis* PXN21 and *B. coagulans* lactospore on the baking, *B. subtilis* Bn1 and *B. coagulans* BC30 on the irradiation. With thermal shock (CH), *B. subtilis* PB6 and *B. coagulans* lactospore on the drying, *B. subtilis* PXN21 and *B. coagulans* BC30 on the acidification/fermentation, *B. licheniformis* Me1 on the supercritical carbon dioxide and *B. coagulans* BC30 on the irradiation were the most negatively highlighted. In the other processes, all eight strains behaved in a similar way (Table 5).

Table 5

Strains that stood out positively and negatively in each process.

Process	↑ Resistence	↑ Resistence (CH)	↓ Resistence	↓ Resistence (CH)
Pasteurization (Milk)	-	-	-	-
Pasteurization (juice)	-	-	-	-
Cooking (meatballs)	-	-	-	-
Baking (bread)	*B. subtilis* Bn1, *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. coagulans* BC30	-	*B. flexus* Hk1, *B. subtilis* PB6, *B. subtilis* PXN21, *B. coagulans* lactospore	-
Drying (crystallized pineapple)	-	*B. flexus* Hk1, *B. subtilis* Bn1, *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. subtilis* PXN21, *B. coagulans* BC30	-	*B. subtilis* PB6, *B. coagulans* lactospore
Acidification/fermentation (yogurt)	-	*B. flexus* Hk1, *B. subtilis* Bn1, *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. subtilis*	-	*B. subtilis* PXN21, *B. coagulans* BC30

		PB6, *B. coagulans* lactospore		
Supercritical carbon dioxide (juice)	-	*B. flexus* Hk1, *B. subtilis* Bn1, *B. mojavensis* KJS3, *B. subtilis* PB6*, B. subtilis* PXN21, *B. coagulans* lactospore, *B. coagulans* BC30	-	*B. licheniformis* Me1
Irradiation (powder pepper)	*B. flexus* Hk1, *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. subtilis* PB6, *B. subtilis* PXN21	*B. flexus* Hk1, *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. subtilis* PB6, *B. subtilis* PXN21	*B. subtilis* Bn1, *B. coagulans* BC30	*B. coagulans* BC30

(CH) Samples that were subjected to thermal shock.

- All strains, there no significant difference.

Additionally, based on the resistence, some strains had better specificity for certain foods when compared to other strains. The small decimal reductions (gamma) are related to higher resistence of strain and higher specificity and affinity to particular process and food matrix. The high resistance and specificity of the strains to the technological processes and foods, respectively, possibly is influenced by the composition of the food matrix (Christiansen et al., 2006).

The table 6 shows the specificity of the strains to processes and food matrices in descending order (low gamma value→higher resistence). All eight strains showed greater decimal reductions (gamma) to the irradiation process compared to the other processes. Thus,

all eight strains showed low resistance and low specificity to irradiation, with reductions varying from 1.96-5.23 log CFU/g without thermal shock and 1.92-4.93 log CFU/g with thermal shock (CH) (Table 5). Additionally, the strain *B. flexus* Hk1 and *B. mojavensis* KJS3 showed low resistance and low specificity to acid pH (pasteurization-juice) and high temperatures in both wet and dry heat (cooking and baking) compared to other processes, with reductions varying from 0.65-2.38 log CFU/g without thermal shock and 0.83-1.92 log CFU/g with thermal shock (CH). The strain *B. subtilis* PB6 showed low resistance and low specificity to acid pH (pasteurization-juice), high temperatures in both wet and dry heat (cooking and baking) and to water activity around 0.65-0.75 (drying-crystallized pineapple), compared to other processes, with reductions varying from 0.70-3.02 log CFU/g without thermal shock and 1.09-3.23 log CFU/g with thermal shock (CH). The strain *B. subtilis* PXN21 showed low resistance and low specificity to acid pH (pasteurization-juice and acidification/fermentation), high temperatures in both wet and dry heat (cooking and baking) compared to other processes, with reductions varying from 0.97-2.13 log CFU/g without thermal shock and 1.08-2.17 log CFU/g with thermal shock (CH). The strain *B. coagulans* BC30 showed low resistance and low specificity to acid pH (pasteurization-juice) compared to other processes, with reductions from 2.30 log CFU/g without thermal shock and 2.50 log CFU/g with thermal shock (CH). The strain *B. coagulans* lactospore showed low resistance and low specificity to acid pH (pasteurization-juice) and to water activity around 0.65-0.75 (drying) compared to other processes, with reductions from 2.30 log CFU/g without thermal shock and 2.50 log CFU/g with thermal shock (CH). The other strains, the resistence/specificity to the processes varied according to the application or not of thermal shock (CH). The strain *B. coagulans* lactospore showed low resistance and low specificity to high temperatures in both wet and dry heat (cooking with thermal shock (CH) and baking without thermal shock) compared to other processes, with reductions from 1.23 log CFU/g and 1.64 log CFU/g, respectively. The strain *B. subtilis* Bn1 showed low resistance and low specificity to pH (pasteurization-juice and acidification/fermentation), water activity around 0.65-0.75 (drying), high temperatures in wet heat (cooking), pressure (supercritical carbon dioxide) with thermal shock (CH), with reductions varying from 0.27-1.92 log CFU/g. The strain *B. licheniformis* Me1 showed low resistance and low specificity to pH (pasteurization-juice) without thermal shock and to high temperatures in both wet and dry heat (cooking and baking with thermal shock) compared to other processes, with reductions from 1.52 log CFU/g and 1.51-1.61 log CFU/g, respectively (Table 6).

The strain *B. coagulans* BC30 was the only one that showed similar behavior in six processes (pasteurization-milk, cooking, baking, drying, acidification/fermentation and supercritical carbon dioxide) with small decimal reductions ranging from 0-1.02 log CFU/g without thermal shock and 0.07-1.06 log CFU/g with thermal shock (CH). At the pasteurization (juice) and irradiation (pepper) processes, gamma ranged from 2.30-5.23 log CFU/g without thermal shock and 2.50-4.93 log CFU/g with thermal shock (CH). However, the strain *B. subtilis* PXN21 showed low resistance and low specificity in five processes (pasteurization-juice, cooking, baking, acidification/fermentation and irradiation), with small decimal reductions ranging from 0.97-2.23 log CFU/g without thermal shock and 1.08-2.94 log CFU/g with thermal shock (CH) (Table 6). Thus, *B. coagulans* BC30 was the strain that showed greater resistance and greater specificity to the studied processes and *B. subtilis* PB6 and *B. subtilis* PXN21 were the strain that stood out negatively, showing lower resistance and lower affinity to the processes, presenting significant decimal reductions ($p<0.05$) in five processes studied with decimal reductions ranging from 0.70-3.02 log CFU/g without thermal shock and 1.08-3.23 log CFU/g with thermal shock (CH) (Table 6).

Table 6

Specificity of the strains to processes/food matrices.

Process (food matrices)	**Without thermal shock γ (log CFU/mL ou g)**	**Process (food matrices) (CH)**	**With thermal shock (CH) γ (log CFU/mL ou g)**
B. flexus Hk1		*B. flexus* Hk1	
Acidification/Fermentation (yogurt)	0.10±0.03[A]	Acidification/Fermentation (yogurt)	0.09±0.00[A]
Pasteurization (milk)	0.12±0.03[A]	Supercritical carbon dioxide (juice)	0.12±0.04[A]

Supercritical carbon dioxide (juice)	0.14±0.06^{A}	Drying (crystallized pineaplle)	0.12±0.22^{A}
Drying (crystallized pineaplle)	0.15±0.06^{A}	Pasteurization (milk)	0.15±0.03^{A}
Cooking (meatballs)	0.89±0.02^{B}	Pasteurization (juice)	1.04±0.39^{B}
Pasteurization (juice)	1.40±0.00^{C}	Baking (bread)	1.43±0.34^{B}
Baking (bread)	1.42±0.23^{C}	Cooking (meatballs)	1.46±0.49^{B}
Irradiation (powder pepper)	1.98±0.04^{D}	Irradiation (powder pepper)	1.92±0.25^{B}
B. subtilis Bn1		*B. subtilis* Bn1	
Pasteurization (milk)	0.20±0.10^{A}	Baking (bread)	0.00±0.29^{A}
Drying (crystallized pineaplle)	0.42±0.13^{A}	Pasteurization (milk)	0.03±0.00^{A}
Acidification/Fermentation (yogurt)	0.52±0.18^{A}	Acidification/Fermentation (yogurt)	0.27±0.02^{B}
Cooking (meatballs)	0.79±0.17^{A}	Supercritical carbon dioxide (juice)	0.33±0.07^{B}
Supercritical carbon dioxide (juice)	0.92±0.04^{A}	Pasteurization (juice)	0.48±0.06^{C}
Baking (bread)	1.04±0.08^{A}	Drying (crystallized pineaplle)	0.66±0.03^{D}
Pasteurization (juice)	1.94±0.40^{A}	Cooking (meatballs)	1.92±0.09^{E}
Irradiation (powder pepper)	5.01±0.03^{B}	Irradiation (powder pepper)	4.01±0.09^{F}
B. mojavensis KJS3		*B. mojavensis* KJS3	
Pasteurization (milk)	0.08±0.00^{A}	Pasteurization (milk)	0.14±0.07^{A}
Drying (crystallized pineaplle)	0.09±0.00^{A}	Supercritical carbon dioxide (juice)	0.26±0.00^{A}
Supercritical carbon	0.16±0.00^{A}	Drying (crystallized	0.33±0.07^{A}

dioxide (juice)		pineaplle)	
Acidification/Fermentation (yogurt)	0.30±0.06^{A}	Acidification/Fermentation (yogurt)	0.45±0.00^{A}
Baking (bread)	0.65±0.15^{B}	Pasteurization (juice)	0.83±0.06^{B}
Cooking (meatballs)	0.78±0.26^{B}	Cooking (meatballs)	0.94±0.21^{B}
Irradiation (powder pepper)	1.96±0.28^{C}	Baking (bread)	1.51±0.08^{C}
Pasteurization (juice)	2.38±0.21^{C}	Irradiation (powder pepper)	2.48±0.29^{C}
B. licheniformis Me1		*B. licheniformis* Me1	
Pasteurization (milk)	0.25±0.09^{A}	Pasteurization (milk)	0.05±0.00^{A}
Drying (crystallized pineaplle)	0.31±0.02^{A}	Acidification/Fermentation (yogurt)	0.19±0.04^{A}
Supercritical carbon dioxide (juice)	0.39±0.37^{A}	Drying (crystallized pineaplle)	0.23±0.00^{A}
Acidification/Fermentation (yogurt)	0.41±0.13^{A}	Pasteurization (juice)	0.43±0.40^{A}
Baking (bread)	0.67±0.03^{A}	Supercritical carbon dioxide (juice)	0.67±0.22^{A}
Cooking (meatballs)	1.15±0.41^{A}	Baking (bread)	1.51±0.39^{B}
Pasteurization (juice)	1.52±0.44^{B}	Cooking (meatballs)	1.61±0.59^{B}
Irradiation (powder pepper)	1.96±0.28^{C}	Irradiation (powder pepper)	2.48±0.29^{C}
B. subtilis PB6		*B. subtilis* PB6	
Acidification/Fermentation (yogurt)	0.16±0.02^{A}	Acidification/Fermentation (yogurt)	0.19±0.02^{A}
Supercritical carbon dioxide (juice)	0.25±0.07^{A}	Supercritical carbon dioxide (juice)	0.29±0.03^{A}
Pasteurization (milk)	0.32±0.02^{A}	Pasteurization (milk)	0.50±0.08^{A}

Drying (crystallized pineaplle)	0.70±0.16^{B}	Pasteurization (juice)	1.09±0.46^{B}
Cooking (meatballs)	0.96±0.03^{B}	Cooking (meatballs)	1.28±0.04^{B}
Baking (bread)	1.78±0.02^{C}	Drying (crystallized pineaplle)	1.84±0.17^{C}
Irradiation (powder pepper)	1.98±0.35^{C}	Irradiation (powder pepper)	2.61±0.39^{D}
Pasteurization (juice)	3.02±0.13^{D}	Baking (bread)	3.23±0.37^{D}
B. subtilis PXN21		*B. subtilis* PXN21	
Pasteurization (milk)	0.00±0.00^{A}	Supercritical carbon dioxide (juice)	0.09±0.00^{A}
Supercritical carbon dioxide (juice)	0.29±0.09^{A}	Drying (crystallized pineaplle)	0.10±0.02^{A}
Drying (crystallized pineaplle)	0.30±0.02^{A}	Pasteurization (milk)	0.21±0.07^{A}
Cooking (meatballs)	0.97±0.14^{B}	Acidification/Fermentation (yogurt)	1.08±0.51^{B}
Acidification/Fermentation (yogurt)	1.23±0.25^{C}	Pasteurization (juice)	1.48±0.11^{C}
Baking (bread)	1.76±0.31^{D}	Cooking (meatballs)	2.00±0.32^{C}
Pasteurization (juice)	2.13±0.11^{E}	Baking (bread)	2.17±0.57^{C}
Irradiation (powder pepper)	2.23±0.08^{E}	Irradiation (powder pepper)	2.94±0.09^{D}
B. coagulans lactospore		*B. coagulans* lactospore	
Pasteurization (milk)	0.10±0.00^{A}	Supercritical carbon dioxide (juice)	0.01±0.00^{A}
Supercritical carbon dioxide (juice)	0.22±0.06^{A}	Pasteurization (milk)	0.33±0.16^{A}
Cooking (meatballs)	0.44±0.18^{A}	Acidification/Fermentation	0.59±0.29^{A}

		(yogurt)	
Acidification/Fermentation (yogurt)	0.77±0.32^{A}	Baking (bread)	0.69±0.13^{A}
Drying (crystallized pineaplle)	1.12±0.16^{B}	Cooking (meatballs)	1.23±0.49^{B}
Pasteurization (juice)	1.19±0.27^{B}	Pasteurization (juice)	1.64±0.40^{B}
Baking (bread)	1.64±0.18^{B}	Drying (crystallized pineaplle)	2.26±0.30^{C}
Irradiation (powder pepper)	2.23±0.08^{C}	Irradiation (powder pepper)	2.94±0.09^{D}
B. coagulans BC30		*B. coagulans* BC30	
Drying (crystallized pineaplle)	0.00±0.00^{A}	Supercritical carbon dioxide (juice)	0.07±0.00^{A}
Acidification/Fermentation (yogurt)	0.04±0.00^{A}	Cooking (meatballs)	0.36±0.11^{A}
Cooking (meatballs)	0.33±0.17^{A}	Drying (crystallized pineaplle)	0.51±0.09^{A}
Supercritical carbon dioxide (juice)	0.41±0.37^{A}	Pasteurization (milk)	0.64±0.15^{A}
Pasteurization (milk)	0.51±0.12^{A}	Acidification/Fermentation (yogurt)	0.97±0.02^{A}
Baking (bread)	1.02±0.38^{A}	Baking (bread)	1.06±0.15^{A}
Pasteurization (juice)	2.30±0.08^{B}	Pasteurization (juice)	2.50±0.41^{B}
Irradiation (powder pepper)	5.23±0.04^{C}	Irradiation (powder pepper)	4.93±0.46^{C}

– (CH) Samples that were subjected to thermal shock; (γ=No-Nf); No (Initial count); Nf (Final count); Means ± standard deviation of two replicates; Means with different superscript capital letters on the same column indicate significant difference between processes of the same strain, by Scott-Knott ($p<0.05$).

Therefore, as expected, *Bacillus* showed survivability/resistance to all technological processes, due to its ability to form spores (resistant structures) (Cutting, 2011). However, the behavior/resistence of the *Bacillus* strains varied from process to process. In addition, the

resistence and specificity of *Bacillus* at the technologic process and food, respectively, ranged from strain to strain. Thus, the response of *Bacillus* could be considered as strain-dependent, possibly due to specific characteristics/properties and mechanisms of action of each strain. However, such characteristics/properties and mechanisms of action are not fully understood.

3.10. Proteomic analysis

The proteomic analysis are being done with the objective of evaluating the protein differences between the strains and thus to know/understand the specific characteristics and mechanisms of the *Bacillus* strains responsible for their better or worse resistance and specificity. The data of the proteomic analysis are under analysis and will be subsequently added to the publication.

4. Conclusions

In conclusion, *Bacillus* showed survivability/resistance to all technological processes. However, the behavior/resistence of the *Bacillus* strains varied from process to process. Additionally, the survival and specificity of *Bacillus* at the technologic process and food, respectively, ranged from strain to strain, so the response could be considered as strain-dependent.

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Whole wheat bread is a good matrix for delivering probiotic spore-forming bacteria

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Abstract

The objective of this study as to evaluate the resistance of *Bacillus coagulans* GBI-30, 6086 (BC) spores through the processing and storage of wheat and whole wheat breads. Wheat and whole wheat bread formulations, containing or not BC were prepared. Throughout the processing, samples were collected (after mixture, after fermentation, after baking and for all storage time) for enumeration of BC. In addition, BC were enumerated in different parts of breads (bark, crumb and whole slice) collected after baking and at different storage times (0, 3.0, 7.0 and 10 days). Quality characteristics analysis of pan breads were also conducted (moisture, specific volume, texture, color, water activity and pH). As for the stages of the pan bread making process, after mixing and after fermentation there were no significant decimal reductions (gamma). After baking, the bark was the part of the pan bread that presented the highest number of decimal reductions (gamma) in the four formulations analyzed. The survival of *Bacillus coagulans* GBI-30, 6086 on probiotic pan breads without thermal shock was significantly reduced ($p<0.05$) in bark around 2 cycle log, crumb and whole slice 1 cycle log. In wheat probiotic pan bread with thermal shock (CH) in the bark, crumb and whole slice around 2 cycle log, 2 cycle log, 1 cycle log, respectively and whole wheat probiotic pan bread with thermal shock (CH) in the bark, crumb and whole slice at around 2 cycle log, 1 cycle log and 1 cycle log, respectively. The wheat whole probiotic pan bread with thermal shock showed better results than wheat probiotic pan bread with thermal shock, probably due to the presence of fibers in the composition and higher water activity. Thus, the total process

showed decimal reductions of up to approximately 2 log in the four formulations analyzed. These results show that pan bread can be used as an important matrix for thourough deliver of probiotics to human diet.
Keywords: *Bacillus*; fibers; pan bread; process; quality characteristics; spore-forming probiotic.

1. Introduction

The probiotics have been defined as "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" (Hill et al., 2014). Several microbial genera have been reported to present probiotic properties (Foligné, Daniel, Pot, 2013). Among these, specific *Bacillus* strains have been claimed to present probiotic properties (Cutting, 2011; Kim et al., 2011; Nithya, Halami, 2013), *Bacillus coagulans* GBI-30, 6086 is a patented strain sold under the brand name GanedenBC$^{30®}$ (Ganeden Biotech, Mayfield Heights, OH). It is a non-toxic, non-pathogenic, non-antibiotic resistant *Bacillus* strain, which is safe for consumption (Endres et al., 2009). Several health benefits related to consumption of *B.coagulans* GBI-30, 6086 have been reported. For instance, patients showed improvement in symptoms of irritated bowel syndrome (IBS), such as bloating, abdominal pain and decreased bowel movements after administration of *B. coagulans* GBI-30, 6086 (Dolin, 2009; Hun, 2009). The administration of *B.coagulans* GBI-30, 6086 provided relief in patients with gas symptoms, reducing abdominal pain and other gastrointestinal symptoms (Kalman et al., 2009) and also relief of symptoms of lactose intolerance due to the ability of *B. coagulans* GBI-30, 6086 to digest lactose, fructose and milk protein (*in vitro*) has been reported (Maathuis et al., 2010). Jensen et al. (2010) demonstrated immune modulating and anti-inflammatory effects associated to administration of *B. coagulans* GBI-30, 6086 and Kimmel et al. (2010) observed increased levels of immunological markers such as cytokines (IL-6, IL-8, and INF-γ) and CD3CD69 cells in patients treated with *B. coagulans* GBI-30, 6086. Benefits in autoimmune diseases, such as pain reduction in patients with rheumatoid arthritis, have also been associated to administration of *B. coagulans* GBI-30, 6086 (Mandel et al., 2010).

In addition to the probiotic properties of a strain, the food matrix is recognized to be of vital importance for the delivery of probiotics through foods as well as for the survival of probiotics in the gastrointestinal tract (GIT). This is because the foods assist in the regulation

of GIT colonization by the probiotic microorganism. This occurs as food matrices may contain other ingredients, such as fiber, that protect and interact with probiotics in the GIT, resulting in the alteration of their functionality and efficacy (Ranadheera et al., 2010).

Even though *Lactobacillus* and *Bifidobacterium* are the most studied genera as probiotics (Sanders, Morelli, Tompkins, 2003), their delivery via several food matrices is compromised as these bacteria do not survive harsh processing conditions. For instance, bread is prepared through the mixture of ingredients (flour, water, and yeast, sodium chloride, with or without fat, sugar and milk powder), modeling, fermentation and baking in the oven (Pico et al., 2015). Despite the continuing consumer interest in bakery products that offer health benefits, which includes the production of probiotic bakery products (Haros et al., 2006), the use of bread as a probiotic carrier has been limited by the baking step. Probiotic strains of *Lactobacillus* and *Bifidobacterium* can not survive the high temperature achieved during baking (~200°C) (Zhang et al., 2014). Therefore, the application of spore-forming bacteria (*Bacillus*) (Cutting, 2011) can be considered a feasible alternative to allow the development of probiotic foods that during processing experience harsh conditions that would kill *Lactobacillus* and *Bifidobacterium*. In addition to the resistance to tough processing conditions, probiotic *Bacillus* can remain viable for a long time during storage and survive the gastrointestinal tract conditions when consumed (Cutting, 2011; Durkee, 2010; Hong et al., 2005).

As bread is daily consumed since ancient times, standing among the most eaten foods in the world (Pico et al., 2015; Pico et al., 2016), it could serve as an important matrix to deliver probiotics to human diet.Therefore, the objectives of this study were to evaluate the resistance of *B. coagulans* GBI-30, 6086 (BC) spores through the processing (after mixture, after fermentation and after baking) and during storage of wheat and whole wheat pan breads. In addition, the survival of BC in different parts of pan breads (bark, crumb and whole slice) collected after baking and at different storage times (0, 3.0, 7.0 and 10 days) was studied.

2. Materials and Methods

2.1. Probiotic strain

B. coagulans GBI-30, 6086 spores (Ganeden Biotech Inc, Mayfield Heights, Ohio, USA) were used in this study.

2.2. Pan bread formulations

Wheat and whole wheat pan breads were produced based on the following formulations (flour basis): i) wheat pan bread: White flour (100%), water (60%), sugar (4%), salt (2%), biological yeast (2%), whole milk powder (4%), hydrogenated vegetable fat (4%), sodium propionate (0.5%) and flavor enhancer (2%); ii) whole wheat pan bread: White flour (49%), whole wheat flour (51%), water (62%), sugar (4%), salt (2%), biological yeast (2%), whole milk powder (4%), hydrogenated vegetable fat (4%), sodium propionate (0.5%) and flavor enhancer (2%).

2.3. Pan bread processing

The pan breads were prepared using the modified straight dough method (Schmiele et al., 2012).The production can be divided in different stages: ingredients mixture, fermentation (32°C/60 minutes) and baking (200°C/18 minutes). The dry ingredients were mixed in an A30 model mixer (Guaraituba, Brazil) in slow speed (90 rpm, 1 minute). The water was added at 4°C, the dough was mixed in slow (90 rpm, 7 minutes) and high speed (180 rpm, 6 minutes), until development of the gluten network. Then, the dough was divided in portions of 250 g that were left to rest (5 minutes), following moulding in C-Ret Trif model dough moulder (Curitiba, Brazil). Then, the dough was placed in greased bread tins (10.25 × 6.75 × 22 cm) and further kept in evolution-CCKU-8040-182 model proofing chamber (Poços de Calda, Brazil), 32°C, for 60 minutes (controlled hum moisture 80%). The doughs were baked (200°C/18 minutes) in vipinho-0448 trif model oven (Curitiba, Brazil). After baking, the pan breads were cooled down at room temperature and further stored for 10 days at 25°C.

Two independent experiments (process of pan bread fabrication) were made for each pan bread formulation. BC spores were added at the mixture stage. Samples for enumeration of BC spores were collected at the following stages of the process (after mixture, after fermentation, after baking and in different periods of the shelf life). In addition, different parts of pan breads (bark, crumb and whole slice) were collected after baking and in different periods of the shelf life (0, 3, 7 and 10 day) aiming to determine the number of BC spores

(section 2.6). Samples (bark, crumb and whole slice) were also collected at 1, 4, 7 and 10 day of shelf lifefor quality characteristics analysis of pan breads.

2.4. Enumeration of BC spores

Samples collected at different processing steps (after mixture, after fermentation, after baking and during storage) were or were not submitted to thermal shock (thermal process at 80°C/10 minutes and cooling). Then, decimal dilutions were prepared and samples without thermal shock and with thermal shock (CH) were inoculated through the Pour-plate technique in BC agar (g/L: yeast extract powder (5g), peptone (5g), glucose (5g), potassium phosphate dibasic (0.5g), potassium phosphate monobasic (0.5g), magnesium sulfate (0.3g), trace mineral solution (1mL)) (Kasvi, Curitiba, PR, Brazil). All reagents were from Dinamica quimica (Diadema, SP, Brazil), with the exception of yeast extract powder and pepton that were from kasvi (Curitiba, PR, Brazil). Then, the inoculated Petri dishes were incubated at 42°C for 48 hours, the colonies were counted and the results expressed as $\log_{10}$ CFU/g. The number of survivors in the samples analyzed was calculated based on initial inoculum level (10^9 per 25 g portion of bread) and the count of BC after each stage of process. The culture medium BC and incubation conditions were formulated and performed according to the supplier's guidelines of the BC strain used.

2.5. Rheological properties

The rheological evaluation of the flour samples (refined and whole wheat), in triplicate, was performed according to the official methods of the American Association of Cereal Chemist International (AACCI, 2010) for Farinographic - method 54-21.02, using Brabender farinograph model 810130 (Duisburg, HE GAVE); Alveografic - method 54-30.02, using Chopin alveograph, model MA95 (Chopin, Villeneuve-la-Garenne, FRA).

2.6. Quality characteristics assessment of pan bread

The pan bread quality characteristics (bark, crumb and whole slice) analyzed on day 1 were moisture, specific volume, texture and color. Water activity and pH were also measured

on days 1, 3, 7 and 10. The pH was determined using an Akso AK103 model pHmetrer (Rio Grande do Sul, Brazil). The water activity was measured using a water activity analyzer, Aqualab digitaL 4TEV (Decagon, Pullman, USA), both according to manufacturer's instructions. Bread crumb color was evaluated using the MiniScan XE portable spectrophotometer (Hunter Associates Laboratory, Inc, Reston, Virginia, USA) with D65 illuminant and 10º observation angle. The values of color (L*) ranging from 0 (black) to 100 (white), a* varying from green (negative) to red (positive), b* varying from blue (negative) to yellow (positive), C* representing the chroma and h* the hue angle were evaluated. Moisture content was determined based on 44-15.02 method (AACCI, 2010), while the specific volume was determined according to 10.05.01 method (AACCI, 2010). Texture was determined in texture analyzer, TAXT2i model (Stable Micro Systems, Haslemere/England), using the 74-09.01 method (AACCI, 2010), with eighteen repetitions. Two 12.5 mm thick two-slice crumbs were compressed in each test (discarded bread slices). The test conditions were pre-test velocity = 1 mm/s, test velocity = 1.7 mm/s, post-test velocity = 10 mm/s, distance = 40% and time = 60 s. The measurements of firmness are expressed in N. All these analyses were made in triplicate.

2.7. Statistical analysis

All data were presented as means ± standard deviation and analyzed by ANOVA, followed by the t test and Scott-Knott using the Sisvar software 5.6 (Lavras, MG, Brazil). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. BC survival throughout processing and storage of different types of pan breads

The survival of BC through different steps of pan bread processing is shown in table 1. In general, *B. coagulans* GBI-30, 6086 had high resistance to the baking process. The four formulations of probiotic pan breads showed decimal reductions (gamma) after the baking process (day 0) from 1.88, 1.06 and 1.06 log CFU/g for bark, crumb and whole slice of wheat

probiotic pan bread without thermal shock, respectively and 1.84, 1.19 and 1.32 log CFU/g for bark, crumb and whole slice of whole wheat probiotic pan bread without thermal shock, respectively. With thermal shock (CH) the decimal reductions were from 1.88, 1.60, 1.51 CFU/g for bark, crumb and whole slice of wheat probiotic pan bread with thermal shock (CH), respectively and 1.62, 1.41 and 1.52 CFU/g for bark, crumb and whole slice of whole wheat probiotic pan bread with thermal shock (CH), respectively, there was no significant difference ($p<0.05$) in survival of *Bacillus* between bark, crumb and whole slice in the four formulations of probiotic pan breads in day 0, individually, respectively (Table 1).

When compared the gamma of wheat probiotic pan bread without thermal shock and gamma of wheat probiotic pan bread with thermal shock (CH) there was significant difference ($p<0.05$) in crumb and whole slice in day 0, presenting higher decimal reductions (gamma) with thermal shock (CH). However, when compared the gamma of whole wheat probiotic pan bread without thermal shock and gamma of whole wheat probiotic pan bread with thermal shock (CH), there was no significant difference ($p<0.05$) in the bark, crumb and whole slice in day 0 (Table 1).

As for the steps of after mixture and after fermentation, the decimal reductions (gamma) were of 0.00, 0.00, 0.02 and 0.01 log CFU/g for after mixture and 0.05, 0.24, 0.05 and 0.20 log CFU/g for after fermentation in the wheat probiotic pan bread without thermal shock, whole wheat probiotic pan bread without thermal shock, wheat probiotic pan bread with thermal shock (CH) and whole wheat probiotic pan bread with thermal shock (CH), respectively, there was no significant difference between the four types of pan breads analyzed both after mixture and after fermentation. However, when comparing the gamma of the step after baking (bark, crumb, whole slice) with the gamma after mixture or with the gamma after fermentation there was a significant difference ($p<0.05$) in the four types of pan breads analyzed, individually (Table 1). In this way, all stage of after baking (bark, crumb and whole slice) differed significantly ($p<0.05$) of stage of after mixture and after fermentation in the four types of pan breads analized individually. However, the stages of after mixture and after fermentation did not differ significantly between them in the four types of pan breads analized individually. Thus, the after baking step presented the largest reductions compared to the other steps in the pan bread production process.

Table 1

Survival of *Bacillus coagulans* GBI-30, 6086 in different processing steps of pan bread.

	Types of pan breads			
Processing steps	**Wheat probiotic pan bread γ (log CFU/g)**	**Whole wheat probiotic pan bread γ (log CFU/g)**	**Wheat probiotic pan bread CH γ (log CFU/g)**	**Whole wheat probiotic pan bread CH γ (log CFU/g)**
After Mixture	0.00±0.00 ^{aA*}	0.00±0.00 ^{aA*}	0.02 ± 0.01 ^{aA*}	0.01±0.00 ^{aA*}
After fermentation	0.05±0.04 ^{aA*}	0.24±0.03 ^{aA*}	0.05 ± 0.09 ^{aA*}	0.20±0.13 ^{aA*}
After baking (bark) day 0	1.88±0.81 ^{aB*}	1.84±0.19 ^{aB*}	1.88±0.84 ^{aB*}	1.62±0.15 ^{aB*}
After baking (crumb) day 0	1.06±0.03 ^{aB*}	1.19±0.09 ^{aB*}	1.60±0.18 $^{aB\#}$	1.41±0.09 ^{aB*}
After baking (whole slice) day 0	1.06±0.52 ^{aB*}	1.32±0.02 ^{aB*}	1.51±0.54 $^{aB\#}$	1.52±0.02 ^{aB*}

– (CH) Samples that were subjected to thermal shock; (γ=No-Nf); (No) Initial count; Wheat probiotic pan bread: 7.38; Whole wheat probiotic pan bread: 7.59; Wheat probiotic pan bread (CH): 7.24; Whole wheat probiotic pan bread (CH): 7.25. Means ± standard deviation of two replicates; Means with different superscript lowercase letters on the same line indicate significant difference between samples of wheat and whole wheat pan breads without thermal shock and samples of wheat and whole wheat pan breads with thermal shock (CH), respectively, by test t ($p<0.05$). Means with different superscript capital letters on the same column indicate significant difference between samples, by Scott-Knott ($p<0.05$). Means with different symbols on the same line indicate significant difference between samples of wheat pan bread without thermal shock and with thermal shock (CH) and samples of whole wheat pan bread without thermal shock and with thermal shock (CH), respectively, by test t ($p<0.05$).

The survival of the strain in the four pan breads formulations analyzed varied according to the type of pan bread between day 0 and 10 of storage, respectively (Table 2). The four formulations of probiotic pan breads showed decimal reductions (gamma) during the 10 days of storage ranging from 1.88-2.94, 1.06-1.25 and 1.06-1.37 log CFU/g for bark, crumb and whole slice of wheat probiotic pan bread without thermal shock, respectively and 1.84-2.17, 1.00-1.19 and 1.18-1.52 log CFU/g for bark, crumb and whole slice of whole wheat probiotic pan bread without thermal shock, respectively. With thermal shock (CH) the decimal reductions were from 1.87-2.86, 1.49-2.00, 1.51-2.22 CFU/g for bark, crumb and whole slice of wheat probiotic pan bread with thermal shock (CH), respectively and 1.62-2.06, 1.19-1.58 and 1.31-2.20 CFU/g for bark, crumb and whole slice of whole wheat probiotic pan bread with thermal shock (CH), respectively. Regarding the four types of pan bread samples (in the form of bark, crumb and whole slice) compared individually between the days of storage, there was no significant difference in the samples of wheat probiotic pan bread, wheat probiotic pan bread (CH) and whole wheat probiotic pan bread. However, in the whole wheat

probiotic pan bread (CH), day 7 differed significantly ($p<0.05$) from the other days (0, 3 and 10) in crumb and whole slice, showing greater decimal reductions (gamma) (Table 2). Additionaly, there was no significant difference between the samples of wheat probiotic pan bread and whole wheat probiotic pan bread without thermal shock and between the samples of wheat probiotic pan bread and whole wheat probiotic pan bread with thermal shock (CH) at all days of storage. When samples without thermal shock and with thermal shock (CH) were compared, there was a significant difference ($p<0.05$) in crumb and whole slice (day 0, 3 and 10) between wheat probiotic pan bread and wheat probiotic pan bread (CH) and in whole slice (day 7) between whole wheat probiotic pan bread and whole wheat probiotic pan bread (CH), showing greater decimal reductions (gamma).

Table 2

Survival of *Bacillus coagulans* GBI-30, 6086 in samples of pan bread during storage (0-10 days).

	Types of pan breads			
Storage times	**Wheat probiotic pan bread γ (log CFU/g)**	**Whole wheat probiotic pan bread γ (log CFU/g)**	**Wheat probiotic pan bread CH γ (log CFU/g)**	**Whole wheat probiotic pan bread CH γ (log CFU/g)**
Bark (day 0)	1.88±0.81 ^{aA*}	1.84±0.19 ^{aA*}	1.88±0.84 ^{aA*}	1.62±0.15 ^{aA*}
Crumb (day 0)	1.06±0.03 ^{aA*}	1.19±0.09 ^{aA*}	1.60±0.18 $^{aA\#}$	1.41±0.09 ^{aA*}
Whole slice (day 0)	1.06±0.52 ^{aA*}	1.32±0.02 ^{aA*}	1.51±0.54 $^{aA\#}$	1.52±0.02 ^{aA*}
Bark (day 3)	2.94±0.06 ^{aA*}	2.15±0.59 ^{aA*}	2.86±0.05 ^{aA*}	1.90±0.62 ^{aA*}
Crumb (day 3)	1.21±0.19 ^{aA*}	1.00±0.09 ^{aA*}	2.00±0.34 $^{aA\#}$	1.20±0.08 ^{aA*}
Whole slice (day 3)	1.20±0.39 ^{aA*}	1.18±0.15 ^{aA*}	2.22±0.33 $^{aA\#}$	1.31±0.20 ^{aA*}

Bark (day 7)	2.73±0.04 ^{aA*}	2.17±0.57 ^{aA*}	2.60±0.20 ^{aA*}	1.81±0.61 ^{aA*}
Crumb (day 7)	1.25±0.01 ^{aA*}	1.14±0.41 ^{aA*}	1.49±0.56 ^{aA*}	1.58±0.31 ^{aB*}
Whole slice (day 7)	1.37±0.17 ^{aA*}	1.36±0.01 ^{aA*}	1.70±0.70 ^{aA*}	2.20±0.08 $^{aB\#}$
Bark (day 10)	2.01±0.17 ^{aA*}	2.05±0.17 ^{aA*}	1.87±0.23 ^{aA*}	2.06±0.10 ^{aA*}
Crumb (day 10)	0.95±0.16 ^{aA*}	1.12±0.06 ^{aA*}	1.89±0.05 $^{aA\#}$	1.19±0.20 ^{aA*}
Whole slice (day 10)	1.20±0.27 ^{aA*}	1.52±0.05 ^{aA*}	1.72±0.36 $^{aA\#}$	1.45±0.31 ^{aA*}

– (CH) Samples that were subjected to thermal shock; (γ=No-Nf); (No) Initial count: Wheat probiotic pan bread: 7.38; Whole wheat probiotic pan bread: 7.59; Wheat probiotic pan bread (CH): 7.24; Whole wheat probiotic bread (CH): 7.25. Means ± standard deviation of two replicates; Means with different superscript lowercase letters on the same line indicate significant difference between samples of wheat and whole wheat pan breads without thermal shock and samples of wheat and whole wheat pan breads with thermal shock (CH), respectively, by test t ($p<0.05$). Means with different superscript capital letters on the same column indicate significant difference between days in samples of bark, crumb and whole slice, respectively, by Scott-Knott ($p<0.05$). Means with different superscript symbols on the same line indicate significant difference between samples of wheat pan bread without thermal shock and with thermal shock (CH) and samples of whole wheat pan bread without thermal shock and with thermal shock (CH), respectively, by test t ($p<0.05$).

3.2. Rheological properties

The characteristics obtained with the farinograph and extensograph are shown in Table 3. The water absorption, stability, resistance and extensibility varied significantly ($p<0.05$) between refined and whole wheat flour.

Table 3

Rheological properties (water absorption, stability, resistence and extensibility) of flours used for pan bread making.

Type of flour	Water absorption (%)	Stability (min)	Resistance (BU)	Extensibility (mm)
Refined flour	58.2±0.12 ^{a}	23.1±0.8 ^{a}	525±35 ^{a}	125±7 ^{a}
Whole wheat Flour	65.3±0.12 ^{b}	15.5±0.9^{b}	434±28^{b}	105±7.5^{b}

BU - Brabender Units.Means with different superscript lowercase letters in the same column indicate significant difference between samples, by Scott-Knott ($p<0.05$).

3.3. Quality characteristics of pan bread

The quality characteristics of pan bread (moisture, volume, texture and color) are presented in table 4. Moisture and texture did not vary significantly between pan breads added of BC and control pan breads (with no BC). Regarding the volume, samples of whole wheat pan bread and whole wheat probiotic pan bread showed higher values when compared to wheat pan bread and wheat probiotic pan bread ($p<0.05$). The color parameters B and C did not differ significantly among the samples of pan bread analyzed. However, the A and h parameters was increased ($p<0.05$) and the L parameter was reduced ($p<0.05$) in whole wheat pan bread and whole wheat probiotic pan bread. The four pan bread formulations showed water activity around 0.94 to 0.95 and pH around 5.7 to 5.9. The activity water and pH did not vary significantly between pan breads added of BC and control pan breads (with no BC). The pH did not vary significantly among four pan bread formulations analyzed throughout the shelf-life (1, 3, 7 and 10) (Table 4). The water activity did not vary significantly in the same formulation between different days analyzed (1, 3, 7 and 10). However, varied significantly ($p<0.05$) among four pan bread formulations analyzed in the same day (1, 3, 7 and 10), respectively (Table 5) The whole wheat probiotic pan bread showed significant increases in water activity on days 3, 7 and 10, respectively, when compared with wheat probiotic pan bread.

Table 4
Moisture, volume, texture and color in different pan bread types.

Types						**Color**		
	Moisture (%)	**Volume (L/Kg)**	**Texture (Firmness) (N)**	**L**	**a**	**B**	**C**	**h**
Wheat pan Bread	34.6 ± 0.17[a]	4.2 ± 0.15[a]	1.7 ± 0.10 [a]	84.5 ± 0.60[a]	1.6 ± 0.02[a]	19.7 ± 0.23[a]	19.8 ± 0.23[a]	4.8 ± 0.01[a]
Wheat	34.8 ±	4.1 ±	1.9 ±	84.8	1.6	18.8	18.8	4.9

probiotic pan Bread	0.85[a]	0.28[a]	0.19 [a]	± 0.42[a]	± 0.03[a]	± 0.00[a]	± 0.00[a]	± 0.09[a]
Whole wheat pan Bread	35.8 ± 0.38[a]	3.4± 0.02[b]	2.4 ± 0.16[a]	75.2 ± 0.37[b]	4.5 ± 0.03[b]	18.8 ± 0.01[a]	19.4 ± 0.01[a]	13.5 ± 0.08[b]
Whole wheat probiotic pan Bread	35.5 ± 0.56[a]	3.3 ± 0.16[b]	2.2 ± 0.77[a]	74.9 ± 0.31[b]	4.6 ± 0.23[b]	18.5 ± 0.15[a]	19.1 ± 0.09[a]	14.1 ± 0.79[b]

Means ± standard deviation of two replicates (3 replicates); Means with different superscript letters in the same column indicate significant difference between samples, by Scott-Knott ($p<0.05$).

Table 5

Water activity and pH of samples of pan bread in the days 1, 3,7 and 10.

Samples	Days	Water activity	pH
Wheat pan bread	1	0.943 ± 0.0005 [aA]	5.89 ± 0.007 [aA]
	3	0.944 ± 0.002 [aA]	5.88 ± 0.02 [aA]
	7	0.945 ± 0.001 [aA]	5.83 ± 0.09 [aA]
	10	0.943 ± 0.003 [aA]	5.74 ± 0.12 [aA]
Wheat probiotic pan bread	1	0.944 ± 0.002 [aA]	5.91 ± 0.02 [aA]
	3	0.944± 0.001 [Aa]	5.88 ± 0.04 [aA]
	7	0.945 ± 0.0002 [aA]	5.83 ± 0.09 [aA]
	10	0.943± 0.0004 [aA]	5.70 ± 0.03 [aA]
Whole wheat pan Bread	1	0.947 ± 0.0001 [aA]	5.89 ± 0.02 [aA]
	3	0.948 ± 0.002 [bA]	5.86 ± 0.02 [aA]
	7	0.949 ± 0.0008 [bA]	5.79 ± 0.00 [aA]
	10	0.945 ± 0.0001 [bA]	5.76 ± 0.007 [aA]
Whole wheat probiotic pan bread	1	0.945 ± 0.001 [aA]	5.82 ± 0.03 [aA]
	3	0.949 ± 0.001 [bA]	5.86 ± 0.02 [aA]
	7	0.953 ± 0.003 [cA]	5.81 ± 0.02 [aA]
	10	0.946 ± 0.002 [bA]	5.76 ± 0.10 [aA]

Means ± standard deviation of two replicates (3 replicates); Means with different superscript lowercase letters in the same column indicate significant difference among types of pan breads for the same day, by Scott-Knott ($p<0.05$). Means with different superscript capital letters in the same column indicate significant difference among the same type of pan bread for different days according to Scott-Knott ($p<0.05$).

4. Discussion

In last years, the growth of the diversify bread market and bakery industry has been evident mainly due to the search for healthy diets and products with functional ingredients. Such as, probiotic bacteria and/or whole-grain flour that contains higher content of fibre, protein, and other nutrients, including minerals (calcium, magnesium, and potassium) and many phytochemicals (Geng et al., 2016, Santos et al., 2016). The pan bread present has a structure different from other types of bread, thin crust and a crumb with regular porosity and texture soft and elastic. The quality is defined according to the texture, moisture, specific volume, crust color, bark/crumb ratio, crumb firmness and flour characteristics (Besbes et al., 2014).

The higher values of water absorption and lower values of stability, resistance and extensibility of the whole wheat flour are explained by the fibers present that require a larger amount of water, greater resistance of the dough to mixing and longer hydration times, retain more water in the final product (Schmiele et al., 2012). Additionally, the fibers greatly compete for water with other polymers resulting in the dilution of the gluten, which makes it more difficult to form a gluten network (viscoelastic properties) capable of attributing a higher resistance to the dough, leading to weaker doughs (Rosell et al., 2001). Corroboring with our study, Schmiele et al. (2012) reported that the use of bran and whole grain flour changes dough rheology and the quality produced pan bread. The replacement of the refined wheat flour for wheat bran and whole grain wheat flour increased water absorption and resistance to extension and decreased stability and extensibility and also increased the moisture content, firmness and hardness and decreased specific volume of pan bread.

As regards quality parameters, no significant differences was observed between the probiotics pan breads (breads with *Bacillus* probiotics) and control pan breads (pan breads without *Bacillus* probiotics). The whole wheat pan breads showed higher values of moisture and texture (firmess) and lower values of volume when compared with wheat pan breads. However, there was no significant difference in moisture and texture (firmness) among the four pan breads formulations. A lower volume in the whole wheat pan breads is expected owing to the dilution of the gluten and weakening of the gluten network formed, because this contains a greater amount of fiber (Katina, 2003). Breads with higher fiber concentrations show higher moisture contents and lower volumes, reflected in greater firmness and hardness owing to their more compact nature (Schmiele et al., 2012). Significant changes in color

parameters among wheat and whole wheat pan breads are acceptable, as whole wheat pan breads present darker coloring than wheat pan breads, due also to the presence of fibers in the composition of the whole wheat flour. According with Khalid et al. (2017) the presence of fiber in dough caused an increase on water absorption, decrease on bread loaf volume and negative influence on farinograph stability. The fibers are the main component that to have highly significant ($p<0.05$) and negative influence on most bread making characteristics.

The four pan bread formulations showed water activity and pH around 0.94 to 0.95 and 5.7 to 5.9, respectively. The results for water activity and pH are expected, as the pan bread is classified in foods with high water activity ($0.94<0.97$) and low acidity ($pH>4.5$) (Tortora et al., 2010). Possibly, the pH did not change between the four formulations analyzed, because the strain did not germinate and did not multiply in the pan breads during the storage. As for the activity water, the significant increase in whole wheat pan breads (day 3, 7 and 10) can be justified by the presence of fibers, because the larger the fiber content, the higher the moisture content. Corroborating with our study, Jao et al., 2011 showed that the pH no significantly altered ($pH>4.5$) in the probiotic bakery products containing *B. coagulans* GBI-30, 6086 after 9 days of storage.

The survival of *B. coagulans* GBI-30, 6086 in the four pan breads formulations analyzed was significantly reduced on the pan breads manufacturing process ($p<0.05$). However, in general, the probiotic strain presented good resistance in both wheat and whole wheat pan breads, because at the end of manufacturing process, it was observed falls that varied of 1 to 2 log in wheat pan breads and whole wheat pan breads. After mixing and fermentation there were no significant drops, which was already expected, since the mixture is a mechanical process only and in the fermentation the temperature is low (32°C) and the time short (60 minutes).

Regarding the baking process, the largest reductions were demonstrated in bark in both wheat pan breads and whole wheat pan breads without and with thermal shock, approximately 2 log cycle drop. Larger reductions in the bark were already expected, because in the bark the strain is more in contact with the high temperature of baking (~190-200°C), whereas in the crumb the strain is more protected (temperature in the center of the bread ~70-80°C). In addition, in the wheat pan bread with thermal shock there was a significant increase in decimal reductions (gamma) in the crumb and whole slice when compared to wheat pan bread without thermal shock after baking. These larger reductions show that the spores are stressed with the high temperature of baking and when the thermal shock occurs the hindrance of the recovery of these. However, these significant increase in decimal reductions was not observed

in crumb and whole slice of whole wheat pan bread, possibly due to higher activity water and the presence of fibers in their composition, guaranteeing a greater protection to the strain.

As for the storage, when compared probiotic pan breads without termal shock and probiotic pan breads with termal shock (CH), the significant increase in decimal reductions on days 3 and 10 in crumb and whole slice in wheat probiotic pan breads did not occur in whole wheat probiotic pan breads. Thus, the presence of fiber in the composition of the whole wheat pan breads seems to increase the protection to the strain, reducing the decimal reductions (gamma) in pan breads that were submitted to thermal shock, because breads with higher fiber concentrations show higher moisture contents and activity water, possibly, reflected in greater survival of strain. Therefore, it is recommended not to apply thermal shock to wheat pan breads. However, for whole wheat pan breads both the application or not of thermal shock can be used.

Study of Zhang et al. (2014) reported that the viable counts of *Bifidobacterium lactis* BB12 declined significantly during baking at various temperatures (165, 185, 205°C) for 12 minutes. The survival rate of bacteria (initial count 10^6 CFU/g) was affected by heating temperature and heating time. At the end of 12 minutes still had survival, but very low (10^2 CFU/g). While, Jao et al. (2011) observed the viability of *B. coagulans* GBI-30, 6086 in baking process of eight different baking products (chrysanthemum cookies, egg pastry cakes, mooncakes, muffins, polo breads, soda cookies, sponge cakes, and toasts) and the viability during the storage at 4 or 25°C for 15 and 6 days, respectively. The values of strain counts in the eight baking products were less than their raw dough after the baking process and storage. The results showed that the strain viability of baking products decreased with storage days, both 4 and 25°C. However, the probiotic strain survived in significant amounts to the baking process and storage. Thus, *B. coagulans* GBI-30, 6086 was defined as good candidates for baking product use, different from *Bifidobacterium lactis* BB12 that showed 4 cycle log drops.

Additionally, as there were only 1 to 2 log drop in the total process of pan breads production and there were no significant changes in the survival of the strain during the 10 days of storage in the four formulations analyzed, it is possible to produce probiotic pan breads with the strain of *B. coagulans* GBI-30, 6086, which is suitable for use in pan breads, both wheat and whole wheat pan bread, with counts of probiotics at a level higher than the recommended level (10^6 CFU/g) (Sarkar, 2013). It is important to take into account the number of decimal reductions (gamma) during the total process for the initial inoculum calculation and thus have possible the beneficial effects. Such as, improvement of irritated

bowel syndrome (IBS) (Dolin, 2009), colitis (Fitzpatrick et al., 2012) and lactose intolerance (Maathuis et al., 2010), immune modulating and anti-inflammatory effects (Jensen et al., 2010).

5. Conclusions

In general, *B. coagulans* GBI-30, 6086 had high resistance to the baking process and also during storage (1 to 2 log drop in the total process of pan breads production and there were no significant changes in the survival of the strain during the storage). Therefore, both whole wheat and wheat pan breads are promising food matrices as probiotic carriers. However, whole wheat pan bread has shown greater capacity to protect the probiotic strain, possibly by presence of fiber in their composition.

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In vitro evaluation of survival/functionality of the *Bacillus coagulans* GBI-30, 6086 spores in juice and yogurt in simulated gastrointestinal tract conditions

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Abstract

In this study an *in vitro* dynamic model of intestinal tract with the simulated gastric and intestinal conditions was used to evaluate the impact/efficacy of orange juice and yogurt as carriers of probiotic *Bacillus coagulans* GBI-30, 6086 (BC). The dynamic system allowed to control the environmental conditions (pH, temperature and residence time) mimicking *in vivo* gastric and intestinal (GIT) conditions. The count of BC was performed of juice and yogurt added of BC collected in different steps: 1-food matrix before passage in the system; 2-content after initial contact with gastric compartment (30 min, low pH-2); 3 and 4-content after resting (1h15min) and dynamic (2h) stage in gastric compartment; 5 and 6-content in rest (3h) and dynamic (4h) stage in duodenal compartment; 7 and 8-content in rest (5h) and dynamic (6h) stage in jejunal compartment; 9-content in end collector (6h). The results indicated decimal reductions approximately 1 log cycle during passage through the gastrointestinal tract simulator (6h). In this way, there was no significant difference in the survival of BC in the samples of juice, yogurt, juice with thermal shock (CH) and yogurt with thermal shock (CH) in total experiment *in vitro*, respectively. Therefore, *B. coagulans* GBI-30, 6086 presented a high survival (decimal reductions approximately 1 log cycle) in both food matrices (juice and yogurt), proving that for sporulated probiotics the food matrix does not have as much influence as for non-sporulated probiotics. In addition, both juice and yogurt are excellent food matrices to function as carriers of BC, since they do not interfere in the survival of the strain.

Keywords: *Bacillus*; *in-vitro*; juice; spore-forming probiotic; gastrointestinal tract; yogurt.

1. Introduction

"*Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" (Hill et al., 2014). Strains of *Lactobacillus* (De Gregorio et al., 2015; Fooladi et al., 2015) and *Bifidobacterium* (Jeong et al., 2015; Kim et al., 2008) with probiotic properties have been studied and added to several foods. Even though recent literature has been reporting on probiotic potential of strains of *Bacillus coagulans*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilis* and *Bacillus polyfermenticus* (Cutting, 2011; Huang et al., 2010; Im et al., 2009; Sun et al., 2011). *Bacillus coagulans* GBI-30, 6086 (BC) is the spore-forming bacteria with generally recognized as safe (GRAS) status for food applications (Endres et al., 2009).Several health benefits have been reported to BC, such as as irritable bowel syndrome (Dolin, 2009; Hun, 2009), intestinal gas (Kalman et al., 2009), colitis (Fitzpatrick et al., 2012) in lactose intolerance (Maathuis et al., 2010), in rheumatoid arthritis (Mandel et al., 2010), in immune modulating and anti-inflammatory action (Jensen et al., 2010) and in common viral infections of the respiratory tract (Jurenka, 2012).

The recognition of GRAS status for BC comprised an important milestone in the development of probiotic foods as spore-forming bacteria are known to present greater resistance to processing and GIT conditions (Cutting, 2011). The better survivability of BC throughout food processing and digestion is highly desirable as positive effects of probiotics on hosts are dependent on the intake (Hong et al., 2005; Nithya and Halami, 2013). Even though the ability to form spores is key for the survival of BC, probiotic efficacy is also dependent upon the food matrix (Ranadheera et al., 2010). An adequate food matrix (vehicle) should be able to protect, carry, deliver and overcome the physical and chemical barriers in the GIT (Piano et al., 2006; Possemiers et al., 2010; Ranadheera et al., 2010).

The gastrointestinal tract (GIT) is a dynamic microecosystem with a large diversity of microbial species in equilibrium (Collado et al. 2009; Gerritsen et al. 2011). Despite this, the consumption of probiotics can modulate the intestinal microbiota and maintain or restore human health (Ng et al., 2013). Therefore, the introduction of probiotics in foods may comprise a wise strategy to modify human microbiota and improve health parameters. For simulate the survival of probiotics in the GIT, several *in vitro* digestion models exist, such as

TIM-1 dynamic system (Martinez et al., 2011), dynamic gastrointestinal digester (DIDGI) (Adouard et al., 2016) and simulator of the human intestinal microbial ecosystem (SHIME) (Possemiers et a., 2010). These dynamic models operate simulating the physiological conditions of digestion, such as gastric and/or intestinal, pH and temperature regulation, peristaltic movements, transit time and gastrointestinal secretions (digestive enzymes, mineral solutions and acid or base solutions). Additionally, *in vitro* models allow a greater reproducibility, few ethical issues and the ability to collect samples throughout the experiment compared to *in vivo* models (Adouard et al., 2016; Villemejane et al., 2015).

Dairy products, such as, yogurt, fermented milk, cheese and ice-cream are amongst the mostly used vehicles for delivering probiotics (Cruz et al., 2009; Cruz et al., 2012; Guo et al., 2016; Lollo et al., 2015; Seppo et al., 2003). Yogurt is a very popular food matrix and highly used to deliver probiotic microorganisms mainly due it is recognized in the consumer's mind as a product healthy (Lourens-Hattingh, Viljoen, 2001). On the other hand, fruit juices are not frequently considered adequate matrices to deliver probiotic microorganisms such as *Lactobacillus* and *Bifidobacterium*. The low pH of fruit juices limit the application of most probiotic microorganisms, which loose viability very quickly when exposed to acidic conditions during storage (Yoon et al., 2006). In this way, strategies to increase probiotic viability such as encapsulation has been tested (Antunes et al., 2013; Chaikham et al., 2017). Hurdles such low pH, high temperature, low water activity among others, have been limiting the application of probiotic in a series of foods (Zhang et al., 2014 ; Zheng et al., 2014), despite the consumer demands on functional foods (Fares et al., 2015; Martins, 2013). In view of the foregoing, the use of spore-forming bacterias representing an ideal choice for the development of functional products. Therefore, considering the importance to assess the impact of food matrix on probiotic efficacy, in this study an *in vitro* dynamic model was used to evaluate the viability of BC in orange juice and yogurt during simulated gastric and intestinal conditions.

2. Materials and Methods

2.1. Probiotic Bacillus Strains

Bacillus coagulans GBI-30, 6086 spores were used this study. This probiotic strain was donated by Ganeden Biotech Inc, Mayfield Heights, Ohio, USA.

2.2. Food preparation

2.2.1. Orange juice

The orange juices were prepared using commercial concentrated pulp (65.2° brix). The juice acidity was adjusted to 11°brix with water. Then, the juice (pH 3.6) was pasteurized in water bath Quimis, model 0334M-28 (Diadema, SP, Brazil), at 95°C for 30 seconds. For the preparation of the probiotic juice, BC spores were inoculated after thermal processing at concentration 10^8 CFU/ mL. The juice samples were prepared and stored at 4°C.

2.2.2. Yogurt

The yogurts were prepared according to procedures of Tamime and Robinsons (1999). The homogenized and sterilized milk was standardized (total solids 13%) through the addition of skimmed milk powder. The mixture was subjected to thermal treatment (90°C for 5 minutes) in water bath Quimis, model 0334M-28 (Diadema, SP, Brazil), cooled down to 42°C, added 2.5% (v/v) traditional lactic culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus,* CHR-Hansen, Brazil) and fermented (45°C) in kiln Marconi, model MA 032 (Piracicaba, SP, Brazil) until pH 4.6. Then, the yogurt was cooled down to 10°C. For the preparation of the probiotic yoghurt, BC spores were added at 1% (v/v) proportion after fermentation at concentration 10^8 CFU/ mL.The yogurt samples were prepared and stored at 4°C.

2.3. Experimental design

2.3.1. Dynamic gastrointestinal model and conditions tested

The dynamic system (Martinez et al., 2011 and Minekus et al., 1995 with modifications) was used to evaluate the fate of BC in different food matrices (juice and yogurt) through the passage in the gastrointestinal tract. The environmental conditions (pH, temperature and residence time) were controlled to mimic *in vivo* conditions. The system consisted of three flasks simulating the stomach, duodenum and jejunum, respectively. The total process time for food to pass through the three flasks (compartments) was 6h. Each flask representing a

compartment was placed within a container with circulating water to maintain the required temperature (37°C). The circulation of water in the containers was ensured through a water bath Fanem (Sao Paulo, Brazil), connected to the system with constant water flow. The homogenization of contents within compartments was made by magnetic stirrer placed within each flask. The pH was monitored by using potentiometers (pH electrode) Akso AK103 model pHmetrer (Rio Grande do Sul, Brazil), coupled to each compartment. Acidic (HCl, 1M) and basic (NaOH, 1M) solutions were used to adjust the pH within each compartment. Peristaltic pumps Masterflex (Sao Paulo, Brazil) ensured the flow of contents between compartments. The flow of the peristaltic pumps were 2.22 mL / min (pump1 and 2) and 1.66 mL / min (pump 3 and 4) (Fig. 1). Pepsin and lipase were secreted in gastric compartment. Bile and pancreatic juice were secreted in the duodenal and jejunal compartment.

The simulated secretion in the gastric compartment comprised of a gastric electrolyte solution (NaCl [47.2 mmol/L], KCl [6.9 mmol/L], KH_2PO_4 [0.9 mmol/L], $MgCl_2(H_2O)_6$ [0.1 mmol/L] and $NaHCO_3$ [25 mmol/L]) with 600 U per ml of pepsin and 60 U per ml of lipase. The pH was controlled at 2.0. The residence time in the gastric compartment was 90 min. The duodenal and jejunal secretions comprised the electrolyte solution (NaCl [38.4 mmol/L], KCl [6.8 mmol/L], KH_2PO_4 [0.8 mmol/L], $MgCl_2(H_2O)_6$ [0.33 mmol/L] and $NaHCO_3$ [25 mmol/L]) with bile (1.2 g/L) and pancreatin (0.18 g/L) (Sigma-Aldrich, Brazil). In the duodenum and jejunum the pH were maintained at 6.0 and 7.0, respectively. The residence time was 2h in each compartment, i.e.,duodenum and jejunum. For each experiment, 100 mL of food matrix (juice or yogurt) was used in a ratio of 1:5 (food matriz: solutions). For each food matrix (juice and yogurt) two independent experiments were conducted. All reagents were from Dinamica quimica (Diadema, SP, Brazil), with the exception of pepsin, lipase, bile and pancreatin that were from Sigma-Aldrich (Sao Paulo, Brazil).

Fig. 1. Dynamic gastrointestinal simulator.

2.4. Enumeration of BC

The samples of juice and yogurt were collected before (N_0) and during the simulated dynamic gastrointestinal conditions. During the passage of juice and yogurt through the dynamic gastrointestinal system, eight samples were collected in different intervals. The sampling points were: 1-content (food matrix + compartment solution) immediately contact with the gastric compartment (low pH-2, 30 min); 2 and 3-content in rest (1h15min) and dynamic (2h) stage in gastric compartment; 4 and 5-content in rest (3h) and dynamic (4h) stage in duodenal compartment; 6 and 7-content in rest (5h) and dynamic stage (6h) in jejunal compartment; 8-content in end collector (6h). The samples were collected (1 mL) and for the enumeration were used samples without and with thermal shock (thermal process at 80°C/ 10 min and cooling). Next, decimal dilutions were prepared in 0.1% peptone water. Then, aliquots of 1mL were inoculated in BC agar (g/L: yeast extract powder (5g), peptone (5g), glucose (5g), potassium phosphate dibasic (0.5g), potassium phosphate monobasic (0.5g), magnesium sulfate (0.3g), trace mineral solution (1mL)). All reagents to prepare the medium were from Dinamica quimica (Diadema, SP, Brazil), with the exception of yeast extract powder and pepton that were from kasvi (Curitiba, PR, Brazil). The plates were incubated in kiln Marconi, model MA 032 (Piracicaba, SP, Brazil) at 42°C for 48h. The counts of BC were

reported as log_{10} colony forming units (CFU) per ml of juice or g of yogurt. The culture medium BC and incubation conditions were formulated and performed according to the supplier's guidelines of the BC strain used.

2.5. Statistical analysis

The results were expressed as means ± standard deviation and analyzed by ANOVA, followed by the t test and Scott-Knott using the Sisvar software 5.6 (Lavras, MG, Brazil). The differences were considered statistically significant when $p < 0.05$. Two replicates were made for each food matrix.

3. Results

The fate of BC in probiotic orange juice and yogurt was evaluated under in *vitro* GIT conditions. The results of the present study showed high survival for *B. coagulans* GBI-30, 6086 in the samples of juice and yogurt (Table 1). When analyzed each one of the samples (juice, yogurt, juice (CH) and yogurt (CH)), considering the total *in vitro* assay (0 min up to 6h) there was no significant difference in the survival of BC, with only 1 log drop (Table 1). The table 1 showed that there was no significant difference in survival of BC both between the samples of juice and yogurt without thermal shock and between juice and yogurt with thermal shock (CH) in the different steps tested (0 min, 30 min, 1h15 min, 2h, 3h, 4h, 5h, 6h and end collector). When evaluating juice and yogurt samples separately, there was no significant difference in survival of BC in the samples of both juice and yogurt, without and with thermal shock, respectively, among the different steps tested (0 min, 30 min, 1h15 min, 2h, 3h, 4h, 5h, 6h and end collector). Additionally, the table 1 showed that there was no significant difference in survival of BC between the samples of juice without and with thermal shock (CH) and between yogurt without and with thermal shock (CH) in the different steps tested (0 min, 30 min, 1h15 min, 2h, 3h, 4h, 5h, 6h and end collector), respectively.

Table 1

Survival of *B. coagulans* GBI-30, 6086 in the samples of juice and yogurt without and with thermal shock, collected from the *in vitro* GIT model at 0 min, 30 min, 1h15 min, 2h, 3h, 4h, 5h, 6h and end collector.

	Juice (log CFU/mL)	Yogurt (log CFU/mL)	Juice (CH) (log CFU/mL)	Yogurt (CH) (log CFU/mL)
Zero	8.37 ± 0.11 ^{aA*}	8.59 ± 0.01 ^{aA*}	8.14 ± 0.13 ^{aA*}	8.37 ± 0.25 ^{aA*}
30 min	7.67 ± 0.30 ^{aA*}	7.77 ± 0.01 ^{aA*}	7.48 ± 0.31 ^{aA*}	7.74 ± 0.00 ^{aA*}
1h15min	7.53 ± 0.12 ^{aA*}	7.86 ± 0.07 ^{aA*}	7.40 ± 0.09 ^{aA*}	7.62 ± 0.23 ^{aA*}
2h	7.87 ± 0.01 ^{aA*}	7.89 ± 0.07 ^{aA*}	7.71 ± 0.19 ^{aA*}	7.56 ± 0.35 ^{aA*}
3h	7.55 ± 0.37 ^{aA*}	7.48 ± 0.01 ^{aA*}	7.44 ± 0.26 ^{aA*}	7.22 ± 0.24 ^{aA*}
4h	7.82 ± 0.10 ^{aA*}	7.50 ± 0.04 ^{aA*}	7.46 ± 0.33 ^{aA*}	7.35 ± 0.18 ^{aA*}
5h	7.69 ± 0.40 ^{aA*}	7.54 ± 0.21 ^{aA*}	7.30 ± 0.00 ^{aA*}	7.21 ± 0.08 ^{aA*}
6h	7.71 ± 0.38 ^{aA*}	7.32 ± 0.29 ^{aA*}	7.31 ± 0.22 ^{aA*}	7.22 ± 0.20 ^{aA*}
End collector	7.56 ± 0.54 ^{aA*}	7.25 ± 0.32 ^{aA*}	7.25 ± 0.22 ^{aA*}	7.10 ± 0.28 ^{aA*}

-(CH) Samples that were subjected to thermal shock; Means ± standard deviation of two replicates; Means with different superscript lowercase letters on the same column indicate significant difference between samples by Scott-Knott ($p<0.05$). Means with different superscript capital letters on the same line indicate significant difference between samples of juice and yogurt without thermal shock and juice and yogurt with thermal shock (CH), respectively, by test-t ($p<0.05$). Means with different superscript symbols on the same line indicate significant difference between samples of juice without thermal shock and with thermal shock (CH) and yogurt without thermal shock and with thermal shock (CH), respectively, by test-t ($p<0.05$).

The juice and yogurt showed decimal reductions (gamma) ranging in the stomach from 0.50 to 0.84 log CFU/g and 0.69 to 0.81 log CFU/g without thermal shock, respectively. While, with thermal shock (CH) ranged from 0.43 to 0.66 log CFU/g and 0.63 to 0.81 log CFU/g, respectively. In the duodenum compartment ranging from 0.55 to 0.82 log CFU/g and 1.09 to 1.10 log CFU/g without thermal shock, respectively. While, with thermal shock (CH) ranged from 0.67 to 0.70 log CFU/g and 1.02 to 1.15 log CFU/g, respectively. Finally, in the jejunum compartment ranging from 0.66 to 0.68 log CFU/g and 1.04 to 1.27 log CFU/g without thermal shock, respectively. While, with thermal shock (CH) ranged from 0.83 to 0.83 log CFU/g and 1.15 to 1.17 log CFU/g, respectively. There was a approximately 1 log decrease in stomach, probably due to the low pH. In the other compartments (duodenum and jejunum) and until the end of the passage in the gastrointestinal tract (6h) the probiotic bacteria strain remained with decimal reductions (gamma) around 1 log CFU/mL in the samples of juice, yogurt, juice (CH) and yogurt (CH). There was no significant difference

($p<0.05$) in decimal reductions (gamma) between the stages of each juice and between juice without thermal shock and juice with thermal shock (Table 2). As for yogurt, there was no significant difference ($p<0.05$) in decimal reductions (gamma) between the stages of each yogurt and between yogurt without thermal shock and juice with thermal shock (Table 2). Additionally, there was no significant difference ($p<0.05$) in decimal reductions (gamma) between juice without thermal shock and yogurt without thermal shock and between juice with thermal shock and yogurt with thermal shock, respectively.

Table 2

Decimal reductions (gamma) of *B. coagulans* GBI-30, 6086 in the samples of juice and yogurt without and with thermal shock, collected from the *in vitro* GIT model at 0 min, 30 min, 1h15 min, 2h, 3h, 4h, 5h, 6h and end collector.

	Juice γ **(log CFU/mL)**	**Yogurt** γ **(log CFU/mL)**	**Juice (CH)** γ **(log CFU/mL)**	**Yogurt (CH)** γ **(log CFU/mL)**
30 min	0.70±0.20 ^{aA*}	0.81±0.00 ^{aA*}	0.66±0.18 ^{aA*}	0.63±0.27 ^{aA*}
1h15min	0.84±0.01 ^{aA*}	0.73±0.09 ^{aA*}	0.64±0.04 ^{aA*}	0.75±0.02 ^{aA*}
2h	0.50±0.12 ^{aA*}	0.69±0.09 ^{aA*}	0.43±0.33 ^{aA*}	0.81±0.10 ^{aA*}
3h	0.82±0.49 ^{aA*}	1.10±0.02 ^{aA*}	0.70±0.40 ^{aA*}	1.15±0.51 ^{aA*}
4h	0.55±0.01 ^{aA*}	1.09±0.06 ^{aA*}	0.67±0.46 ^{aA*}	1.02±0.45 ^{aA*}
5h	0.68±0.30 ^{aA*}	1.04±0.21 ^{aA*}	0.83±0.13 ^{aA*}	1.17±0.34 ^{aA*}
6h	0.66±0.28 ^{aA*}	1.27±0.30 ^{aA*}	0.83±0.09 ^{aA*}	1.15±0.47 ^{aA*}
End collector	0.81±0.44 ^{aA*}	1.34±0.33 ^{aA*}	0.89±0.09 ^{aA*}	1.17±0.54 ^{aA*}

- (CH) Samples that were subjected to thermal shock; (γ=No-Nf); (No) Initial count: Juice: 8.37; Yogurt: 8.59; Juice (CH): 8.14; Yogurt (CH): 8.37; Means ± standard deviation of two replicates; Means with different superscript lowercase letters on the same column indicate significant difference between samples by Scott-Knott ($p<0.05$). Means with different superscript capital letters on the same line indicate significant difference between samples of juice and yogurt without thermal shock and juice and yogurt with thermal shock (CH), respectively, by test-t ($p<0.05$). Means with different superscript symbols on the same line indicate significant difference between samples of juice without thermal shock and with thermal shock (CH) and yogurt without thermal shock and with thermal shock (CH), respectively, by test-t ($p<0.05$).

4. Discussion

Currently, there is a large scientific interest in food digestion. Studies that address survival and persistence/functionality of probiotic bacteria are of fundamental importance for the development of new probiotic food. Also to know the possible mechanisms of action and how these probiotics might affect human health (Kailasapathy, 2006; Mainville et al., 2005; Uriot et al., 2016) it is important to understand survival and persistence/functionality of probiotic bacteria. As probiotic bacteria should survive the food processing and the passage in the gastrointestinal tract to deliver health benefits (Silva et al., 2017), several strategies have been studied to increasing probiotic survival throughout GIT. These approaches include selection of strains adapted to stressing conditions (Collado, Sanz, 2007), use of microencapsulation (Eratte et al., 2017) and prebiotics (Martinez et al., 2011), among others. Nonetheless, the application of probiotic spore-forming bacteria seems to comprise the best approach to expand the delivery of probiotics to a wide range of foods (Cutting, 2011).

Similarly to our study, Uriot et al. (2016) observed significantly survival of *Streptococcus thermophilus* (strains LMD9, PB18O and EBLST20) in all digestive compartments of the gastrointestinal tract simulator, which may be related to the presence of urease and heat shock protein functions. In addition, when *S. thermophilus* LMD9 was delivered in a fermented milk, a significant improvement of survival in the gastrointestinal tract simulator was observed compared to non-fermented milk. However, other strains (*Lactobacillus acidophilus*, *L. casei, L. helveticus, L. rhamnosus, Bifidobacterium lactis* and *Bif. Longum*) tested *in vitro* showed a much lower survival (Caillard and Lapointe, 2017). The resistance to simulated gastric juices of several commercially available probiotics products were studed (sixteen strains, incluiding eleven *Lactobacillus*, four *Bifidobacterium* and one *Pediococcus* and ten oral forms such as enteric/non-enteric capsules/tablets and microencapsulated strains). The results demonstrated that all tested strains showed high sensitivity to acidic conditions and suggested that most of these microorganisms would not show any viability when immersed in the stomach.

The results of this study are consistent with other studies involving other strains and dairy matrix. Madureira et al. (2011) observed the survival of the strains (*L. casei* LAFTI®L26, *L. acidophilus* LAFTI®L10 or *Bifidobacterium animalis* Bo) during transit throughout the simulated gastrointestinal system. When tested the survival in the MRS system

L. casei LAFTI®L26, *L. acidophilus* LAFTI®L10 and *B. animalis* Bo showed decimal decreases of 4 log cycle, 5 log cycle and 0 log cycle in stomach, respectively. In duodenum, *L. casei* LAFTI®L26 vanished, *L. acidophilus* LAFTI®L10 did not change and *Bifidobacterium animalis* Bo fell 1 cycle. However, when inoculated strains (*L. casei* LAFTI®L26, *L. acidophilus* LAFTI®L10 and *B. animalis* Bo) in whey cheese matrix, the decimal reductions decreased for 1.5 log cycle, 3 log cycle and 0 log cycle in stomach, respectively. In duodenum, *L. casei* LAFTI®L26 and *L. acidophilus* LAFTI®L10 decreased 1 log cycle and *B. animalis* Bo did not change. Thus, the whey cheese matrix appeared to protect both the strains, being promising carriers of those probiotic bacteria. In addition, the largest reductions occurred in the stomach, as this is considered the critical phase due to the low pH. However, this was not observed in our study, possibly because of high spore resistance.

Study of Oliveira et al. (2014) revealed that *Lactobacillus acidophilus* LA-5, *Lactobacillus casei* paracasei 01 and *Bifidobacterium lactis* BB 12, added in a goat coalho cheese, survived (decimal reductions of 2-3 log cycles) the simulated gastrointestinal digestion in the upper gastrointestinal tract model, suggesting that these probiotic strains are able to pass through the gastrointestinal tract when delivered in the assayed cheese matrix. The survival of three commercial probiotic strains (*L. casei* shirota, *L. casei* Immunitas, *L. acidophilus* Johnsonii) in water and milk, using a dynamic gastric model of digestion followed by incubation under duodenal conditions was observed. When tested the survival in water, *L. acidophilus* Johnsonii, *L. casei* shirota and *L. casei* Immunitas showed decimal decreases of 5 log cycle, 5 log cycle and 8 log cycle in stomach (1h) and duodenum (2h), respectively. However, when inoculated strains (*L. acidophilus* Johnsonii, *L. casei* shirota and *L. casei* Immunitas) in milk, the decimal reductions decreased for 4 log cycle, 0 log cycle and 0 log cycle in stomach (1h) and duodenum (2h), respectively (Curto et al., 2016). Thus, the decimal reductions for *Lactobacillus* and *Bifidobacterium* (non-sporulated probiotics) were much higher than for *Bacillus*, because in our study the reductions were around 1 log whereas in the studies described the reductions reached up to 8 log.

Studies approaching other food matrix also were reported. Klindt-Toldam et al. (2016) showed the survival of *L. acidophilus* NCFM and *Bif. lactis* HN019 in two types of chocolate (milk chocolate and dark chocolate) during *in vitro* simulated passage. The two probiotics strains showed good survival during simulated passage of gastrointestinal tract. The viability of *Bif. lactis* was slightly higher (decimal reductions approximately 0 log CFU/g) than *L.*

acidophilus (decimal reductions approximately 0.6 log CFU/g), however, the difference was non-significant, with milk chocolate being the most protective carrier. Possemiers et al. (2010) have also shown that chocolate is excellent probiotic delivery (*Lactobacillus helveticus* CNCM I-1722 and *Bifidobacterium longum* CNCM), both chocolates (milk and dark chocolate) offered superior protection to milk. In this way, showing the importance of the food matrix (composition) in survival of non sporulated bacteria during passage through gastrointestinal tract.

The survival of *Bacillus* strain *in vitro* simulated gastrointestinal conditions (static system) has also been reported. Hong et al. (2008) reported that spores of strains of *Bacillus indicus* HU36, *Bacillus subtilis* PY79 and *B. subtilis* Natto PY79 were completely resistance (decimal reductions approximately 0 log CFU/g) to exposure to simulated gastric fluid and simulated intestinal fluid, thus corroborating with our study. The survival and persistence of *Bacillus* strains in gastrointestinal tract is justified for the germination of the spore in region of the jejunum and ileum where the pH of the gastric juices has been sufficiently reduced. That could allow production of antimicrobial agents, such as bacteriocin-like inhibitory substances, thereby contributing to the competitive exclusion of pathogens, and thus present probiotic effect (Duc et al., 2004). Nevertheless, the mechanisms of action of probiotic *Bacillus* strains are not fully understood yet. Regarding the survival of *Bacillus* (strain itself or inoculated in some alimentary matrix) to conditions of the gastrointestinal tract in dynamic system, this has not yet been reported until then.

Therefore, as no significant difference was observed in the decimal reductions (gamma) between samples (juice or yogurt) without thermal shock and with thermal shock, to evaluate the survival of BC, thermal shock can be applied or not, both in juice and yogurt. In this study, the matrix may not be as important in the survival of sporulated probiotics (strain BC) as it is in non-sporulated probiotics. The high survival of BC in the gastrointestinal tract simulator may be due to high spore resistance, as there were only a drop of approximately 1 log cycle during total passage (6h) through the gastrointestinal tract simulator (*in vitro*), both juice and yogurt. Additionaly, no significant difference was observed in the decimal reductions (gamma) between samples of juice and yogurt, both without thermal shock and with thermal shock. In this way, both juice and yogurt can be considered probiotic after total passage (6h) by the gastrointestinal tract simulator. Acording to Uriot et al. (2016) the high survival capacities are generally associated with probiotic efficacy. Thus, *B. coagulans* GBI

30, 6086 is likely to exhibit excellent probiotic efficacy given its high ability to survive gastrointestinal conditions.

5. Conclusions

In conclusion, a high survival (decimal reductions approximately 1 log cycle) for *B. coagulans* GBI-30, 6086 was observed in two food matrices (juice and yogurt), it seems that for sporulated probiotics (strain BC) the food matrix does not have as much influence as for non-sporulated probiotics. Additionaly, both are excellent food matrices to function as probiotic carriers during passage through the gastrointestinal tract. The development of food products containing *Bacillus* may be excellent strategies for transporting probiotics in foods whose characteristics are deleterious to *Lactobacillus* and *Bifidobacterium* and/or foods whose process may inactivate or lead to reduced viability of *Lactobacillus* and *Bifidobacterim,* thus contribute to growth of functional food area.

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Bacillus coagulans GBI-30, 6086 spores modulates intestinal microbiota and changes health parameters in rats

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Abstract

The aim of this study was to investigate the impact of the type different of probiotic food matrixes (orange juice and yogurt) on the persistence/functionality of spore-forming probiotic microorganism (*Bacillus coagulans* GBI-30, 6086) on microbial ecology and health parameters of male rats. For this purpose, 48 male rats were divided into 6 groups: control group received distilled water; juice group received orange juice; yogurt group received yogurt; probiotic *Bacillus* group received *Bacillus* probiotic suspended in distilled water; juice probiotic group received probiotic orange juice and yogurt probiotic group received probiotic yogurt. All animals received 4mL/day of each food matrix by gavage during twenty one days. Then, the animals were sacrificed and the gastrocnemius muscle, cecal content and blood were collected and analyzed. The results of the *in vivo* tests on health parameters showed that the group fed with probiotic yogurt exhibited significantly lower glucose and triglycerides levels ($p<0.05$) and higher abundance and diversity on gut microbiota profile when compared to the control group ($p<0.05$). Additionally, the probiotic yogurt group demonstrated a increase of the class of Actinobacteria and Bacillales and Bifidobacteriales orders when compared to the control group ($p<0.05$). Our findings suggest that consumption of probiotic yogurt can be potentially useful, since it decreased the glicose and triglycerides levels and modulated the intestinal microbiota. Regarding probiotic juice group, there was no significant beneficial effect ($p<0.05$) on any of the evaluated health parameters and intestinal microbiota. In conclusion, our study proved that yogurt is an excellent probiotic carrier matrix, acting in

the reduction of glucose and triglycerides levels and modulation of the intestinal microbiota. Therefore, yogurt as a matrix had a fundamental influence on the survival and effects of probiotic bacteria (*in vivo*).
Keywords: *Bacillus*; health parameters; intestinal microbiota; juice; spore-forming probiotic; yogurt.

1. Introduction

The probiotics have been defined as "*live microorganisms that when administered in adequate amounts confer health benefits on the host*" (Hill et al., 2014). If on one hand probiotic strains of *Lactobacillus* and *Bifidobacterium* have been the main microorganisms applied in foods (Saxelin, Tynkkynen, Mattila-Sandholm & De Vos, 2005), there is a growing interest on the addition of probiotic spore-forming bacteria such as *Bacillus coagulans* and *Bacillus subtilis* in foods (Cutting, 2011; Hung at al., 2012; Paik, Park, & Park, 2005). This interest is based on the fact that *Bacillus* are resistant to several unit operations used during food processing and they also survive better adverse gastric and intestinal conditions due to the greater resistance of the spores (Hong, Duc, & Cutting, 2005). Among several potentially probiotic spore-forming strains, *Bacillus coagulans* GBI-30, 6086 can be highlighted because it is a non-toxic, non-pathogenic and safe for human use probiotic strain (Endres et al., 2009).

Several health benefits have been related to the consumption of probiotics (*in vivo*). Benefical effects involving non-sporuled probiotics without food matrix carrier: Prevention and treatment of gastrointestinal diseases as irritable bowel syndrome (Kajander et al., 2008) and inflammatory bowel diseases (Amit-Romach, Uni & Reifen, 2010), modulation of immune system and treatment/improvement of respiratory and food allergies and atopic diseases such as asthma (Hougee et al., 2010) and atopic dermatitis (Kirjavainen, Salminen, & Isolauri, 2003), decrease in cholesterol levels (Huang, Wang, Cheng, & Zheng, 2010), attenuation in the development of hypertension and reduction of blood pressure (fermented milk) (Jauhiainen et al., 2005, Seppo, Jauhiainen, Poussa, & Korpela, 2003), antidiabetic effect (Tabuchi et al., 2003), improvement of oral health (Maekawa & Hajishengallis, 2014; Teughels et al. 2013) and reduction of symptoms of stress-related disorders, such as anxiety and depression (Bravo et al., 2011; Rao et al., 2009); Non-sporuled probiotics in food matrix: Modulation of the immune system (probiotic in malted milk) (O'Mahony et al., 2005), attenuation in the development of hypertension and reduction of blood pressure (probiotic in

cheese) (Lollo et al., 2015), relief of lactose intolerance symptoms (probiotic in yogurt) (He et al., 2008), combat of infection by pathogens (probiotic in fermented milk) (Mirzaei, Shahirfar, & Mobayen, 2012) and treatment/improvement of respiratory diseases (probiotic in fermented milk) (Wang, Lin, Wang, & Hsu, 2004); Spore-forming probiotics without food matrix carrier: prevention and treatment of gastrointestinal diseases as irritable bowel syndrome (Dolin, 2009; Hun, 2009) and inflammatory bowel diseases (Im, Choi, Pothoulakis, & Rhee, 2009); modulation of the intestinal microbiota (Sun, Yang, Ma, Song, & Lin, 2011), immune system and relief of lactose intolerance symptoms (Kimmel, Keller, Farmer, & Warrino, 2010); Spore-forming probiotics in food matrix: Modulation of the intestinal microbiota (probiotic in cereals-mix fermented) (Ng et al., 2013) and imunne system (probiotic in milk) (Sun, Wang & Zhang, 2010). However, there are still few studies reporting on the health effects (*in vivo)* of foods containing probiotic spore-forming bacteria.

For beneficial effects to be observed, the consumption of a minimum concentration of probiotics of 10^6 CFU/g is deemed, which represents a daily dose of 10^8 CFU /g. This dose is required to compensate for a possible reduction in the concentration of probiotics during passage through the gastrointestinal tract (Granato, Branco, Cruz, Faria, & Shah , 2010; Shah, 2007). As the food matrix has an important role on probiotic efficiency (Ranadheera, Baines, & Adams, 2010), the choice of not all food matrixes may be considered suitable for all probiotic strains. In addition to being responsible for transporting the probiotics, food matrix also impact on probiotic colonization of the gastrointestinal tract. Another important aspect of food matrix on probiotic efficacy is that food may contain functional ingredients, which combined with metabolites of the microorganisms can act synergistically and beneficially impact on the health of the host (Ranadheera, Baines, & Adams, 2010). In this way, yogurt is the most commonly applied food matrix to deliver probiotics (Abadía-García et al., 2013; Lourens-Hattingh & Viljoen, 2001; Ross, Fitzgerald, Collins, & Stanton, 2002). Despite this, there is a growing interest in application of probiotics to non-dairy products (Heenan, Adams, Hosken, & Fleet, 2004). The diversification of food matrixes to deliver probiotics is key for the given the above and considering that juices and dairy products are amongst the most consumed foods throughout the world, the aim of this study was to investigate the impact of addition of *Bacillus coagulans* GBI-30, 6086 to these food matrixes on the microbial ecology of the gut and health status of male rats.

2. Materials and Methods

2.1. Probiotic Bacillus Strain

The probiotic strain used in our study was *Bacillus coagulans* GBI-30, 6086 kindly donated by (Ganeden Biotech Inc, Mayfield Heights, Ohio, USA).

2.2. Food matrix preparation and inoculation of Bacillus coagulans GBI-30, 6086

The orange juices were prepared using commercial concentrated pulp. The juice soluble solids content was adjusted to 11°brix with water, following pasteurization in water bath Quimis, model 0334M-28 (Diadema, SP, Brazil) at 95°C for 30 seconds. For the preparation of the probiotic juice, *Bacillus coagulans* GBI-30, 6086 spores were inoculated after thermal processing at concentration 10^8 CFU/ mL. The probiotic juice were prepared weekly and stored at 4°C.

The production of yogurts was performed according to procedures described by Tamime and Robinsons (1999). The sterilized and standardized (total solids 13%) milk was subjected to thermal treatment (90°C/5 minutes) in water bath Quimis, model 0334M-28 (Diadema, SP, Brazil) and cooled down to 42°C. Then, traditional lactic culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus,* CHR-Hansen, Brazil) was added at 2.5% (v/v), following fermentation in kiln Marconi, model MA 032 (Piracicaba, SP, Brazil) at 45°C until pH 4.6 and cooling to 10°C. For the preparation of probiotic yoghurt, *Bacillus coagulans* GBI-30, 6086 spores were added after fermentation to reach a concentration of 10^8 CFU/mL. The probiotic yogurt samples were prepared weekly and stored at 4°C.

The probiotic bacteria strain presented counts around 8 log CFU/mL throughout the storage, 21 days in juice and yogurt.

2.3. Determination of food matrix centesimal composition

Moisture and ash were determined according to AOAC (2012) methods and total lipids following to IAL (2005) method in all food matrix. Total proteins were determined in juices according to AOAC (2012) method and in yogurts following to IAL (2005) method. The total carbohydrates content was inferred by difference.

2.4. Assessment of health benefits due to consumption of probiotic foods

2.4.1. Animals and diets

A total of 48 male (21-d-old, specific pathogen free) Wistar rats were obtained from the Multidisciplinary Center for Biological Research (University of Campinas, SP, Brazil). The animals remained in individual cages (22 ± 1°C, 60-70% humidity, inverted 12 h light/12 h dark cycle), for adaptation with free access to water and commercial chow (Nuvital, Brazil) for 3 weeks. The experiment was approved by the Ethics Commission on Animal Use (CEUA, UNICAMP, protocol n° 3456-1). After adaptation, the animals were randomized into 6 groups (n-8 per group): "*control*" group received distilled water; "*juice"* group received orange juice; "*yogurt"* group received yogurt; "*probiotic Bacillus"* group received *Bacillus* probiotic suspended in distilled water (10^9 CFU for portion (4mL)); "*juice probiotic"* group received probiotic orange juice (10^9 CFU for portion of juice (4mL)) and "*yogurt probiotic"*group received probiotic yogurt (10^9 CFU for portion of yogurt (4mL)). All animals received 4mL/day of their food matrix respectively, by gavage during twenty-one days. Weight gains was monitored weekly and the food intake every 2 days. Then, the animals were sacrificed and the gastrocnemius muscle and cecum fecal samples were removed and stored at -80°C. Similar experiment design were performed using probiotic fermented milk (Lollo et al., 2015b) and probiotic dairy dessert (Moura et al., 2016) and green propolis (Roquetto et al., 2015).

2.4.1.1. Blood analyses

Blood samples were collected and centrifuged at 3,000 x g (4°C, 10 min) to obtain serum. Serum biochemical parameters were determined using commercial kits according to the manufacturer's instructions (Labcenter, Brazil): aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, high density lipoprotein (HDL), triacylglycerols, uric acid, creatinine, glucose, total protein and albumin.

2.4.1.2. Western Blot

Muscle tissue samples were homogenized in the extracting buffer. The extracts were subjected to SDS-PAGE, transfer to nitrocellulose membranes, blocked with BSA (bovine

serum albumin) and incubated with specific primary antibodies. Immunoreactive bands were detected by chemiluminescence (Super Signal West Pico Chemiluminescent Substrate Kit, Thermo Scientific, USA). The bands were visualized using UNITEC instrument (model Alliance LD2) and blots were quantified using UN SCAN IT software. The western blotting analyzes were performed following a methodology described by Moura et al. (2016).

2.5. Intestinal microbiota profile

2.5.1. Identifying the instestinal microbiota

Total DNA was extracted from the cecal contents with the QIAmp DNA Stool Kit. For each sample, the 16S rRNA (V3-V4) was amplified using the direct amplifier corresponding to the following sequence: 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACTCCTACGGGAGGCAGCA G** 3′

In this sequence, the portion in bold font corresponds to the adapter Nextera® transposase sequences A, and the sequence in bold is the initiator widely conserved 338F. The reverse initiator used was: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TTACCGCGGCTGCTGGCA C**3′

The portion in bold corresponding to the adapter Nextera® transposase sequences B and the sequence in bold is the initiator of ample utilization 533R. For the preparation of the libraries, the kit “Illumina TruseqDNA Sample Preparation v2” was used, labeling each sample with a bar code. Sequencing was performed on an equipment Illumina Hiseq2000 at the Laboratório Central de Tecnologias de Alto Desempenho em Ciências da Vida (LaCTAD-Unicamp, Campinas, SP, Brazil). In order to identify the intestinal microbiota, it was used a methodology described by Roquetto et al. (2015) with modifications.

2.5.2. Taxonomic assignment obtained by 16S rRNA gene sequencing analysis

Initial sequences quality check were peformed by FASTQC and then quality filtering using Trimmomatic (0.36). The search for chimera was performed using UCHIME2 (Edgar, Haas, Clemente, Quince, & Knight, 2011). The sequences were then analyzed using QIIME (quantitative insights into microbial ecology), version 1.9.0, software (Caporaso et al., 2010).

OTUs were clustered at 97% identity using an open reference approaches (UCLUST algorithm) (Edgar, 2010) and identity against the Green genes bacterial 16S rRNA database (13_5 release) (McDonald et al., 2012) using RDPII classifier (Wang, Garrity, Tiedje, & Cole, 2007) and PyNast for align sequences (Caporaso et al., 2010). For annotation analysis was discardall OTUs that are observed fewer than 2 times (i.e., singletons). After that, the rare faction of the samples was performed (normalization for the same number of OTUs - 45035 OTUs). The rarefied data were used for alpha diversity evaluation through QIIME to generate rarefaction curves, Good's coverage, Chao1 richness (Chao & Bunge, 2002), Shannon and Simpson diversity indices (Shannon, 1948; Simpson, 1949). Beta diversity was evaluated with UniFrac (Lozupone & Knight, 2005). In addition, principal component analysis (PCA) (Gaze et al., 2015) was performed to emphasize variation and show patterns in the datasets. The matrix data set was composed of 6 lines and 19 columns, being the former the samples and the latter the values of the analyses (treatments and bacterial orders). For the PCA analysis, only OTUs at orders level with abundance values above 0.1% in at least six samples were used. Auto scaling of data was performed before the analysis in order to give the variables the same relevance. In order to provide a better separation of the samples Varimax rotation was used. PCA was performed with the software XLSTAT version 2015.2 (Adinsoft, Paris, France).The heat map exploratory data analysis tool to analyze feature and sample clusterings simultaneously was performed with the software XLSTAT version 2015.2 (Adinsoft, Paris, France).

2.6. Statical analysis

The datas of centesimal composition were presented as means ± SD and analyzed by ANOVA, followed by the Scott-Knott test using the Sisvar software 5.6 (Lavras, MG, Brazil). All others datas were presented as means ± SEM and analyzed by ANOVA, followed by the Duncan post-hoc test using the statistical package for social sciences (SPSS, Chicago, IL, USA) software, version 23.0 for windows. The level for significance was set to $p < 0.05$.

3. Results

3.1. Centesimal composition food matrix

The centesimal composition food matrix (juice, probiotic juice, yogurt and probiotic yogurt) is described below (Table 1). The parameters of moisture, ashes, total lipids, proteins and total carbohydrates did not differ significantly ($p<0.05$) between juice and probiotic juice and between yogurt and probiotic yogurt, respectively. However, there was a significant difference ($p<0.05$) between juice and yogurt and between probiotic juice and probiotic yogurt for the parameters of total lipids, proteins and total carbohydrates, respectively.

Table 1. Centesimal composition food matrix (juice, probiotic juice, yogurt and probiotic yogurt).

Parameters (g/100 mL)	Juice	Probiotic juice	Yogurt	Probiotic yogurt
Moisture	91.5±0.95 [a]	91.9±0.04 [a]	87.9±0.09 [a]	87.9±0.20[a]
Ashes	0.43±0.01 [a]	0.60±0.00 [a]	0.82±0.01 [a]	0.88±0.02 [a]
Total lipids	ND <0.10 [a]	ND <0.10 [a]	2.8±0.01 [b]	2.8±0.03 [b]
Proteins	0.79±0.00 [a]	0.82±0.00 [a]	3.3±0.01 [b]	3.4±0.02 [b]
Total Carbohydrates	11.2[a]	11.6[a]	5.0[b]	4.8[b]

Data are expressed as means ± SD. ND= Not detected. Different superscript letters on the same line indicate statistical differences by Scott-Knott test ($p<0.05$).

3.2. Diet intake and body weight

The consumption of probiotic juice or yogurt did not cause significantly difference in the body weight and food intake (commercial feed) of the rats, when compared to the control groups ($p<0.05$) (Table 2).

Table 2. Body weight and food intake of rats, at the end of twenty one experiment days.

Parameters (g)	Control	Juice	Yogurt	Probiotic *Bacillus*	Probiotic juice	Probiotic yogurt
Body weight	335.9 ± 8.0[a]	331.7 ± 6.2[a]	341.1± 6.9[a]	329.6 ± 7.8[a]	334.0 ± 9.0[a]	330.2 ± 7.7[a]
Food intake	593.6 ±20.2[a]	597.6 ±13.2[a]	551.4±18.2[a]	582.2 ±24.6[a]	568.9±23.3[a]	537.6 ±20.5[a]

Data are expressed as means ± SEM. Different superscript letters on the same line indicate statistical differences by Duncan test ($p<0.05$).

3.3. Biochemical parameters

The continued ingestion of probiotic yogurt (4 mL/day) during 21 days was able to significantly reduce classical health parameters in rats, such as glucose (9.82%) and triacylglycerols (34.66%) levels when compared to the control group ($p<0.05$). Thus, the administration of probiotic yogurt caused a reduction in these two parameters to smaller levels than the control group ($p< 0.05$). In addition, the probiotic *Bacillus* group also showed a significant reduction in triglycerides (23.85%) levels when compared to the control group ($p<0.05$). It was interesting to note, however, that levels of glucose and triacylglycerols did not vary in the rats fed with probiotic juice. (Fig. 1). The other parameters (ALT, AST, creatinine, uric acid, cholesterol, HDL, albumin and total protein) did not change significantly in the probiotic groups (rats fed with probiotic *Bacillus* in water, probiotic yogurt or probiotic juice) when compared to the control groups (rats fed with conventional yogurt or juice or control diet) ($p<0.05$).

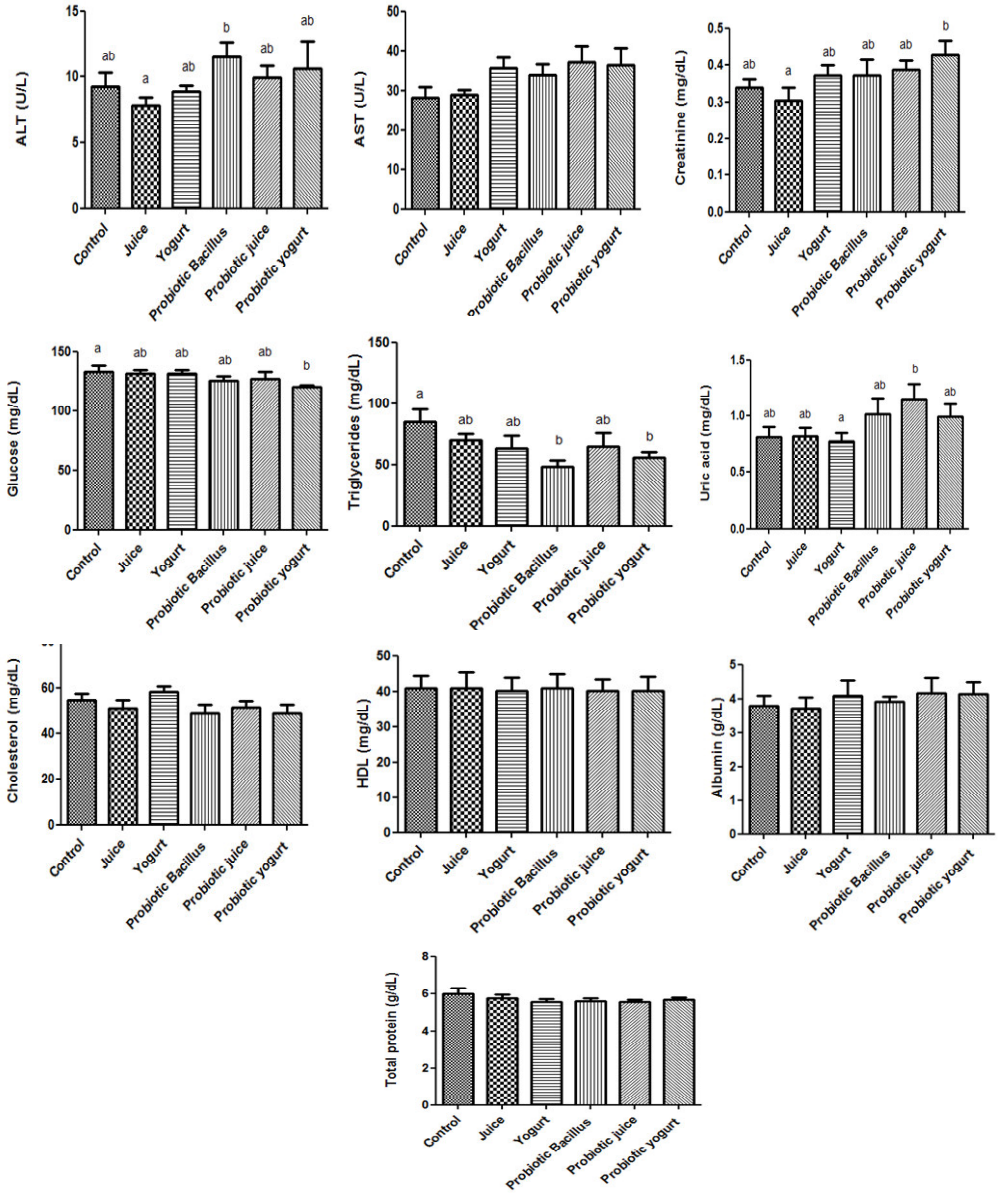


Fig. 1. Effect of administration of probiotic yogurt and juice during 21 days on biochemical parameters of rats. Data are expressed as means ± SEM. Different superscript letters indicate statistical differences by Duncan test ($p<0.05$).

3.4. Proteins expression

The consumption of probiotic juice and probiotic yogurt did not cause significantly difference on the expression of stress-related enzymes (SOD, GPx, catalase and thermal shock proteins (HSP 70) in the rats, when compared to the control groups ($p<0.05$) (Fig. 2).

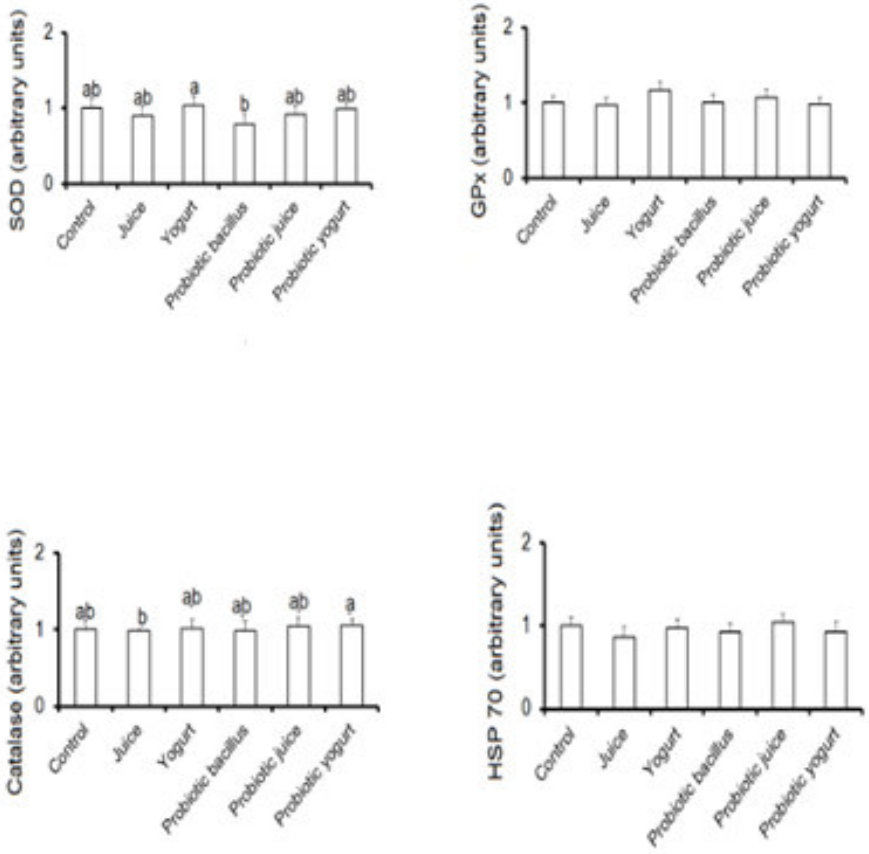


Fig. 2. Effect of administration of probiotic yogurt and juice during 21 days on expression of enzymes and thermal shock proteins of rats. Data are expressed as means ± SEM. Different superscript letters indicate statistical differences by Duncan test ($p<0.05$).

3.5. Gut microbiota profile

3.5.1. 16S rRNA gene sequencing-based structure of the microbiota

A total of 19.771.488 reads passed the sequence quality filters applied through the Trimmomatic (0.36) software, with an average value of 681.775 reads per sample after the quality filtering were obtained. The number of ace, Chao1, Good's estimated sample coverage (ESC), OTUs, Shannon and Simpson indices were obtained for all the samples (Table 3). In

general, the alpha diversity showed that "probiotic yogurt" samples showed higher values of diversity indices than the other samples (Table 3). The estimated sample coverage was satisfactory for 90% of the samples. The results of analysis of the beta diversity, based on unweighted Uni-Frac analysis, indicated that the "probiotic yogurt" samples formed a discrete group distinguished from the other five different types of samples (yogurt, juice, probiotic juice, probiotic *Bacillus* and control) (Fig. 3).

Table 3. The number of ace, Chao1, Good's estimated sample coverage (ESC), OTUs, Shannon and Simpson indices obtained for all the samples.

	Ace	chao1	ESC	Otus	Shannon	Simpson
Yogurt	15144.3	14089.4	0.94	3895.6	7.7	0.98
Probiotic yogurt	18788.5	17518.4	0.92	4852.2	8.2	0.99
Juice	15966.8	15141.7	0.94	4069.0	7.9	0.99
Probiotic juice	16191.7	15171.4	0.94	4014.6	7.8	0.98
Probiotic *Bacillus*	15197.9	14053.6	0.94	3983.0	7.7	0.98
Control	15204.3	14245.5	0.94	3905.8	7.7	0.98

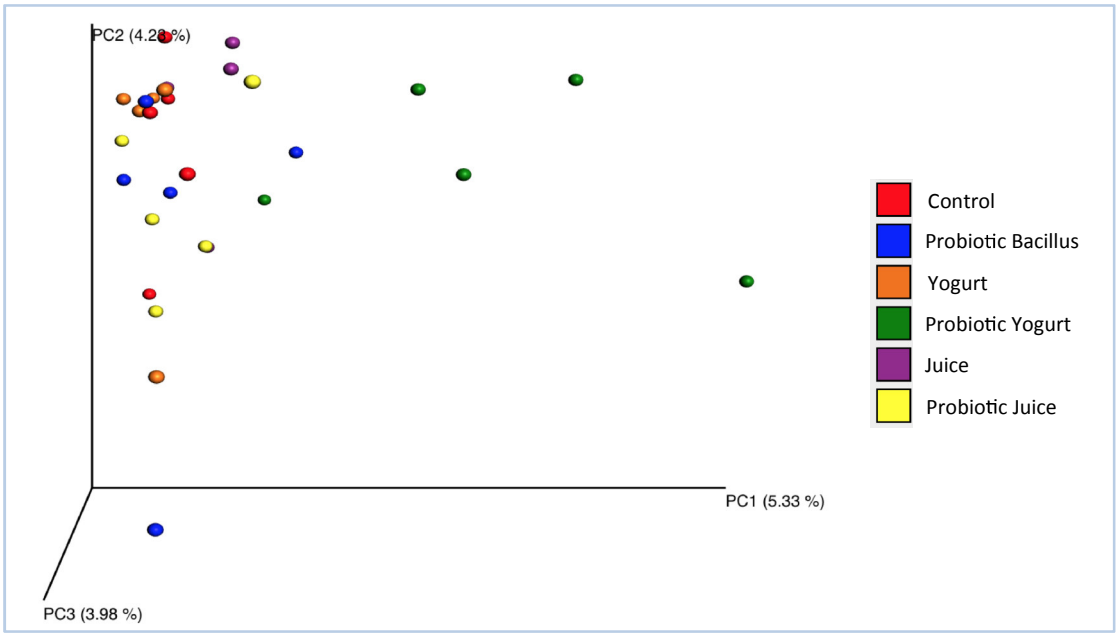


Fig. 3. Principal coordinate analysis of unweighted UniFrac distances for the 16S rRNA gene sequence data.

3.5.2. Taxonomic assignment obtained by 16S rRNA gene sequencing analysis

For the entire study, the majority of OTUs were attributed to the classes in the six treatments (yogurt, probiotic yogurt, juice, probiotic juice, probiotic *Bacillus* and control) were Clostridia followed by Bacteroidia and Bacilli (Table 4). The probiotic yogurt group showed significant abundance ($p<0.05$) of classes such as Actionacteria, Betaproteobacteria and Gammaproteobacteria compared to the control group. An abundance of three bacterial major orders (Clostridiales, Bacteroidales and Lactobacillales) was observed in all treatments (Table 5 and Fig 4A). In addition, probiotic yogurt treatment showed also Bifidobacteriales (0.1%) (Fig. 4B). However, besides bifidobacteriales a significant abundance ($p<0.05$) of the orders Enterobacteriales, Vibrionales and Alteromonadales was found.

To better visualize and explore the taxonomic assignment obtained by 16S rRNA gene sequencing analysis at bacterial orders level, a principal component analysis was performed (Fig. 5). Only OTUs with abundance values above 0.1% in at least six samples are shown were selected for PCA analysis. PCA explained approximately ~78% of the data variation, with 49.48% and 28.01% at the first and second dimensions, respectively, suggesting that the analysis contains relevant information on the issue under study. Indeed, PCA showed that the bacterial orders were clustering associated in different patterns with treatments (Fig. 5). The PCA showed that orders such as, Bifidobacteriales, Alteromonadales, Vibrionales, Bacillales, Burkholderiales, Coriobacteriales, Turicibacterales, Erysipelotrichales, Enterobacteriales clearly clustering with the "Probiotic Yogurt" treatment. On the other hand, Verrucomicrobiales, Cyanobacteria (Other), Lactobacillales, Clostridiales and Mollicutes were closely associated with "Yogurt" and "Juice" treatments. In case of "Probiotic Juice" and "Probiotic *Bacillus*" treatments, the same bacterial orders (Bacteroidales, Campylobacterales and Desulfovibrionales) were closely associated with "Control" treatment.

Table 4. Relative abundance of bacterial classes inferred from 16S rRNA gene sequencing analysis.

Taxonomy (%)	Control	Juice	Yogurt	Probiotic Bacillus	Probiotic Juice	Probiotic Yogurt
Clostridia	47.5±4.7[a]	49.2±2.1[a]	51.8±2.5[a]	49.2±4.3[a]	46.9±3.2[a]	46.3±3.7[a]
Bacteroidia	41.6±4.4[a]	38.9±2.1[a]	33.7±2.5[a]	39.9±3.3[a]	43.3±3.1[a]	39.3±2.3[a]
Bacilli	7.7±1.7[a]	8.0±1.2[a]	9.2±0.66[a]	7.0±1.1[a]	7.02±0.49[a]	5.4±1.2[a]
Verrucomicrobiae	0.00±0.00[a]	0.00±0.00[a]	0.96±0.96[a]	0.94±0.91[a]	0.02±0.02[a]	0.04±0.02[a]
Mollicutes	0.34±0.15[a]	0.32±0.10[a]	0.86±0.54[a]	0.20±0.07[a]	0.30±0.10[a]	0.28±0.10[a]
Unassigned;Other	0.38±0.06[a]	0.42±0.02[a]	0.42±0.03[a]	0.46±0.04[a]	0.48±0.03[a]	0.40±0.03[a]
Cyanobacteria;Other	0.56±0.17[a]	0.57±0.38[a]	0.42±0.25[a]	0.20±0.07[a]	0.26±0.08[a]	0.14±0.06[a]
Alphaproteobacteria	0.06±0.02[a]	0.07±0.02[a]	0.08±0.02[a]	0.06±0.02[a]	0.04±0.02[a]	0.14±0.05[a]
Betaproteobacteria	0.42±0.09[a]	0.65±0.19[a]	0.34±0.08[a]	0.36±0.10[a]	0.28±0.03[a]	1.6±0.57[b]
Deltaproteobacteria	0.20±0.03[a]	0.50±0.16[b]	0.28±0.06[ab]	0.34±0.10[ab]	0.28±0.07[ab]	0.30±0.03[ab]
Gammaproteobacteria	0.36±0.02[a]	0.45±0.08[a]	0.32±0.04[a]	0.40±0.13[a]	0.26±0.04[a]	2.7±0.87[b]
Epsilonproteobacteria	0.34±0.11[a]	0.25±0.09[a]	0.16±0.06[a]	0.30±0.20[a]	0.40±0.18[a]	0.20±0.03[a]
Erysipelotrichi	0.18±0.04[ab]	0.47±0.13[b]	0.10±0.00[a]	0.22±0.10[ab]	0.12±0.04[a]	0.48±0.19[b]
Coriobacteria	0.100±0.00[a]	0.12±0.03[a]	0.10±0.03[a]	0.10±0.00[a]	0.08±0.02[a]	0.14±0.02[a]
Actinobacteria	0.02±0.02[a]	0.02±0.02[a]	0.02±0.02[a]	0.06±0.02[ab]	0.00±0.00[a]	0.16±0.08[b]

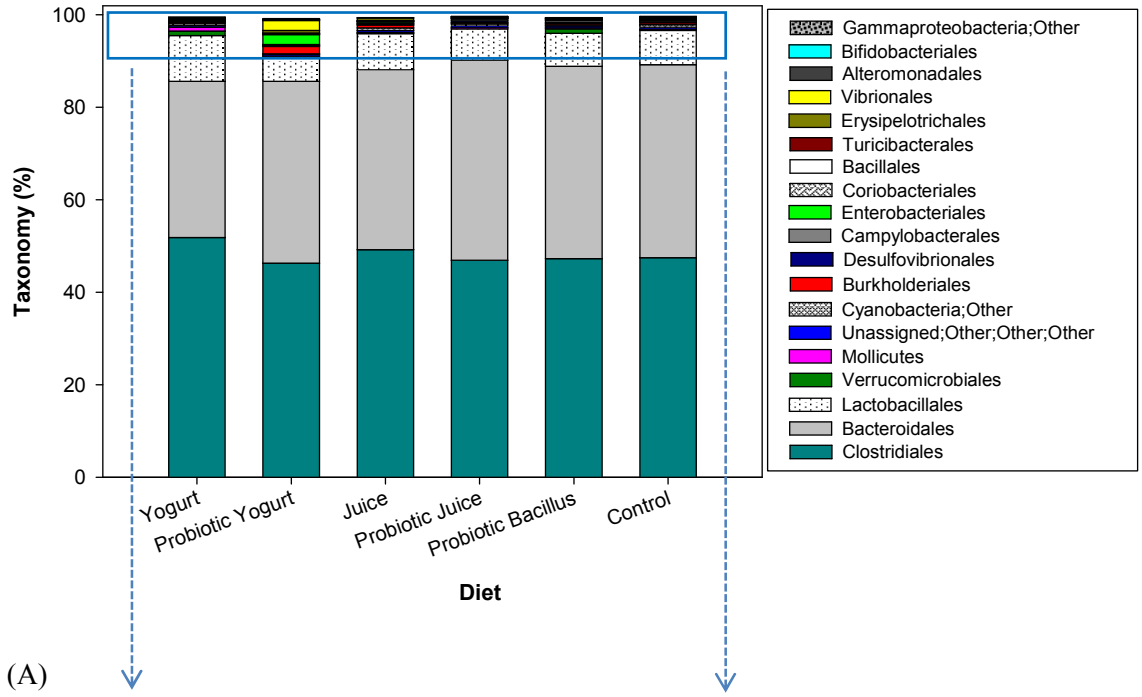
Data are expressed as means ± SEM. Different superscript letters on the same line indicate statistical differences by Duncan test ($p<0.05$).

Table 5. Relative abundance of bacterial orders inferred from 16S rRNA gene sequencing analysis.

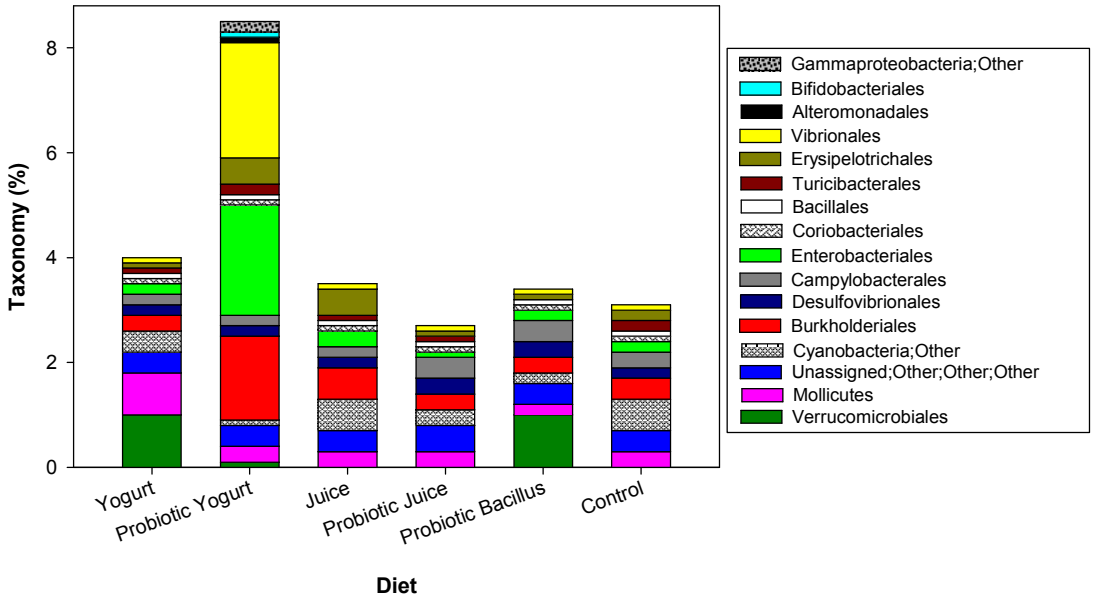
Taxonomy (%)	Control	Juice	Yogurt	Probiotic Bacillus	Probiotic Juice	Probiotic Yogurt
Clostridiales	47.4±4.7[a]	49.1±2.1[a]	51.8±2.5[a]	47.3±4.3[a]	46.8±3.2[a]	46.3±3.7[a]
Bacteroidales	39.0±3.9[a]	38.9±2.1[a]	33.7±2.5[a]	41.5±3.6[a]	43.3±3.1[a]	39.3±2.3[a]
Lactobacillales	7.4±1.8[a]	7.7±1.2[a]	8.9±0.65[a]	7.1±1.07[a]	6.8±0.52[a]	5.1±1.1[a]
Bacillales	0.08±0.02[a]	0.1±0.00[ab]	0.14±0.02[ab]	0.14±0.02[ab]	0.14±0.02[ab]	0.16±0.009[b]
Bifidobacteriales	0.02±0.02[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.10±0.05[b]
Coriobacteriales	0.10±0.00[a]	0.12±0.03[a]	0.10±0.03[a]	0.10±0.00[a]	0.08±0.02[a]	0.14±0.02[a]

Verrucomicrobiales	0.00±0.00[a]	0.00±0.00[a]	0.96±0.96[a]	0.94±0.91[a]	0.02±0.02[a]	0.04±0.02[a]
Mollicutes	0.34±0.15[a]	0.32±0.10[a]	0.86±0.54[a]	0.22±0.06[a]	0.30±0.10[a]	0.28±0.10[a]
Unassigned;Other	0.38±0.06[a]	0.42±0.02[a]	0.42±0.03[a]	0.44±0.04[a]	0.48±0.03[a]	0.40±0.03[a]
Cyanobacteria	0.56±0.17[a]	0.57±0.38[a]	0.42±0.25[a]	0.22±0.07[a]	0.26±0.08[a]	0.14±0.06[a]
Burkholderiales	0.42±0.09[a]	0.65±0.19a	0.32±0.09[a]	0.34±0.09[a]	0.24±0.02[a]	1.62±0.55[a]
Desulfovibrionales	0.18±0.03[a]	0.27±0.06[a]	0.26±0.07[a]	0.34±0.07[a]	0.24±0.05[a]	0.22±0.03[a]
Campylobacterales	0.34±0.11[a]	0.25±0.09[a]	0.16±0.06[a]	0.38±0.19[a]	0.40±0.18[a]	0.20±0.03[a]
Enterobacteriales	0.20±0.00[a]	0.25±0.08[a]	0.20±0.03[a]	0.20±0.07[a]	0.14±0.02[a]	2.1±0.89[b]
Turicibacterales	0.20±0.09[ab]	0.10±0.04[ab]	0.06±0.04[ab]	0.02±0.02[a]	0.08±0.02[ab]	0.22±0.07[b]
Erysipelotrichales	0.18±0.04[a]	0.47±0.13[b]	0.10±0.00[a]	0.12±0.04[a]	0.12±0.04[a]	0.30±0.15[ab]
Vibrionales	0.10±0.00[a]	0.10±0.00[a]	0.08±0.02[a]	0.08±0.03[a]	0.06±0.02[a]	2.1±1.0[b]
Alteromonadales	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.06±0.02[b]

Data are expressed as means ± SEM. Different superscript letters on the same line indicate statistical differences by Duncan test ($p<0.05$).



(A)



(B)

Fig. 4. Abundance (in percent) of all bacterial orders (A). Abundance of bacterial families observed with out to the three major orders (i.e., Clostridiales, Bacteroidales and Lactobacillales) obtained by 16S rRNA gene sequencing analysis (B). Only OTUs with abundance values above 0.1% in six samples are shown.

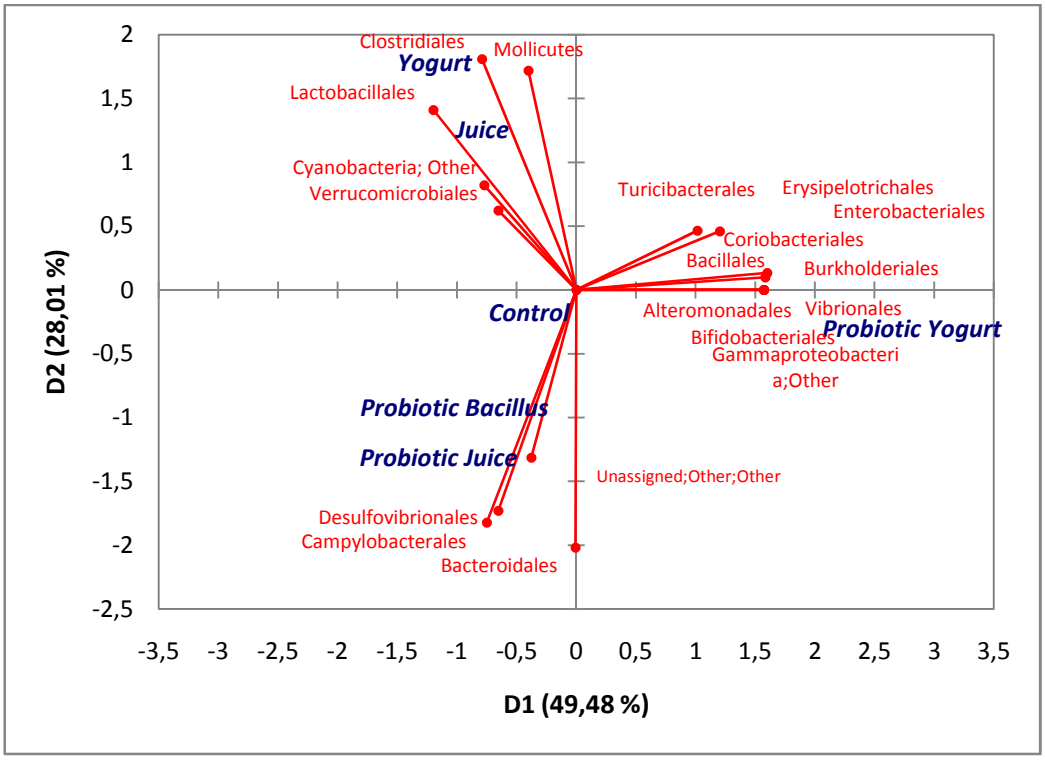


Fig. 5. Principal component analysis diagram for the abundance (in percent) of all bacterial orders obtained by 16S rRNA gene pyrosequencing analysis. Only OTUs with abundance values above 0.1% are shown.

4. Discussion

4.1. Biochemical parameters

In recent years, studies report that probiotic microorganisms may confer several beneficial effects to the host's health such intestinal microbiota modulation (Toscano, Grandi, Miniello, Mattina, & Drago, 2016), in cancer (Aragón, Carino, Perdigón, & LeBlanc, 2014; Desrouillères, Millette, Vu, Touja, & Lacroix, 2015), gastrointestinal (Kumar et al., 2015) metabolic (Kumar et al, 2013; Kumar, Tomar, Thakur, & Singh, 2017), and mental diseases (Yeon et al., 2010), periodontitis (Messora et al., 2016), infections for pathogens (Mirzaei, Shahirfar, & Mobayen, 2012), liver injury (Zhang et al., 2017) among others. However, few studies have focused on the evaluation of spore-forming probiotics effects through food

consumption (Haldar & Gandhi, 2016; Ng et al., 2013; Sun, Wang & Zhang, 2010). So this study was done to evaluate the impact of formulated probiotic products with a spore-forming strain. This research evaluated the effect of ingestion of juice or yogurt containing probiotic bacteria *B. coagulans* GBI-30, 6086 on classical health biochemical parameters and intestinal microbiota profile in healthy rats. Regarding the body weight and food intake of the rats, there were no significant changes in the body weight or amount of food ingested of animals with the ingestion of the spore-forming probiotic in water, juice or yogurt during 21 days. Thus, ingestion of the strain did not cause any negative signs, such as weight loss or reduced hunger in animals.

The results of this study showed that the continued ingestion of probiotic added in yogurt for 21 days was able to significantly decrease triglycerides and glucose levels when compared with control group, on the other hand probiotic in juice showed a reduction, but not significant. This is the first work that shows that consumption of yogurt with *Bacillus* probiotics has led to lower levels of triglycerides and glucose. Probably, these effects vary from strain to strain, being considered dependent strain. The high triglyceride and glucose levels are risk factors associated with the development of coronary heart disease and diabetes mellitus, respectively (Karamali et al., 2016; Le & Walter, 2007). Since, these biochemical parameters are usually elevated in unhealthy animals (Lollo et al., 2015a; Roquetto et al., 2015). It is believed that if probiotic yogurt was able to reduce these parameters in healthy animals, possibly the increase of these parameters could be attenuated in diseased animals. Other parameters also such blood pressure and cholesterol were modulated for non-sporuled probiotics food matrices as cheese and cereal mix, respectively (Lollo et al., 2015a; Ogunremi, Sanni, & Agrawal, 2015). Rats fed with probiotic cheese (*Lactobacillus acidophilus* LA 14 and *Bifidobacterium longum* BL 05) exhibited lower blood pressure parameters when compared whit rats fed with cheese without probiotic (Lollo et al., 2015a). Study of Ataie-Jafari, Larijani, Majd and Tahbaz (2009) reported that the cholesterol levels were reduced with the consumption of probiotic yogurt in hypercholesterolemic subjects (*L. acidophilus* e *Bifidobacterium lactis*).

Studies addressing non-sporuled probiotics demonstrated the potential of different strains in reducing of triglycerides and glucose. According to Ahn et al., 2015a and Ahn et al., 2015b the supplementation of dual probiotic strains containing *Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum* KY1032 led to a significant reduction of 18% in the serum triglycerides in non-diabetic subjects with mild to moderate hypertriglyceridemia. The

mechanism that involves the modulation of serum lipid profiles via probiotics is still not clear. Karamali et al., 2016 demonstrated that taking probiotic supplements (capsule that contained three viable strains: *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium bifidum*) for 6 weeks in patients with gestational *diabetes mellitus* had beneficial effects on glycaemic control, triglycerides and VLDL cholesterol concentrations. The possible mechanisms of action involve increase in hepatic "natural-killer" T (NKT)-cell numbers, reduction of inflammatory signalling (Ma, Hua, & Li, 2008), up regulation of adiponectin and down regulation of inflammation and blocking of the suppression of GLUT4 (Nakamura & Omaye, 2012). Additionally, the increase glucagon-like peptide (GLP)-1 secretion from enteroendocrine L cells (influence the improvement of carbohydrate metabolism), decrease glucotoxicity and increase insulin sensitivity of target cells (Tremaroli & Backhed, 2012). The production of short-chain fatty acids, that can prevent low-grade inflammatory responses through maintaining intestinal integrity (Tremaroli & Backhed, 2012) and the protection of pancreatic cells, that are important because dysfunction of these cells decrease the insulin sensitivity in the regulation of blood glucose levels and fat metabolism too can be involved (Boden & Shulman, 2002; Lye, Kuan, Ewe, Fung, & Liong, 2009). For spore-forming probiotics, the mechanisms of action may be the same as those involved in non-sporulated probiotics. However, more studies are needed to prove this relationship.

As the expression of enzymes and thermal shock proteins (stress proteins), it was well established that heat shock proteins (HSP) are able to protect cells against thermal extremes (Wu et al., 2012) and the enzymes against oxidative stress (Kathirvel, Chen, Morgan, French, & Morgan, 2010). In this job, the probiotic yogurt and juice consumption did not significantly alter the expression of enzymes and thermal shock proteins. Few studies report the relationship between probiotics, oxidative stress and heat shock proteins. The possible relationship betweel probiotics and HSPs is not yet elucidated, but it seems that low molecular weight peptides and other soluble factors secreted by probiotics in the intestinal lumen would modulate the production of HSPs (Tao et al., 2016). Moura et al. (2016) showed that the consumption of the dessert (5 g/day) containing non-sporuled probiotic (*L. acidophilus* La5) for 2 weeks did not cause any changes in the HSPs in rats when compared with group control. HSPs are usually expressed in situations of stress and act as a cellular defense, protecting cell proteins against denaturation. Thus, possible that the amount of ingested probiotic bacteria did not increase the stress of the animals. Additionally, Kleniewska, Hoffmann, Pniewska and Pawliczak (2016) reported that a limited number of

probiotic strains may reduce oxidative stress. This way, the response in the expression of the HSPs and enzymes may vary from strain to strain (strain-dependent). Despite these study envolving non-sporuled probiotics and HSPs, studies dealing spore-forming probiotics and HSPs have not yet been found in the literature until then. Thus, more studies are necessary in order to confirm this possible relationship of HSPs with spore-forming probiotics and to understand its possible mechanisms of action.

4.2. Taxonomic assignment obtained by 16S rRNA gene sequencing analysis

The microbiota study indicated that the consumption of the probiotic yogurt resulted in higher abundance and diversity of gut microbiota profile of male rats than the other samples (Table 3 and Fig. 3). Our results indicate also that the classes Clostridia and Bacteroidia and the orders Clostridiales and Bacteroidales were not significantly altered amongst the six groups studied. Many bacteria of classes of Clostridia and Bacteroidia are normally commensals of the human gut microbiota and can also be responsible for infections (Collado, Isolauri, Salminen, & Sanz, 2009; Toscano, Grandi, Miniello, Mattina, & Drago, 2016). Thus, the not to alteration of these classes and orders observed in the present study can be considered a positive result of both probiotic juice and probiotic yogurt which contributes maintaining stable the host's health. Additionally, the ingestion of probiotic yogurt caused an increase of the class Actinobacteria and two orders such as: Bacillales and Bifidobacteriales diffent of probiotic juice, showing that the probiotic yogurt was able to positive modulate the intestinal microbiota. Thus, proving the influence of the food matrix.

The changes in gut microbiota profile observed in this study is consistent with the results of other studies previously reported. Haldar & Gandhi, 2016 observed that oral administration of two *Bacillus* strains (*B. coagulans* B37 ou *B. pumilus* B9) in skim milk reduced fecal coliforms count and increased *Lactobacillus* and *Bacillus* counts in the intestinal flora in rats. Possibly, the *Bacillus* adhere to the intestine of the rat and acts beneficially. Thus, as in our study there was an increase in order Bacillales and the bacterium *B. coagulans* GBI-30, 6086 belongs to these order, we can suggest that probably the *B. coagulans* survive in the human gastrointestinal tract, thereafter germinates, grows, and multiplies as vegetative form. Consequently, the *Bacillus* possible adhere to the intestine of

the rat and help to exert beneficial effects (Ghelardi et al., 2015; Haldar & Gandhi, 2016).

Study of Wang et al. (2014) showed an increase in *Bifidobacterium* and other beneficial bacteria, whereas opportunistic pathogens decreased in adults after of consumption of probiotic (*Lactobacillus planatarum* P-8). The consumption of a fermented milk containing non-sporuled probiotic (*Lactobacillus casei* Shirota) in healthy adults increased the *Bifidobacterium* count, suggesting the potent features of strain as a probiotic (Matsumoto et al., 2010). Chaikham, Apichartsrangkoon, Jirarattanarangsri and Van de Wiele, (2012) showed that juice added of non-sporuled probiotics (*Lactobaillus acidophilus* LA5 or *Lactobacillus casei* 01) benefically modulated the intestinal microbiota, increasing bifidobacteria and decreasing pathogenic bacteria (i.e. clostridia and fecal coliforms) (*in vitro*). In addition, diets rich in dairy product containing non-sporuled probiotics have been reported to confer beneficial effects detectable by the modulation of microbiota. The ingestion of yogurt containing *Bifidobacterium animalis* DN-173010 modified the composition of the intestinal microbiota (increase of *Bifidobacterium*) in subjects (He et al., 2008). Consequently, the significant elevation observed in the our study of the bacterial class Actinobacteria and order Bifidobacteriales in probiotic yogurt group can be considered as a positive probiotic mark, once the class Actinobacteria includes the order Bifidobacteriales, that includes the family Bifidobacteriaceae, that includes the genera *Bifidobacterium* that is already well known as beneficial to the host (Collado, Isolauri, Salminen, & Sanz, 2009).

Even though *B. coagulans* GBI-30, 6086 is added to foods as spores, which present high physical and chemical resistances, it seems clear that the food matrix is also relevant for delivering the efficiency of spore-forminng probiotic bacteria. According to what was exposed, our findings suggest that yogurt can be an efficient vehicle for the delivery of spore-forming probiotic bacteria. Yogurt appears to be a more appropriate food matrix for *B. coagulans* GBI-30, 6086 when compared to juice, probably due to its composition. As shown in table 1, it can be seen that yogurt is richer in fat and protein, while juice content in carbohydrates is higher. In this way, the two food matrices show differences in their composition that may interfere with the efficacy of the matrix as carrier, through the interaction of these components with the probiotic, boosting its beneficial effects (Ranadheera, Baines, & Adams, 2010).

5. Conclusions

Therefore, the yogurt is an efficient food matrix to delivery probiotic spore-forming bacteria as compared to fruit juice. The results obtained in this study showed that the daily consumption of yogurt containing the probiotic *Bacillus coagulans* GBI-30, 6086 during 21 days was able of significantly decrease the glucose and triglycerides levels in rats. In addition, the consumption of this yogurt containing the probiotic *Bacillus coagulans* GBI-30, 6086 resulted in the modulation of the intestinal microbiota, characterized by the increase of class Actinobacteria and orders Bacillales and Bifidobacteriales.

Considering the resistance of spores of *Bacillus coagulans* GBI-30, 6086, it is possible to obtain probiotic yogurt that is stable during processing, storage and consumption. This can be considered a key strategy to overcome important hurdles related to processing of probiotic foods and to increase the diversity of probiotic foods and to ensure that these microorganisms exert beneficial effects on the host.

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4. DISCUSSÃO

Os probióticos são definidos como microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios para a saúde do hospedeiro (Hill et al., 2014). Os principais mecanismos de ação dos probióticos envolvem: melhoria da função de barreira epitelial, melhoria de adesão as células intestinais e inibição de patógenos por competição por aos locais de adesão, produção de substâncias antimicrobianas e modulação do sistema imune (Rijkers et al., 2010). Juntos, estes mecanismos são capazes de modular a composição da microbiota intestinal e prevenir o crescimento de bactérias patogênicas. Dessa forma, a microbiota intestinal exerce um papel importante na saúde humana e na doença. A manipulação desses microorganismos pela ingestão de probióticos é uma abordagem atraente, uma vez que atuam modulando a microbiota intestinal e podem contribuir com manutenção e restauração da saúde (Gerritsen et al., 2011). O trato gastrointestinal (TGI) é um micro-ecossistema que contém uma microbiota diversificada de 500-1000 espécies microbianas diferentes. Os seres humanos dependem da microbiota intestinal para realizar funções vitais e, portanto, o equilíbrio entre os grupos intestinais de microrganismos é essencial (Collado et al., 2009).

As perturbações da composição da microbiota, também conhecidas como disbiose (Gerritsen et al., 2011), têm sido associadas a um maior risco para doenças específicas, incluindo doenças crônicas inflamatórias do TGI (Joossens et al., 2011), diarréias (Young, Schmidt 2004), síndrome do intestino irritado (Maukonen et al., 2006), alergias (Suzuki et al., 2007), diabetes (Wu et al., 2010) e obesidade (Turnbaugh et al., 2006). Os alimentos funcionais não apenas satisfazem a fome e fornecem os nutrientes básicos, como também melhoram o bem-estar do hospedeiro (Vergari et al., 2010). Entre estes, alimentos com novos nutrientes ou componentes adicionais, como os probióticos, fazem parte de um mercado altamente lucrativo para as indústrias de alimentos devido ao seu potencial de saúde valioso (Bigliardi, Galati 2013). Uma variedade de efeitos benéficos dos probióticos no TGI e em outros locais tem sido relatado, tais como, doenças intestinais inflamatórias (Im et al., 2009), síndrome do intestino irritável (Dolin, 2009; Hun, 2009), doenças metabólicas (obesidade, diabetes, hipercolesterolemia, hipertensão) (Ataie-Jafari et al., 2009; Jauhiainen et al., 2005; Tabuchi et al., 2003), doenças alérgicas (Wang et al., 2004), doenças mentais (Yeon et al., 2010), doenças periodentais (Maekawa, Hajishengallis, 2014), infecções bacterianas (Mirzaei

et al., 2012), câncer colorretal (Lee et al., 2007) e modulação do sistema imune (Medici et al., 2004).

Assim, o consumo de probióticos parece ser uma abordagem interessante e viável para modular a microbiota intestinal e para manter ou restaurar a saúde humana (FAO/OMS, 2002). Portanto, o objetivo do artigo de revisão (primeiro artigo) foi discutir as interações dos probióticos com a microbiota intestinal e seus impactos na saúde humana. Em primeiro lugar, as técnicas utilizadas para analisar a microbiota intestinal foram apresentadas, seguidas de um resumo das características dos probióticos e seus mecanismos de ação. Finalmente, estudos que correlacionam os probióticos, a microbiota intestinal e interações/impactos a saúde foram discutidos. Quanto aos métodos para caracterizar a microbiota intestinal na presença ou não de probióticos temos os tradicionais (métodos dependentes de cultivo) e moleculares (métodos independentes de cultivo). Os métodos tradicionais são técnicas que avaliam a composição da microbiota intestinal pelo uso de diferentes meios de cultura seletivos para populações bacterianas específicas. Porém, considerando as limitações desses métodos dependentes de cultivo, técnicas moleculares baseadas na análise dos genes ribossômicos RNAr 16S, um marcador de diversidade genética, tem emergido, tais como, seqüenciamento de Sanger, pirosequenciamento, microarrays DNA, hibridização *in situ* por fluorescência (Fish), polimorfismo do comprimento do fragmento de restrição terminal (TRFLP) reação de cadeia polimerase quantitativa em tempo real (qPCR), electroforese em gel de gradiente de desnaturação (DGGE) e eletroforese em gel com gradiente de temperatura (TGGE), análise espacial intergénica ribossomal (RISA), metagenômica, metatranscriptômica, metabolômica e metaproteômica (Sekirov et al., 2010). Um melhor conhecimento da composição e diversidade da microbiota intestinal e como as mudanças nesse microecosistema podem causar ou estão associados com doenças permanece longe de ser completamente compreendido. Portanto, uma melhor compreensão das populações microbianas do trato gastrointestinal é crucial e contribuirá certamente para o desenvolvimento de novas estratégias para a prevenção e/ou tratamento de várias doenças. A manipulação da microbiota TGI por consumo de probióticos é uma abordagem interessante para manter e restaurar a saúde humana.

Dessa forma, a busca pela saúde, bem-estar e alimentação saudável tem levado a indústria de alimentos e pesquisadores a estudar e desenvolver alimentos funcionais como probióticos (Martins, 2013). Pesquisas que abordam a sobrevivência de microorganismos probióticos em alimentos são de fundamental importância para o desenvolvimento de novos

alimentos probióticos (Kailasapathy, 2006). Desta forma, o segundo artigo avaliou a sobrevivência de cepas de *Bacillus* (*B. subtilis* PXN21, *B. subtilis* PB6, *B. coagulans* lactospore, *B. coagulans* BC30, *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. flexus* Hk1 and *B.subtilis* Bn1) em vários processos tecnológicos, envolvendo várias matrizes alimentares, tais como pasteurização (leite e suco de laranja), cozimento (almôndega), forneamento (pão), secagem (abacaxi cristalizado), acidificação/fermentação (iogurte), CO_2 supercrítico (suco de laranja), irradiação (pimenta em pó) e extrusão a quente (cereal matinal) e a frio (macarrão espaguete). Nosso estudo mostrou que a resistência das cepas variou de processo para processo. Quanto aos processos de pasteurização (leite), pasteurização (suco) e cozimento (almôndegas), as oito cepas comportaram-se de forma semelhante tanto sem choque térmico quanto com choque térmico (CH). Nos demais processos (forneamento (pão), secagem (abacaxi cristalizado), acidificação/fermentação (iogurte), CO_2 supercrítico (suco de laranja)) a resistência das cepas variou de acordo com a cepa e aplicação de choque térmico (CH). Quanto ao processo de irradiação (pimenta em pó), as cepas *B. flexus* Hk1, *B. mojavensis* KJS3, *B. licheniformis* Me1, *B. subtilis* PB6 e *B. subtilis* PXN21 foram as menos afetadas em pimenta em pó e *B. subtilis* Bn1 e *B. coagulans* BC30 foram a mais afetados. No processo de extrusão, somente *B. coagulans* BC30 foi utlizada e esta apresentou excelente resistência ao processo de extrusão tanto a quente quanto a frio, mostrando reduções decimais (gama) não significativas. Diversos estudos mostraram a sobrevivência de cepas probióticas a processos tecnológicos. A resistência de nove cepas de *Lactobacillus paracasei* (4R4, 61H4, 61R3, 7R1, 8I2, 8R2, 162M6, 171R4 e 171R7) em tampão e leite integral UHT no processo de pasteurização (73°C, 15 s) foi relatada. Nenhuma das nove cepas de *L. paracasei* sobreviveu após pasteurização em tampão. Quando cultivadas no leite, sete das nove cepas de *L. paracasei* (61H4, 61R3, 7R1, 8I2, 8R2, 171R4 e 171R7) sobreviveram, mas em números baixos. Os componentes do leite podem proteger as células bacterianas da lesão por calor (Christiansen et al., 2006). Estudo de Zheng et al. (2014) mostrou que o tratamento térmico (95 ° C / 1 min) no suco de litchi tomou as bactérias ácido láticas em níveis não detectáveis. No entanto, diferentes das bactérias ácido láticas, as oito cepas de *Bacillus* apresentaram resistência considerável ao pH neutro do leite (~ 6,6-6,8) e ao pH ácido do suco (~ 3,6) em nosso estudo. Barbosa et al. (2015) reportaram a sobrevivência de *Lactobacillus plantarum* 299v, um probiótico comercial (Probis Probiotika) e *Pediococcus acidilactici* HA-6111-2 em pós de laranja obtidos por processo de secagem (pulverização-spray, congelamento e secagem por convecção com ar quente 40°C/48h) e armazenamento subseqüente. Não houve diminuição do número de células na pulverização e congelamento, mas houve uma redução de

ciclos de ~ 2 log na secagem por ar quente por convecção durante o armazenamento à temperatura ambiente, e não houve diferenças significativas durante o armazenamento a 4 ° C. Kailasapathy (2006) relatou que *Lactobacillus acidophilus* e *Bifidobacterium lactis* em iogurte foram capazes de sobreviver à fermentação tanto livre como microencapsulado com alginato de cálcio-amido. A sobrevivência aumentou de 2 e 1 números de células log de *L. acidophilus* e *Bif. Lactis*, respectivamente, devido à proteção das células por microencapsulação. Desta forma, em nosso estudo, as cepas de *Bacillus* analisadas mostraram boa sobrevivência aos processos sem a necessidade de microencapsulação ou adição de agentes protetores.

Com base na resistência, algumas cepas tiveram uma melhor especificidade para certos alimentos quando comparados com outras cepas. A alta resistência e especificidade das cepas aos processos tecnológicos e alimentos, respectivamente, possivelmente é influenciada pela composição da matriz alimentar (Christiansen et al., 2006). A cepa *B. coagulans* BC30 foi a única que mostrou comportamento semelhante em seis processos (pasteurização - leite, cozimento, forneamento, secagem, acidificação / fermentação e dióxido de carbono supercrítico) com pequenas reduções decimais que variam de 0-1,02 log UFC / g sem choque térmico e 0,07-1,06 log UFC / g com choque térmico (CH). Nos processos de pasteurização (suco) e irradiação (pimenta), o gama variou de 2,30-5,23 log UFC / g sem choque térmico e 2,50-4,93 log UFC / g com choque térmico (CH). No entanto, a cepa *B. subtilis* PXN21 mostrou baixa resistência e baixa especificidade em cinco processos (pasteurização - suco, cozimento, forneamento, acidificação / fermentação e irradiação), com reduções decimais variando de 0,97-2,23 log UFC / g sem choque térmico e 1,08-2,94 log UFC / g com choque térmico (CH). Assim, *B. coagulans* BC30 foi a cepa que apresentou maior resistência / maior especificidade aos processos estudados e *B. subtilis* PB6 e *B. subtilis* PXN21 foram as cepas que se destacaram negativamente, mostrando menor resistência e menor afinidade aos processos, apresentando reduções decimais significativas em cinco processos estudados com reduções decimais que variaram de 0,70-3,02 log UFC / g sem choque térmico e 1,08-3,23 log UFC / g com choque térmico (CH). Portanto, como esperado, *Bacillus* mostrou sobrevivência/resistência a todos os processos tecnológicos, devido à sua capacidade de formar esporos (estruturas resistentes) (Cutting, 2011). No entanto, o comportamento/resistência das cepas de *Bacillus* variou de processo para processo. Em adição, a sobrevivência e a especificidade do *Bacillus* ao processo tecnológico e alimento, respectivamente, variaram de cepa para cepa. Assim, a resposta do *Bacillus* pode ser

considerada como cepa dependente, possivelmente devido a características/propriedades específicas e mecanismos de ação de cada cepa, porém, tais características/propriedades e mecanismos ainda não são totalmente compreendidas. Para compreensão de tais características e também mecanismos específicos das cepas de *Bacillus* responsáveis pela sua resistência e especificidade estão sendo feito análises proteômicas e os dados serão posteriormente adicionados para publicação.

Ademais, nos últimos anos, o crescimento da diversificação do mercado de pão e da indústria de panificação tem sido evidente principalmente devido à busca de dietas saudáveis e produtos com ingredientes funcionais, como bactérias probióticas e/ou farinha de grãos integrais que contém maior teor de fibras, e outros nutrientes, incluindo os minerais (cálcio, magnésio e potássio) e muitos fitoquímicos (Geng, Harnly, Chen, 2016; Santos et al., 2016). O pão de forma apresenta uma estrutura diferente de outros tipos de pão, crosta fina e um miolo com porosidade regular e textura macia e elástica. A qualidade do pão é definida de acordo com a textura, umidade, volume específico, cor da casca, relação da casca/miolo, firmeza do miolo e características da farinha (Besbes et al., 2014). Com isso, nosso terceiro artigo abordou a resistência de *Bacillus* (*B. coagulans* GBI-30, 6086) em pães de forma tradicional e integral ao processo de forneamento e armazenamento. Neste estudo, a farinha integral apresentou aumentos significativos ($p<0,05$) para a absorção de água e redução significativa ($p<0,05$) para estabilidade, resistência e extensibilidade em comparação com a farinha refinada. Os valores mais elevados de absorção de água e valores mais baixos de estabilidade, resistência e extensibilidade da farinha integral são explicados pela presença de fibras em sua composição que requerem uma maior quantidade de água, maior resistência da massa à mistura e tempos de hidratação mais prolongados e também retem mais água no produto final (Schmiele et al., 2012). Além disso, as fibras competem muito pela água com outros polímeros, resultando na diluição do glúten, o que torna mais difícil formar uma rede de glúten (propriedades viscoelásticas) capaz de atribuir uma maior resistência à massa, levando a massas mais fracas (Rosell et al. Al., 2001). Corroborando com o nosso estudo, Schmiele et al. (2012) relataram que o uso de farelo e farinha de grãos inteiros muda a reologia da massa e a qualidade produzida pão de forma. A substituição da farinha de trigo refinada por farelo de trigo e farinha de trigo integral aumentou a absorção de água e resistência à extensão e diminuição da estabilidade e extensibilidade e também aumentou o teor de umidade, firmeza e dureza e diminuiu o volume específico do pão de forma.

Quanto aos parâmetros de qualidade, não foram observados diferenças significativas entre os pães probióticos (pães com *Bacillus* probióticos) e pães controle (pães sem *Bacillus* probióticos). Os pães integrais apresentaram maiores valores de umidade e textura (firmeza) e menores valores de volume quando comparados aos pães tradicionais. No entanto, não houve diferença significativa na umidade e textura (firmeza) entre as quatro formulações de pães. Quanto ao volume, houve diferença significativa ($p<0,05$) entre pães integrais e tradicionais. Espera-se um volume menor nos pães integrais devido à diluição do glúten e ao enfraquecimento da rede de glúten formada, pois contém maior quantidade de fibra (Katina, 2003). Os pães com maiores concentrações de fibras mostram maior conteúdo de umidade e menores volumes, refletidos em maior firmeza e dureza devido à sua natureza mais compacta (Schmiele et al., 2012). Nos parâmetros de cor houve alterações significativas ($p<0,05$) entre os pães tradicionais e integrais, tais mudanças são aceitáveis, já que os pães integrais apresentam coloração mais escura do que os pães tradicionais, devido também à presença de fibras na composição da farinha integral. As quatro formulações de pães mostraram atividade de água e pH em torno de 0,94-0,95, 5,7-5,9, respectivamente, não houve diferenças significativas entre as quatro formulações de pães nos dias analisados (1, 3, 7 e 10), respectivamente. No entanto, não foram observadas diferenças significativas entre os pães probióticos e pães controle. Quanto à atividade da água e pH, os resultados são esperados, já que o pão é classificado no grupo de alimentos com alta atividade de água ($0,94<0,97$) e baixa acidez (pH 4,5) (Tortora, Funke, Case, 2010).

A sobrevivência de *B. coagulans* GBI-30, 6086 nas quatro formulações de pão de forma analisadas foi significativamente reduzida no processo de fabricação de pães ($p<0,05$). No entanto, em geral, a cepa probiótica apresentou boa resistência nos pães de forma tradicional e integral, pois, no final do processo de fabricação, observou-se quedas que variaram de 1 a 2 log em pães de forma tradicional e integral. Após a mistura e a fermentação, não houve quedas significativas, o que já era esperado, uma vez que a mistura é apenas um processo mecânico e, na fermentação, a temperatura é baixa (32°C) e o tempo curto (60 minutos). No que diz respeito ao processo de forneamento, as maiores reduções foram demonstradas na casca tanto em pães de forma tradicional como integral sem e com choque térmico, aproximadamente queda de 2 ciclo log. As maiores reduções na casca já eram esperadas, porque na casca a cepa está mais em contato com a alta temperatura de forneamento (~ 190-200°C), enquanto que no miolo a cepa está mais protegida (temperatura no centro da pão ~ 70-80°C). Além disso, no pão de forma tradicional com choque térmico

houve um aumento significativo nas reduções decimais (gama) no miolo e fatia inteira quando comparados ao pão de forma tradicional sem choque térmico após o cozimento. Estas reduções maiores mostram que os esporos são estressados com a alta temperatura de forneamento e com o choque térmico ocorre o impedimento da recuperação destes. No entanto, esse aumento significativo nas reduções decimais não foi observado no miolo e na fatia inteira de pão de forma integral, possivelmente devido à maior atividade de água e à presença de fibras na sua composição, garantindo uma maior proteção à cepa.

Quanto ao armazenamento, quando comparados pães de forma probiótico sem choque térmico e pães de forma probiótico com choque térmico (CH), o aumento significativo nas reduções decimais nos dias 3 e 10 no miolo e fatia inteira em pães forma tradicional não ocorreu nos pães de forma integral. Assim, a presença de fibra na composição dos pães de forma integral parece aumentar a proteção à cepa, reduzindo as reduções decimais (gama) em pães que foram submetidos ao choque térmico, porque os pães com concentrações mais elevadas de fibras apresentam maior conteúdo de umidade e atividade água, possivelmente, refletidos em maior sobrevivência da cepa. Portanto, recomenda-se não aplicar choque térmico aos pães de forma tradicional. No entanto, para pães de forma integral, tanto a aplicação como não de choque térmico podem ser utilizados. Jao et al. (2011) observaram a viabilidade de *B. coagulans* GBI-30, 6086 em processo de forneamento de oito produtos de panificação diferentes (biscoitos de crisântemo, bolos de pastelaria de ovos, mooncakes, muffins, pães de polo, biscoitos de soda, bolos de esponja e torradas) e a viabilidade durante o armazenamento a 4 ou 25°C durante 15 e 6 dias, respectivamente. Os valores das contagens da cepa nos oito produtos de forneamento foram inferiores à sua massa crua após o processo de forneamento e armazenamento. Os resultados mostraram que a viabilidade da cepa nos produtos de panificação diminuiu com os dias de armazenamento, 4 e 25°C. No entanto, a cepa probiótica sobreviveu em quantidades significativas ao processo de forneamanto e armazenamento. Deste modo, *B. coagulans* GBI-30, 6086 foi definido como bom candidato para utilização em produtos de panificação. Além disso, uma vez que houve apenas 1 a 2 log de queda no processo total de produção de pão de forma e não houve mudanças significativas na sobrevivência da cepa durante os 10 dias de armazenamento nas quatro formulações analisadas, é possível produzir pão de forma probiótico com a cepa de *B. coagulans* GBI-30, 6086, que pode ser considerada adequada para uso em pães de forma, tanto tradicional como integral.

Adicionalmente, atualmente, há um grande interesse científico na digestão de alimentos. Estudos que abordam a sobrevivência e persistência/funcionalidade de bactérias probióticas são de fundamental importância para o desenvolvimento de novos alimentos probióticos, também para conhecer os possíveis mecanismos de ação e como esses probióticos podem afetar a saúde humana (Kailasapathy, 2006; Mainville, Arcand, Farnworth, 2005; Uriot et al., 2016). No entanto, as bactérias probióticas devem sobreviver ao processamento de alimentos e à passagem no trato gastrointestinal (estresses tais como: baixo pH estomacal, bile duodenal e jejunal e suco pancreático e secreções digestivas) (Silva et al., 2017). Assim, para aumentar a sobrevivência das bactérias probióticas no trato gastrointestinal têm sido utilizadas muitas alternativas tais como: seleção de cepas adaptadas às condições de estresse (Cutting, 2011), utilização de microencapsulação (Eratte et al., 2017) e prebióticos (Martinez et al., 2011) e administração de cepas probióticas em matriz alimentar (Possemiers et al., 2010). Portanto, no quarto artigo foi testada a sobrevivência da cepa probiótica *B. coagulans* GBI-30, 6086 em duas matrizes alimentares diferentes (suco e iogurte) num modelo *in vitro* que simula o trato gastrointestinal. A cepa de bactéria probiótica apresentou inicialmente cerca de 8 log UFC/mL em todas as amostras. Os resultados mostraram que, no compartimento estomacal, a cepa de bactéria probiótica apresentou contagens em torno de 7 log UFC/mL em todas as amostras, houve uma diminuição de 1 log provavelmente devido ao baixo pH. Nos demais compartimentos (duodeno e jejuno) e até o final da passagem no trato gastrointestinal (6h) a bactéria probiótica permaneceu com contagem em torno de 7 log UFC/mL nas amostras de suco, iogurte, suco (CH) e iogurte (CH). Além disso, não houve diferença significativa na sobrevivência de *B. coagulans* GBI-30, 6086 nas amostras de suco, iogurte, suco (CH) e iogurte (CH) no processo total *in vitro*, respectivamente e no final do processo *in vitro* também não houve diferença significativa entre todas as amostras (suco, iogurte, suco (CH) e iogurte (CH)) analisadas. Similarmente, Uriot et al. (2016) observaram significativa sobrevivência de *Streptococcus thermophilus* em todos os compartimentos digestivos do simulador do trato gastrointestinal, que pode estar relacionado à presença de urease e funções de proteínas de choque térmico. No entanto, outras cepas (*Lactobacillus acidophilus*, *L. casei, L. helveticus, L. rhamnosus, Bifidobacterium lactis* e *Bif. Longum*) testadas *in vitro* mostraram uma sobrevivência muito mais baixa (Caillard, Lapointe, 2017). Estudou-se a resistência a sucos gástricos simulados de vários produtos probióticos comercialmente disponíveis (dezesseis cepas, incluindo onze *Lactobacillus*, quatro *Bifidobacterium* e um *Pediococcus* e dez formas orais tais como cápsulas/comprimidos entéricos/não entéricos e cepas microencapsuladas). Os resultados demonstraram que todas as

cepas testadas mostraram alta sensibilidade a condições ácidas e sugeriram que a maioria destes microorganismos não mostraria qualquer viabilidade quando imersos no estômago. Os resultados deste estudo são consistentes com outros estudos envolvendo outras cepas e matrizes lácteas. Oliveira et al. (2014) revelou que *L. acidophilus* LA-5, *Lactobacillus casei* paracasei 01 e *Bif. lactis* BB 12, adicionados em queijo coalho de cabra, sobreviveram (reduções decimais de 2-3 ciclos log) a digestão gastrointestinal simulada no modelo do trato gastrointestinal superior, Sugerindo que estas cepas probióticas são capazes de passar através do trato gastrointestinal quando administradas na matriz de queijo analisada.

Estudos abordando outras matrizes alimentares também foram relatados. Klindt-Toldam et al. (2016) mostraram a sobrevivência de *L. acidophilus* NCFM e *Bif. Lactis* HN019 em dois tipos de chocolate (chocolate ao leite e chocolate escuro) durante a passagem simulada *in vitro*. As duas cepas probióticas mostraram boa sobrevivência durante a passagem simulada no trato gastrointestinal. A viabilidade de *Bif. Lactis* foi ligeiramente superior (reduções decimais aproximadamente 0 ciclo log) a *L. acidophilus* (reduções decimais aproximadamente 0.6 ciclo log), no entanto, a diferença não foi significativa, sendo o chocolate ao leite o melhor carreador protetor. Assim, a composição da matriz alimentar influencia na sobrevivência das bactérias probióticas durante a passagem através do trato gastrointestinal (Martinez et al., 2011). A sobrevivência da cepa de *Bacillus* em condições gastrointestinais simuladas (*in vitro*) também foi relatado. Hong et al. (2008) relataram que os esporos das cepas de *Bacillus indicus* HU36, *Bacillus subtilis* PY79 e *B. subtilis* Natto PY79 foram completamente resistentes (reduções decimais aproximadamente 0 ciclo log) à exposição a fluido gástrico simulado e fluido intestinal simulado, corroborando assim com o nosso estudo. A sobrevivência e persistência das cepas de *Bacillus* no trato gastrointestinal justifica-se pela germinação do esporo na região do jejuno e íleo onde o pH dos sucos gástricos foi suficientemente reduzido, o que poderia permitir a produção de agentes antimicrobianos como substâncias inibidoras (bacteriocinas), contribuindo assim para a exclusão competitiva de agentes patogênicos e, deste modo, apresentam um efeito probiótico (Duc et al., 2004).

No suco e no iogurte não foi observado diferença significativa nas reduções decimais (gama) entre as amostras sem e com choque térmico. Portanto, para avaliar a sobrevivência de *Bacillus* o choque térmico pode ou não ser aplicado nestes produtos. Neste estudo, a matriz pode não ser tão importante na sobrevivência dos probióticos esporulados (cepa *Bacillus coagulans* GBI 30, 6086) quanto nos probióticos não esporulados. A alta sobrevivência de

Bacillus no simulador do trato gastrointestinal é possivelmente devido à alta resistência dos esporos, uma vez que houve apenas uma queda de aproximadamente 1 ciclo log durante a passagem total (6h) através do simulador do trato gastrointestinal (*in vitro*), tanto no suco quanto no iogurte. Adicionalmente, não houve diferença significativa nas reduções decimais (gama) entre amostras de suco e iogurte, ambos sem choque térmico e com choque térmico. Desta forma, tanto o suco quanto o iogurte podem ser considerados probióticos após a passagem total (6h) pelo simulador do trato gastrointestinal. De acordo com Uriot et al. (2016) as altas capacidades de sobrevivência estão geralmente associadas à eficácia probiótica. Assim, *B. coagulans* GBI 30, 6086 é susceptível a exibir uma excelente eficácia probiótica dada a sua elevada capacidade de sobreviver às condições gastrointestinais.

Para completar o estudo com *Bacillus* foi feito um estudo *in vivo*. Nos últimos anos, vários estudos relatam que os microorganismos probióticos podem conferir efeitos benéficos à saúde do hospedeiro como a modulação da microbiota intestinal (Toscano et al., 2016), no câncer (Aragón et al., 2014; Desrouillères et al., 2015), doenças gastrointestinais (Kumar et al., 2015), metabólicas (Kumar et al, 2013; Kumar et al., 2017), mentais (Yeon et al., 2010), periodentais (Messora et al., 2016), hepáticas (Zhang et al., 2017) e infecções por patógenos (Mirzaei et al., 2012) entre outros. No entanto, poucos estudos se concentraram na avaliação dos efeitos de probióticos formadores de esporos em alimentos (Haldar, Gandhi, 2016; Ng et al., 2013; Sun, Wang, Zhang, 2010). Então, este estudo foi feito para avaliar o impacto de alimentos probióticos formulados com uma cepa formadora de esporos. Portanto, o quinto artigo avaliou o efeito da ingestão de suco ou iogurte contendo bactérias probióticas *B. coagulans* GBI-30, 6086 em parâmetros bioquímicos clássicos de saúde e perfil da microbiota intestinal em ratos saudáveis. Quanto ao peso corporal dos ratos e à ingestão de alimentos por ratos, não houve mudanças significativas no peso corporal ou na quantidade de alimento ingerido pelos animais com a ingestão do probiótico formador de esporos em água, suco ou iogurte durante 21 dias. Assim, a ingestão da cepa não causou sinais negativos, como perda de peso ou fome reduzida nos ratos. Os resultados mostraram que a ingestão contínua de probiótico adicionada em iogurte por 21 dias foi capaz de diminuir significativamente os níveis de triglicerídeos e glicose quando comparados com o grupo controle, por outro lado, o probiótico em suco apresentou redução, mas não significativa. Este é o primeiro trabalho que mostra que o consumo de iogurte contendo *Bacillus* probióticos levou a níveis mais baixos os níveis de triglicerídeos e glicose. Provavelmente, esses efeitos variam de cepa para cepa, sendo considerados como cepa-dependente. Os níveis elevados de triglicerídeos e glicose são

fatores de risco associados ao desenvolvimento de doenças coronárias e diabetes mellitus, respectivamente (Karamali et al., 2016; Le, Walter, 2007). Como esses parâmetros bioquímicos são geralmente elevados em animais não saudáveis (Lollo et al., 2015a; Roquetto et al., 2015). Acredita-se que, se o iogurte probiótico foi capaz de reduzir esses parâmetros em animais saudáveis. Possivelmente, o aumento desses parâmetros poderia ser atenuado em animais doentes. Estudos abordando probióticos não esporulados demonstraram o potencial de diferentes cepas na redução de triglicerídeos e glicose. De acordo com Ahn et al., 2015a e Ahn et al., 2015b, a suplementação de duas cepas probióticas (*Lactobacillus curvatus* HY7601 e *Lactobacillus plantarum* KY1032) conduziu a uma redução significativa de 18% nos triglicerideos séricos em indivíduos não diabéticos com leve a moderada hipertrigliceridemia. O mecanismo que envolve a modulação dos perfis lipídicos do soro via probióticos ainda não está claro.

Karamali et al., 2016 demonstraram que tomar suplementos probióticos (cápsula que continham três cepas viáveis: *L. acidophilus, L. casei* e *Bifidobacterium bifidum*) durante 6 semanas em pacientes com *diabetes mellitus* gestacional tinha efeitos benéficos sobre o controle glicêmico, triglicerídeos e concentrações de colesterol VLDL. Os possíveis mecanismos envolvem: aumento do número de células hepáticas T (NKT) "natural-killer", redução da sinalização inflamatória (Ma, Hua, Li, 2008), regulação positiva da adiponectina, regulação negativa da inflamação e bloqueio da supressão de GLUT4 (Nakamura, Omaye, 2012). Em adição, também envolve aumento da secreção de péptideos tipo glucagon (GLP)-1 das células L enteroendócrinas que influenciam na melhoria do metabolismo dos hidratos de carbono, diminuem a glucotoxicidade e aumentam a sensibilidade à insulina das células alvo (Tremaroli, Backhed, 2012). A produção de ácidos graxos de cadeia curta que podem prevenir respostas inflamatórias de baixo grau através da manutenção da integridade intestinal (Tremaroli, Backhed, 2012) e a proteção das células pancreáticas, que são importantes porque a disfunção destas células diminui a sensibilidade à insulina na regulação dos níveis de glicose no sangue e metabolismo das gorduras também estão envolvidas (Boden, Shulman, 2002; Lye et al., 2009). Para os probióticos formadores de esporos, os mecanismos de ação podem ser os mesmos que os envolvidos em probióticos não esporulados. No entanto, são necessários mais estudos para comprovar esse relacionamento.

Quanto a expressão de enzimas e proteínas de choque térmico (proteínas de estresse), ficou bem estabelecido que as proteínas de choque térmico (HSP) são capazes de proteger as células contra os extremos térmicos (Wu et al., 2012) e as enzimas contra o estresse oxidativo

(Kathirvel et al. Al., 2010). Neste trabalho, o consumo de iogurte e suco probiótico não alterou significativamente a expressão das enzimas e proteínas de choque térmico. Poucos estudos relatam a relação entre probióticos, estresse oxidativo e proteínas de choque térmico. Tao et al. (2006) exploraram uma possível relação entre probióticos e HSPs. O *Lactobacillus* GG, sob condições *in vitro*, foi capaz de aumentar a expressão de HSP25 e HSP70 a partir de uma cultura de células epiteliais intestinais. Os efeitos podem ser mediados por peptideos de baixo peso molecular e outros fatores solúveis ainda não descritos, os quais, quando secretados por probióticos no lúmen intestinal, modulariam a produção de HSPs. Corroborando com nosso estudo, Moura et al. (2016) mostraram que o consumo da sobremesa (5 g/dia) contendo *L. acidophilus* La 5 durante 2 semanas não causou alterações nas HSPs de ratos quando comparado com o grupo controle. As HSPs são geralmente expressas em situações de estresse e agem como uma defesa celular, protegendo as proteínas celulares contra a desnaturação. Assim, é possível que a quantidade de bactérias probióticas ingeridas não aumentasse o estresse dos animais. Além disso, Kleniewska et al. (2016) relataram que um número limitado de cepas probióticas podem reduzir o estresse oxidativo. Desta forma, a resposta na expressão das HSPs e enzimas pode variar de cepa para cepa (cepa-dependente). Apesar destes estudos envolvendo probióticos não esporulados e HSPs, Estudos que tratam probióticos formadores de esporos e HSPs ainda não foram encontrados na literatura até então. Dessa forma, são necessários mais estudos para confirmar esta possível relação de HSPs com probióticos formadores de esporos e compreender seus possíveis mecanismos de ação.

O estudo da microbiota intestinal indicou que o grupo iogurte probiótico mostrou maior abundância e diversidade no perfil da microbiota intestinal do que as demais amostras. Nossos resultados indicam também que as classes Clostridia e Bacteroidia e as ordens Clostridiales e Bacteroidales não foram significativamente alteradas entre os seis grupos. Muitas bactérias da classes de Clostridia e Bacteroidia são normalmente comensais da microbiota do intestino humano e também podem ser responsáveis por infecções (Collado et al., 2009; Toscano et al., 2016). Assim, a não alteração dessas classes e ordens observadas no presente estudo pode ser considerada como resultado positivo de suco probiótico e iogurte probiótico, o que contribui para manter estável a saúde do hospedeiro.

As mudanças no perfil da microbiota intestinal observadas neste estudo são consistentes com os resultados de outros estudos relatados anteriormente. Haldar, Gandhi, 2016 observaram que a administração oral de duas cepas de *Bacillus* (*B. coagulans* B37 ou *B.*

pumilus B9) em leite desnatado reduziu a contagem de coliformes fecais e aumentou as contagens de *Lactobacillus* e *Bacillus* na microbiota intestinal em ratos. Possivelmente, o *Bacillus* adere ao intestino do rato e age de forma benéfica. Assim, como em nosso estudo, houve um aumento na ordem Bacillales e a bactéria *B. coagulans* GBI-30, 6086 pertence a essa ordem. Podemos sugerir que provavelmente os *B. coagulans* sobrevivem no trato gastrointestinal humano, depois germinam, crescem e se multiplicam como forma vegetativa. Conseqüentemente, o *Bacillus* possivelmente aderiu ao intestino do rato e ajudou a exercer os efeitos benéficos (Ghelardi et al., 2015; Haldar, Gandhi, 2016).

O estudo de Wang et al. (2014) mostraram um aumento em *Bifidobacterium* e outras bactérias benéficas, ao passo que os patógenos oportunistas diminuíram em adultos após o consumo de probiótico não esporualdo (*Lactobacillus planatarum* P-8). O consumo de um leite fermentado contendo probióticos não esporulados (*Lactobacillus casei* Shirota) em adultos saudáveis aumentou a contagem de *Bifidobacterium*, sugerindo os potentes traços da cepa como probiótico (Matsumoto et al., 2010). Chaikham et al., (2012) mostraram que o suco adicionado de probióticos não esporulados (*Lactobaillus acidophilus* LA5 ou *Lactobacillus casei* 01) modificou beneficamente a microbiota intestinal, aumentando as bifidobactérias e diminuindo as bactérias patogênicas (ou seja, Clostridias e coliformes fecais) (*in vitro*). Além disso, dietas ricas em produtos lácteos contendo probióticos não esporulados foram relatados por conferir efeitos benéficos detectáveis na modulação da microbiota intestinal. A ingestão de iogurte contendo *Bifidobacterium animalis* DN-173010 modificou a composição da microbiota intestinal (aumento de *Bifidobacterium*) em indivíduos (He et al., 2008). Conseqüentemente, a elevação significativa observada no nosso estudo da classe de Actinobactérias e ordem de Bifidobacteriales no grupo iogurte probiótico pode ser considerada como uma marca probiótica positiva, uma vez que a classe Actinobacteria inclui a ordem Bifidobacteriales, que inclui a família Bifidobacteriaceae, que inclui o gênero *Bifidobacterium* que já é bem conhecido como benéfico para o hospedeiro (Collado et al., 2009).

Embora *B. coagulans* GBI-30, 6086 seja adicionado a alimentos como esporos, que apresentam altas resistências físicas e químicas, parece claro que a matriz alimentar também é relevante para fornecer a eficiência de bactérias probióticas para esporos. De acordo com o que foi exposto, nossos achados sugerem que o iogurte pode ser um veículo eficaz para a entrega de bactérias probióticas formadoras de esporos. O iogurte parece ser uma matriz alimentar mais apropriada para *B. coagulans* GBI-30, 6086 quando comparada ao suco,

provavelmente devido à sua composição. Conforme resultado em nosso estudo, pode-se ver que o iogurte é mais rico em gorduras e proteínas, enquanto o teor de suco em carboidratos é maior. Desta forma, as duas matrizes alimentares mostram diferenças na sua composição que podem interferir na eficácia da matriz como transportadora, através da interação desses componentes com o probiótico, aumentando seus efeitos benéficos (Ranadheera, Baines, Adams, 2010).

5. CONCLUSÃO

Embora os métodos tradicionais e moleculares estejam disponíveis para a caracterização da microbiota intestinal, uma compreensão total da sua composição e diversidade e como as alterações neste microecosistema causam ou estão associadas com o desenvolvimento de doenças parece estar fora de nosso alcance. Assim, o desempenho de mais estudos *in vitro* e *in vivo* que analisam a diversidade, função e mecanismos de ação de microorganismos no TGI e também elucidam como probióticos podem afetar e interagir positivamente com a microbiota intestinal é essencial para o desenvolvimento de novas estratégias para prevenir e gerenciar várias condições patológicas relevantes.

Em conclusão, as cepas de *Bacillus* estudadas mostraram sobrevivência ou resistência a todos os processos tecnológicos. Entretanto, o comportamento ou resistência das cepas de *Bacillus* variou de processo para processo. Além disso, a sobrevivência e a especificidade dos *Bacillus* testados em cada processo tecnológico e alimento, respectivamente, variaram de cepa para cepa, assim a resposta pode ser considerada como cepa-dependente. Estudos complementares devem ser feitos para entender o mecanismo de ação e as diferenças protéicas envolvidas na resistência e especificidade da cepa ao processo e alimento em particular, respectivamente. Portanto, nosso estudo está trazendo contribuições significativas para a diversificação no mercado de alimentos probióticos. Ainda, *Bacillus coagulans* GBI-30, 6086 em pão de forma apresentou elevada resistência ao processo de forneamento e também durante o armazenamento. Portanto, ambos os pães tradicionais e integrais são matrizes alimentares promissoras como transportadoras de probióticos. No entanto, o pão integral mostrou maior capacidade para proteger a cepa probiótica, possivelmente pela presença de fibras na sua composição.

Adicionalmente, de acordo com a elevada sobrevivência (reduções decimais de aproximadamente 1 ciclo log) de *B. coagulans* GBI-30, 6086 observada em duas matrizes alimentares (suco e iogurte) parece que, para probióticos esporulados (cepa *B. coagulans* GBI-30, 6086), a matriz alimentar não tem tanta influência quanto para probióticos não esporulados. Adicionalmente, ambas são excelentes matrizes alimentares para funcionar como carreadoras de probióticos durante a passagem pelo trato gastrointestinal. O desenvolvimento de produtos alimentares que contenham *Bacillus* pode ser uma excelente estratégia para o transporte de probióticos em alimentos cujas características são deletérias para *Lactobacillus* e

Bifidobacterium e / ou alimentos cujo processo pode inativar ou reduzir a viabilidade de *Lactobacillus* e *Bifidobacterim*, contribuindo assim para o crescimento da área de alimentos funcionais. O estudo *in vivo* mostrou que o iogurte apresenta vantagens frente ao suco. Assim, o iogurte pode ser uma matriz alimentar mais eficiente para o fornecimento de probióticos. Os resultados obtidos neste estudo mostraram que o consumo diário de iogurte probiótico contendo *Bacillus coagulans* GBI-30, 6086 durante 21 dias foi capaz de diminuir significativamente os níveis de glicose e triglicerídeos em ratos quando comparado com o grupo controle. Além disso, o consumo deste iogurte probiótico resultou na modulação da microbiota intestinal, caracterizada pelo aumento da classe de Actinobactérias e ordens Bacillales e Bifidobacteriales.

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