UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA

AGRÍCOLA E DO AMBIENTE

ISOLAMENTO E CARACTERIZAÇÃO DE BACTÉRIAS ÁCIDO-LÁCTICAS DE LINGUIÇAS SUÍNAS DEFUMADAS E DESENVOLVIMENTO DE EMBUTIDO POTENCIALMENTE FUNCIONAL

LUCIANA SENTER

Porto Alegre, Rio Grande do Sul, Brasil

Abril de 2014

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente (Área de concentração: Microbiologia de Alimentos Processados e 'In Natura') como requisito para obtenção do grau de Doutor em Microbiologia Agrícola e do Ambiente.

Porto Alegre, Rio Grande do Sul, Brasil

Abril de 2014

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TESE

Submetida como parte dos requisitos
Para obtenção do Grau de

DOUTOR EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente

Instituto de Ciências Básicas da Saúde

Universidade Federal do Rio Grande do Sul

Porto Alegre (RS), Brasil

Aprovada em: 28/04/2015
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AGRADECIMENTOS

- Aos meus professores orientadores Dra. Patricia Valente e Dr. Eduardo César Tondo, pelo apoio, atenção e confiança. Especialmente ao prof. Eduardo, agradeço pela acolhida em seu grupo de trabalho aceitando este desafio de trabalhar com esta nova linha de pesquisa.

- Ao Instituto Federal de Educação, Ciência e Tecnologia de Santa Catarina – IFSC, pela disponibilidade da estrutura laboratorial e pelas bolsas de pesquisa dos Editais nº 13/2012, 07/2013, 12/2013 e 16/2013 da Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação que proporcionaram o desenvolvimento dos projetos supracitados integrantes da tese. À Fundação de Amparo à Pesquisa de Santa Catarina – FAPESC, pelo apoio financeiro pelo edital Universal nº 04/2012.

- Aos colegas do IFSC, Campus Xanxerê pelo incentivo à continuidade do doutorado, especialmente às colegas e amigas Manoela Alano Vieira, Eliane Maria Zandonai Michielin, Samantha Lemke Gonzalez, Carlise Baddin Fritzen Freire e Ieda Rottava pela parceria nos projetos.

- Aos bolsistas do Curso Técnico em Agroindústria Kellen Dalvana Bruchez, Patricia Hirt Sonaglio, Luana Pereira Camargo, Lucas José Martimiano, Leonardo Luiz Busatta, Clara Gorete Feiten Fillipin e Marilda de Lurdes Martini que auxiliaram nas diferentes etapas do projeto.

- Aos Srs. Adelto e Matheus Barrionuevo da Casa do Salame de Xaxim pela disponibilidade da estrutura física da indústria e formulação do produto fornecidos para testes. Ao Jamil Correia da Silva Júnior pela parceria.

- Aos professores Dr. Jacques Robert Nicoli, Dra. Regina Maria Nardi Drummond e Dr. Flaviano dos Santos Martins do Laboratório de Ecologia e Fisiologia de Microrganismos da Universidade Federal de Minas Gerais e aos estudantes do laboratório Dra. Karla Joseane Perez, Me. Rafael Wesley Bastos, Me. Leonardo Borges Acurcio, Me. Samir de Deus Elian Andrade e Dra. Ariane Martins pelo acompanhamento nos experimentos com os animais gnotobióticos.

- Ao Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente;

- À minha família, meus pais Roque e Lourdes Senter, à Luiz Senter, Maria Luiza e Tatiane Azevedo de Vargas Senter, especialmente ao esposo Cassiano Marcon pelo companheirismo, perseverança e paciência nos dias felizes e difíceis deste período. Vocês são bases da minha formação pessoal e profissional, muito obrigada por tudo!

‘Uma cabeça cheia de medos não tem espaço para sonhos’

(Jailson Nascimento)

Isolamento e caracterização de bactérias ácido lácticas de linguiças suínas defumadas e desenvolvimento de embutido funcional[1]

Autor: Luciana Senter

Orientador: Eduardo César Tondo

RESUMO

A demanda por novos produtos alimentícios tem motivado o desenvolvimento da área de embutidos, embora ainda exista certo preconceito com estes produtos, devido ao alto teor de gorduras, aditivos e especiarias neles contidos. A adição de probióticos aos embutidos pode trazer benefícios à saúde, contribuindo para o aumento do consumo destes produtos, os quais podem também se tornar alimentos funcionais. Nesse sentido, o desenvolvimento de um embutido funcional apresenta vantagem adicional, uma vez que a maioria dos alimentos funcionais são produtos lácteos, que não podem ser consumidos por pessoas intolerantes à lactose. Esta tese teve os seguintes objetivos: 1) isolar e caracterizar bactérias ácido lácticas a partir de linguiças suínas defumadas, sem adição de culturas iniciadoras de fermentação, produzidas em uma indústria de embutidos; 2) testar o potencial probiótico das cepas isoladas *in vitro* e *in vivo* e desenvolver uma linguiça suína adicionada da bactéria probiótica selecionada, nativa do produto elaborado. Os resultados demonstraram que dentre as vinte e seis cepas isoladas, *Lactobacillus plantarum* BLS29 apresentou propriedades tecnológicas adequadas para a industrialização de embutidos, como a rápida acidificação do meio de cultura, ausência da produção hemólise e de CO_2, rápida multiplicação em temperaturas de 15°C e 37°C e em presença de sais de cura. Esta cepa também foi capaz de inibir a multiplicação de diversos patógenos *in vitro*, inclusive *Salmonella* Enteritidis SE86 (importante causador de surtos alimentares no estado do RS). Além disso, a BLS29 não causou nenhuma lesão em camundongos gnotobióticos, sugerindo ser segura para a utilização em alimentos. Em um segundo momento desse trabalho, *Lactobacillus plantarum* BLS29 foi utilizado como cultura *starter* funcional para desenvolvimento da linguiça funcional, a qual foi elaborada utilizando a mesma formulação de onde a bactéria foi isolada. Nesse produto, a bactéria persistiu por períodos compatíveis com o período de consumo da região onde foi produzido o embutido. Foi verificada contagem total de 12,9 log UFC/g^{-1} de bactérias lácticas, no produto testado, e 10,6 log UFC/g^{-1}, no controle, sendo que as características físico-químicas de ambos foram muito semelhantes. Baseado nesses resultados a BLS29 pode ser utilizada na produção de embutido fermentado.

[1] Tese de Doutorado em Microbiologia Agrícola e do Ambiente, Instituto de Ciências Básicas e da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (128 p.) Abril de 2014.

Isolation and characterization of lactic acid bacteria of smoked pork sausages and development of embedded functional[1]

Author: Luciana Senter

Advisor: Eduardo César Tondo

ABSTRACT

The demand for new food products has motivated the development of the area of embedded, although there is still some prejudice with these products, due to the high fat, additives and spices contained therein. The addition of probiotics to inline can bring health benefits, contributing to the increased consumption of these products, which may also become functional foods. Accordingly, the development of a functional features embedded additional advantage , since most of the functional food are dairy products, which can't be consumed by lactose-intolerant people. This thesis has the following objectives: 1) to isolate and characterize lactic acid bacteria from smoked pork sausages without addition of starter cultures fermentation, produced in a embedded industry; 2) test the potential of probiotic strains in vitro and in vivo and develop a swine sausage added the probiotic bacteria selected, native to manufactured product. The results showed that among the twenty isolated six strains of *Lactobacillus plantarum* showed BLS29 technological properties suitable for the manufacturing of sausages, such as fast acidification of the culture medium, absence of CO_2 production, multiplying rapidly at temperatures of 15°C and 37°C and in the presence of curing salts. This strain was also able to inhibit the growth of various pathogens in vitro, including *Salmonella* Enteritidis SE86 (important cause of food borne outbreaks in the state of RS). Furthermore, BLS29 caused no damage in gnotobiotic mice, demonstrating to be safe for use in foods. In a second phase of this study, *Lactobacillus plantarum* BLS29 was used as functional starter culture for the development of functional sausage, which was prepared using the same formulation from which the bacterium was isolated. In this product, the bacteria persisted for periods consistent with those of consumption in the region where it was produced the embedded periods. Total count of 12.9 log UFC/g^{-1} of lactic acid bacteria in the tested product was verified, and 10.6 log UFC/g^{-1}, in control, and the physic-chemical characteristics were very similar to the control. Based on these results BLS29 can be used in the production of fermented sausages.

[1] Doctoral Thesis in Agricultural Microbiology, Instituto de Ciências Básicas e da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (128 p.) April, 2014.

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INTRODUÇÃO

O consumo e a exportação de produtos cárneos movimentam de forma significativa a economia do estado catarinense e de todo sul do Brasil. Isso se deve principalmente à colonização italiana e alemã, grandes produtores e consumidores de carne suína, a maioria originada da agricultura familiar (SANTA CATARINA, 2013).

A demanda por novos produtos alimentícios tem influenciado muito o desenvolvimento da área de embutidos, embora ainda se enfrente um determinado preconceito com este tipo de produto, já que, por vezes, os embutidos apresentam alto teor de lipídeos, sal, aditivos e especiarias (VUYST *et al*., 2008). A adição de probióticos aos embutidos podem trazer benefícios à saúde dos consumidores, contribuindo para o aumento do consumo deste produto (MACEDO *et al*., 2011) e melhoria da aceitação dos mesmos. O mercado de probióticos tem se mostrado muito promissor, sendo que são raros os produtos cárneos contendo probióticos. Esse mercado tem sido dominado predominantemente por produtos lácteos, porém estes não podem ser consumido por pessoas intolerantes à lactose. Nesse sentido, produtos cárneos contendo probióticos podem ter vantagem competitiva frente aos produtos lácteos.

O uso de bactérias ácido-lácticas (BAL) como culturas *starter* (iniciadoras de fermentação) tem se mostrado cada vez mais comum na indústria tanto pelas

características sensoriais quanto por seu efeito protetor contra patógenos que possam estar presentes nos alimentos fermentados (MACEDO *et al*., 2011). O uso dessas culturas acelera a acidificação e diminui a atividade de água do produto, dificultando a sobrevivência de micro-organismos patogênicos como *Listeria monocytogenes* e *Salmonella* sp. em salames, associado a outros fatores como condimentos que fazem parte da formulação.

As BAL ocorrem naturalmente em vegetais, no trato gastrintestinal, no leite e carne, sendo responsáveis pela fermentação natural de embutidos curados, como é o caso de salames e linguiças (HUGAS *et al*., 1993; HAMMES & HERTEL, 1998). Muitas espécies de *Lactobacillus* têm sido caracterizadas e têm apresentado atividade probiótica, proporcionando benefícios à saúde do hospedeiro. Por estes motivos, muitas são comercializadas como aditivos alimentares ou como medicamentos.

Neste contexto, os objetivos do presente estudo foram: a) Isolar e identificar bactérias ácido-láticas (BALs) nativas de uma linguiça suína colonial da região oeste de Santa Catarina; b) Testar o potencial de BALs nativas na inibição de patógenos alimentares; c) Testar o potencial probiótico de uma BAL nativa, selecionada por apresentar as melhores características tecnológicas para uso industrial; d) utilizar a BAL no desenvolvimento de linguiça suína fermentada potencialmente funcional.

O presente estudo será apresentado em forma de capítulos, sendo que o Capítulo I traz uma breve revisão bibliográfica sobre o tema e os resultados serão apresentados na forma de três artigos científicos identificados como Capítulo II, Capítulo III e Capítulo IV, respectivamente, descrevendo as metodologias e resultados relacionados em cada estudo.

O primeiro artigo apresenta dados sobre o isolamento e caracterização do potencial probiótico e tecnológico das BAL de linguiças suínas fermentadas naturalmente e tem como título 'Isolation and characterization of *Lactobacillus plantarum* BLS29 as a potential functional starter culture for pork sausage production' e foi submetido para publicação na Revista Científica *Annals of Microbiology* (ANMI-D-15-00244).

No segundo artigo intitulado 'Assessment of the antagonistic effect of *Lactobacillus plantarum* BLS29 on *Salmonella enterica se*rovar Enteritidis SE86 *in vitro* and in gnotobiotic mice', submetido à revista científica Submetido à revista científica Anaerobe (ANAEROBE-S-15-00078), é avaliada a ação antagonista da BAL em co-cultivo com o patógeno alimentar, monitorando-se a contagem populacional, pH e produção de atividade inibitória de BLS29 em diferentes tempos contra SE86. Também são apresentados dados sobre a colonização intestinal dos camundongos gnotobióticos por *L. plantarum* BLS29, além da análise histopatológica do cólon, íleo, baço e fígado dos animais.

No terceiro e último artigo intitulado ‘Fermented pork sausage using *Lactobacillus plantarum* BLS29 as potentially functional starter culture’, submetido à revista científica *Meat Science* (MEATSCI-S-15-00285), são apresentados dados referentes à elaboração do embutido funcional, abrangendo o monitoramento da população total de bactérias lácticas, verificação da presença de patógenos e análises físico-químicas do produto.

CAPÍTULO 1 - REVISÃO BIBLIOGRÁFICA

1.1 Carne suína e embutidos no contexto econômico

Entre os tipos de carnes mais comumente comercializadas no mundo, estão as carnes suína e de frango, sendo que a última vem liderando há décadas o *ranking* de proteína animal (RAMOS & GOMIDE, 2012). A carne suína permite diversificar o processamento, oferecendo grande variedade de produtos sob diversas opções de consumo, ganhando seu espaço num mercado de diversificados hábitos alimentares e de alimentos de preparo rápido e fácil (RAMOS & GOMIDE, 2012), como é o caso dos salames.

Atualmente, estima-se que o rebanho suinícola brasileiro seja da ordem de 38,95 milhões de cabeças, com a maioria do rebanho localizada na região Sul (47,9%). O Estado de Santa Catarina é o maior produtor, responsável por 20,1% do rebanho, sendo os municípios de Concórdia e Seara, localizados no oeste catarinense, responsáveis pela maior participação (IBGE, 2010). O consumo *per capita*/ano de carne suína no Brasil atinge cerca de 12 Kg, sendo considerado baixo, embora 77% da produção destina-se ao consumo interno. Rússia e Hong Kong são os maiores importadores de carne suína brasileira, com 50% e 10% de participação, respectivamente, na forma de cortes (RAMOS & GOMIDE, 2012). Recentemente a carne suína catarinense está competindo também com Estados Unidos, Canadá, Dinamarca, México, Chile e Japão, tendo iniciado suas exportações em agosto de 2013 (CARNETECBRASIL,

2013). O estado de Santa Catarina conta com pelo menos 54 agroindústrias envolvidas na produção de carne suína (PORTAL DO AGRONEGÓCIO, 2010).

De acordo com a Associação Brasileira da Indústria Produtora e Exportadora de Carne Suína (ABIPECS, 2013) a composição da carne suína tem mudado muito nos últimos anos, sendo que a quantidade de gorduras saturadas tem diminuído significativamente. Diferente dos animais ruminantes (bovinos, ovinos e caprinos), os suínos depositam a gordura diretamente nos tecidos sem modificação química, assim a composição dos ácidos graxos pode variar de acordo com a dieta alimentar. Se os animais forem alimentados com milho ou farelo de soja, aumentará a quantidade de ácidos graxos insaturados de sua carne (ácido linoleico, ácido graxo poli-insaturado essencial).

Velazco (2013) ressalta as características da carne suína, que possui 75% de umidade, 20% de proteínas, entre 5 e 10% de gorduras, 1% de carboidratos e 1% de minerais. A quantidade de colesterol varia de 60 a 80 mg por 100 g do produto, não representando um problema nutricional. Em geral a carne de porco contém cerca de 25% de ácido palmítico (16:0), 15% de ácido esteárico (18:0), 45% de ácido oleico (18:1) e cerca de 15% de ácido linoleico (18:2).

1.2 Embutidos cárneos

Os embutidos cárneos tiveram sua origem na região do Mar Mediterrâneo, sendo que os romanos herdaram a técnica dos gregos e

aperfeiçoaram. A produção de embutidos surgiu da necessidade de aproveitar todas as partes como miúdos, sangue e intestino. Cada região possui características particulares de fermentação e desidratação, que dão características próprias aos embutidos (ORDOÑEZ *et al*., 2005). Geralmente são compostos de carnes, miúdos ou gorduras adicionados de condimentos e especiarias.

Dentre os embutidos cárneos mais consumidos no Brasil, destacam-se a linguiça e o salame. Entende-se por Linguiça Colonial, o produto cárneo industrializado, elaborado exclusivamente a partir de carnes suínas, adicionado de toucinho, ingredientes, moído em granulometria variável, embutida em envoltório natural, curado, que sofre um processo rápido de fermentação, defumado e dessecado por tempo indicado pelo processo de fabricação (BRASIL, 2000).

Entende-se por salame, embutidos crus, curados, fermentados, maturados e dessecados, podendo ou não ser defumados (TERRA *et al*., 2004, ORDOÑEZ *et al*., 2005). No Brasil, o produto é classificado em oito diferentes classes, de acordo com a granulometria da carne e do toucinho, tipo de matéria-prima e condimentação (BRASIL, 2000).

Apesar de ambos possuírem o mesmo processo tecnológico o que os diferem basicamente é do tipo de carne que provem e a sua umidade, já que para o salame a umidade máxima permitida por legislação é de 40% e

atividade de água 0,92, entretanto, a umidade da linguiça ultrapassa esta porcentagem dependendo do tempo de maturação (BRASIL, 2000).

1.3 Matéria-prima e ingredientes usados na formulação de linguiças

Além da carne, também é adicionada gordura suína e alguns ingredientes a seguir descritos, que após serem misturados e curados, são embutidos em envoltórios, preferencialmente naturais (tripas), podendo ser defumados e maturados conforme a indicação do fabricante.

1.3.1 Sal (Cloreto de Sódio)

O sal desempenha um papel importante na textura dos produtos cárneos picados, solubilizando as proteínas miofiblilares, mas também potencializa o sabor, atua desidratando e modificando a pressão osmótica, inibindo por consequência a multiplicação microbiana (ORDOÑEZ *et al*., 2005).

1.3.2 Açúcar

Os açúcares criam condições redutoras durante o processo de cura, evitando o desenvolvimento de aromas oxidados. De outro lado, contribuem para a redução de nitritos a nitratos e destes à óxido nítrico, reações que contribuem para a formação do pigmento vermelho característico de embutidos curados (ORDOÑEZ *et al*., 2005). Os açúcares também servem como fonte de energia para bactérias fermentadoras como os *Lactobacillus*, que produzem ácido láctico, diminuindo o pH do produto e agindo em conjunto com o cloreto

de sódio para diminuir a retenção de água nas fibras cárneas, proporcionando a desidratação do produto e dificultando a sobrevivência de micro-organismos deteriorantes e patogênicos como *Pseudomonas* e *Salmonella* (TERRA *et al*., 2004).

1.3.3 Sais de cura

O nitrato de sódio ($NaNO_3$) não tem atividade antioxidante nem antimicrobiana mas torna-se funcional ao ser convertido em nitrito de sódio ($NaNO_2$). As funções desses sais incluem a estabilização da cor, melhoramento da textura, desenvolvimento do *flavor* característico de produtos curados, este último produzido por intermédio da atividade microbiana (JAY, 2005).

O nitrito é convertido na carne em ácido nitroso, o qual é reduzido à óxido nítrico. O óxido nítrico converte a mioglobina em mioglobina nitrosa, um pigmento de cor vermelho característico de produtos curados não cozidos. A adição de ácido ascórbico auxilia na formação do óxido nítrico e protege os pigmentos cárneos da oxidação (TERRA *et al*., 2004).

Ainda de acordo com TERRA *et al*., (2004), a resistência das bactérias ácido lácticas frente à inibição pelo nitrito está relacionada à carência da enzima ferrodoxina, que está presente em clostridios e atua no transporte de elétrons durante a quebra anaeróbica do piruvato para formar ATP, H_2 e CO_2. A atividade do nitrito aumenta à medida que o pH diminui, assim sua ação é

potencializada pela inoculação de BAL. O uso do nitrito em embutidos curados está muito mais ligado à saúde pública pelo fato de inibir um dos principais patógenos em alimentos fermentados, *Clostridium botulinum,* do que relacionado à pigmentação da carne (JAY, 2005).

1.3.4 Temperos

Alho, cebola, pimenta e cominho são os temperos mais comumente usados para realçar o sabor dos embutidos, entretanto também desempenham o papel antioxidante e antimicrobiano nos embutidos (TERRA *et al*., 2004). A alicina é um importante composto antimicrobiano presente no alho e na cebola e contribuem para a inibição microbiana em embutidos fermentados.

1.4 Microbiota fermentadora de embutidos

As bactérias ácido-lácticas (BAL) são responsáveis pelo processo de maturação que os salames e linguiças sofrem. Este processo envolve uma série de transformações bioquímicas como produção de ácido láctico, proteólise e lipólise, todos produzidos principalmente pela ação de BALs, como *Lactobacillus, Micrococcus, Pediococcus, Leuconostoc e Bacillus* (ORDÓÑEZ *et al*., 2005). As BAL são responsáveis pela fermentação láctica que ocorre em produtos cárneos maturados. Sua importância está na produção de ácido lático que baixa o pH do produto, fazendo com que as fibras de carne desidratem, diminuindo também a atividade de água do produto (FIORENTINI *et al*., 2010).

Esses dois fatores associados são determinantes para a sobrevivência da maioria dos patógenos.

Hugas *et al*. (1993) identificaram a presença de *Lactobacillus sake, L. curvatus, L. bavaricus* e *L. plantarum* em embutidos secos de fermentação expontânea na Espanha. Hammes & Hertel (1998) afirmaram que a formação do *flavor* dos embutidos se deve à ação do metabolismo microbiano em conjunto com os condimentos adicionados, defendendo a adição de culturas *starters* como forma de acelerar a maturação dos embutidos devido à ação de proteinases microbianas. Albano *et al*., (2009) isolaram *L. plantarum* e *Enterococcus faecalis* da maioria das amostras de 'Alheira', um embutido fermentado típico de Portugal, entre outras doze espécies.

Entretanto, como demonstram alguns estudos, a ocorrência de bactérias láticas em salames fermentados naturalmente é relativamente baixa, fazendo com que o tempo para atingir o pH adequado permita que patógenos que porventura estejam presentes consigam sobreviver e multiplicar no alimento (SAAD *et al*., 2011; FIORENTINI *et al*., 2010). Dalla Santa (2012), confirma os dados acima, obtendo contagens entre 6 e 8 $\log_{10}$ (UFC/g) de bactérias lácticas, sendo que 24% das amostras de salames analisadas estavam em desacordo com a legislação brasileira vigente (BRASIL, 2001) no que se refere à contaminação por coliformes à 45°C ou *Staphylococcus aureus*.

Algumas culturas *starter*, ou iniciadoras de fermentação, são encontradas no mercado, a fim de contribuírem com a melhoria tanto na qualidade microbiológica quanto na textura e características sensoriais de vários produtos fermentados, incluindo queijos, iogurtes, produtos cárneos, bebidas fermentadas, chucrute, entre outros. Em embutidos como o salame, é possível introduzir culturas iniciadoras de fermentação composta por *Lactobacillus, Micrococcus* ou *Pediococcus* (ORDÓÑEZ *et al*., 2005). Além destes, Macedo *et al*. (2011) destacam o emprego de espécies de *Staphylococcus*, bolores como *Penicillium* e leveduras como *Debaryomyces* e *Candida*.

Culturas *starters* são comercializadas sob a forma liofilizada, contendo mais de um micro-organismo que vão desempenhar funções específicas. Uma das culturas que pode ser empregada na fabricação de embutidos curados é a Bactoferm T-SPX contendo *Pediococcus pentosaceus* e *Staphylococcus xylosus* (Chr Hansen). Os *Lactobacillus* e os *Pediococcus* produzem ácido láctico a partir de açúcares, controlando o desenvolvimento de micro-organismos indesejáveis, melhora a coloração, acelera a desidratação e caracteriza o típico sabor ácido dos produtos cárneos fermentados (TERRA *et al*., 2004).

1.5 Patógenos em embutidos cárneos

Entre os principais patógenos bacterianos que podem se desenvolver em embutidos cárneos curados, estão *Salmonella* sp., que ocupa o primeiro lugar em surtos no Rio Grande do Sul (TONDO e BARTZ, 2011), *Staphylococcus* coagulase positiva e coliformes termotolerantes (à 45ºC), incluindo *Escherichia coli*. Apenas essas três bactérias tem controle exigido pela RDC nº12 de 02 de janeiro de 2001 (BRASIL, 2001). Além destes, existem outros patógenos extremamente importantes como a *Listeria monocytogenes*, uma bactéria que se desenvolve principalmente em carnes cruas e refrigeradas, encontrada em 18 de 60 amostras de salames italianos analisados no sul do Brasil (DALLA SANTA, 2011), capaz de causar aborto e acometer consumidores de sistema imunológico suscetível. *L. monocytogenes* é de difícil controle em alimentos, principalmente naqueles que não sofrem tratamento térmico (BERSOT *et al*., 2001), como é o caso dos salames, sendo que à medida em que aumenta o processamento, aumenta o risco de contaminação (JAY, 2005).

Em um estudo realizado em embutidos artesanais (SENTER *et al*., 2010), se observou que a diminuição dos níveis de contaminação de coliformes termotolerantes à 45ºC, *Salmonella* sp. e *S. aureus*, só foi possível após o treinamento dos manipuladores e comprometimento de toda equipe com a aplicação de BPF. Infelizmente é muito comum encontrar esses patógenos em alimentos minimamente processados (MENDES *et al*., 2011), queijos

(NASCIMENTO *et al*., 2011), mãos de manipuladores (BIONDO *et al*., 2011), linguiças mistas (CHESCA *et al*., 2011) e numa variedade de outros alimentos.

1.6 Probióticos

Em um mercado com consumidores mais exigentes e preocupados com a manutenção da saúde, o consumo de alimentos funcionais vem ganhando espaço cada vez maior. Probióticos caracterizam-se por serem micro-organismos vivos capazes de melhorar o equilíbrio microbiano intestinal produzindo efeitos benéficos à saúde do indivíduo quando administrados em quantidades adequadas (FAO, 2001; BRASIL, 2002). Estes alimentos tem a finalidade de promover a saúde, melhorando o trânsito intestinal dos alimentos, facilitando a digestão, alívio de sintomas de intolerância à lactose, aumento da resposta imune e redução do colesterol sanguíneo (SAAD *et al*., 2011).

Os micro-organismos probióticos geralmente pertencem à algumas espécies dos gêneros bacterianos *Bifidobacterium, Lactobacillus* ou *Enterococcus,* sendo os dois primeiros mais comuns e geralmente mais seguros já que muito raramente apresentam fatores de patogenicidade, enquanto que os *Enterococcus* podem ser oportunistas e em alguns casos podem adquirir resistência à antibióticos ou fatores de patogenicidade por meio de plasmídeos (FRANZ *et al.,* 2003; AMMOR & MAYO, 2006)

Para que um alimento seja considerado probiótico este necessita conter um determinado número de células probióticas viáveis, embora não exista uma

regra que determine qual a concentração fixa de micro-organismos probióticos já que pode variar de acordo com o agente e a recomendação do fabricante, apresentando uma média de 10^9 UFC/g de alimento (WGO, 2008). Grande parcela das culturas probióticas que são amplamente utilizadas na indústria de laticínios tem origem intestinal, alguns deles estão citados na Tabela 1.

Tabela 1.1 Micro-organismos probióticos comerciais.

Micro-organismo	Cepa	Companhia produtora
Bifidobacterium animalis	Bb-12	Chr. Hensen
Bifidobacterium bifidum	Bb-11	Chr. Hensen
Bifidobacterium essencis		Danone® (Activia)
Bifidobacterium infantis	Shirota Immunitas®	Yakult Danone®
Lactobacillus acidophilus	LA-1, LA-5	Chr. Hensen
Lactobacillus casei	Shirota	Yakult (Yakult®)
Lactobacillus plantarum	299v, Lp01	Probi AB
Lactobacillus reuteri	SD2112/MM2	Biogaia
Saccharomyces boulardii		

Fonte: Adaptado de Soccol *et al*. (2010).

O efeito protetor de bactérias probióticas tem sido evidenciado especialmente em estudos com camundongos, quando inoculados com bactérias patogênicas (MAHONEY & HENRIKSSON, 2003; DORTU *et al*., 2008). BANBIRRA *et al*., (2007) demonstrou que a cepa de *Lactobacillus sakei*

2a foi capaz de proteger o trato digestivo contra infecção causada por *L. monocytogenes*, apresentando potencial para utilização como cultura probiótica em produtos fermentados.

O número de pedidos de depósitos de patentes de cepas probióticas tem aumentado muito nos últimos anos, sendo que a mais conhecida cepa de *L. plantarum* (299v) foi patenteada pela PROBI AB em 2010 (PROBI AB, 2010), sendo registrados outros pedidos referentes às propriedades terapêuticas desta cepa ou de outras da mesma espécie (AB-BIOTICS, 2011; IBPRS, 2013). Entre alguns dos benefícios desta cepa estão: atividade antimicrobiana contra *Listeria monocytogenes, Bacillus cereus, Escherichia coli, Salmonella enterica* subsp. *enterica* e *Helicobacter pylori,* redução dos níveis de colesterol sanguíneo, modulação da resposta imune e melhora nos sintomas causados por diarreias e síndromes inflamatórias intestinais (PROBI AB, 2010). Outra cepa de *L. plantarum* NR74 (YOON *et al*., 2013) também demonstrou efeitos benéficos à saúde diminuindo os níveis de colesterol e suprimindo inflamação em células THP-1 em camundongos.

1.6.1 Requisitos para ser considerado probiótico

Entre as principais características para ser considerados probióticos, as bactérias precisam resistir ao pH ácido, aos sais biliares, serem aderentes à parede intestinal no hospedeiro, não apresentar patogenicidade, não ter

atividade hemolítica, ter capacidade de inibir patógenos, produzir bacteriocinas (FAO, 2001).

Algumas espécies do gênero *Lactobacillus* (*L. rhamnosus, L. paracasei*) são comprovadamente probióticas em produtos cárneos fermentados, exercendo também efeito protetor, preservando características tecnológicas e sensoriais do produto (SAMESHIMA *et al*., 1998; ARIHARA, 2006; MACEDO *et al*., 2008).

1.6.2 Probióticos em carnes

Entre os produtos cárneos possíveis de se empregar cepas probióticas, os embutidos se mostram mais atraentes por geralmente não passarem por nenhum tratamento térmico antes de serem ingeridos. Hugas & Manfort (1997) defenderam o uso de lactobacilos como culturas iniciadoras de fermentação com características probióticas, instigando o desenvolvimento de embutidos fermentados probióticos sugerindo o consumo de 10^7-10^9 células viáveis por dia para ter efeitos benéficos em humanos. O emprego de culturas probióticas em produtos cárneos iniciou-se em 1998 em países como Japão, Itália e Alemanha (ARIHARA, 2006; PENNACCHIA *et al*., 2006), entretanto ainda está pouco difundido, quando comparado à variedade de produtos probióticos lácteos.

A utilização de culturas *starter* com propriedades funcionais tem ganhado impulso com o crescente mercado de probióticos, unindo as características

tecnológicas com a capacidade de produzir compostos antimicrobianos que inibam grande parte dos patógenos que possam estar nos embutidos (LEROY *et al*., 2006). Alguns autores defendem que há certa dificuldade em manter as culturas probióticas vivas em salames devido à baixa atividade de água do produto, o que acabaria por prejudicar o desenvolvimento dos probióticos. Entretanto, Erkkilä *et al*. (2001) obtiveram sucesso na elaboração de salame probiótico com cepas comerciais de *Lactobacillus rhamnosus* GG e E-97800 para produzir salame fermentado bem como Macedo *et al.* (2008) que utilizaram cepas de *Lactobacillus casei* e *Lactobacillus paracasei*.

Samelis *et al*. (1998) constatou que embutidos elaborados por fermentação natural no sul europeu apresentavam melhor qualidade sensorial que aqueles elaborados com culturas *starters* do norte da Europa. Isso se deve à pouca habilidade dessa cultura inoculada em competir com a microbiota local do salame. Diante da necessidade de buscar novas cepas com características probióticas e que possam ser aplicadas como culturas *starter* funcionais, mas que preservem as características tecnológicas e sensoriais dos embutidos, este estudo buscou isolar, identificar e testar características probióticas e tecnológicas de uma cepa nativa de linguiça suína fermentada naturalmente para que seja utilizada futuramente como cultura *starter* potencialmente funcional.

CAPÍTULO 2 – Artigo 1

Submetido para publicação na Revista Científica *Annals of Microbiology* (ANMI-D-15-00244).

Isolation and characterization of *Lactobacillus plantarum* BLS29 as a potential functional starter culture for pork sausage production

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Research Paper

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Abstract

The aim of this study was to isolate and characterize lactic acid bacteria (LAB) from naturally fermented pork sausages and test them for the use as a functional starter culture in the production of fermented sausages. LAB (n=26) were isolated from natural fermented pork sausages. Isolates were identified by 16S rDNA or Internal Transcribed Spacer (ITS) region by the sequencing, characterized technological and functional and safety proprieties. Based on those analytical results, one isolate was chosen and its survival was tested in simulated gastric juice and intestinal juice. *Lactobacillus plantarum* (n=15) was the predominant species in fermented sausage, followed by *Enterococcus faecium* (n=8), *Lactobacillus brevis* (n=1), *Enterococcus durans* (n=1) and *Enterococcus hirae* (n=1). *L. plantarum* BLS29 was selected because it was not able to produce CO_2, demonstrated to be homolactic and could multiply at temperatures ranging from 15° C to 37° C, usually practiced during the sausage cure process. This strain showed better growth than other LABs in curing salts and antimicrobial activity against foodborne pathogens. According to our findings, BLS29 can be a promise strain to be used as a functional starter culture for the production of fermented pork sausage.

Keywords: probiotics, *Lactobacillus plantarum,* fermented pork sausage

1 Introduction

The demand for new food products has greatly influenced the development of fermented meat products, despite some prejudice against this type of product, which has sometimes been considered unhealthy because of the high saturated fat, additives and spices (Arihara, 2006; De Vuyst et al. 2008; Macedo et al. 2012). According to Fiorentini et al. (2010) and Raigorodsky (2011), the evolution of fermented sausages is relatively old, having emerged from the need to use all body parts of animals as food, and was introduced in Brazil by Italian immigrants and, more recently, gave rise to small factories, transforming the economy, especially in Southern Brazil. Salamis and sausages are raw embedded, cured, fermented, matured and dry, and may or may not be smoked (Terra et al. 2004; Ordoñez et al. 2005). In Brazil, these products are classified into eight different classes, according to the composition of meat and fat, type of raw material and seasoning (BRAZIL, 2000), considering that salamis have moisture content below 40%.

Probiotics are defined as live microorganisms which, when consumed in adequate amounts as part of the diet, may play an important role in respiratory, immune, and gastrointestinal functions and have a significant effect on the clearance of infectious diseases in children and lactose intolerance (FAO, 2001). Foods containing probiotic are functional foods with great commercial interest whose consumption has increased in recent years. Although Metchnikoff (1907) has described the benefits of microorganisms to consumers'

health, it was only in the last 30 years that the study of probiotics has been intensified, especially in the selection and characterization of probiotic species (FAO, 2001). Numerous studies claim that probiotic species contribute to the reduction of cholesterol levels in the blood, prevention and treatment of food-related allergies, assist in the treatment of infections caused by *Helicobacter pylori*, prevention of cancer and modulation of intestinal flora (Arihara, 2006; El-Ghaish et al. 2011; Yoon et al. 2013).

Probiotics have been extensively used in dairy product production, but few studies demonstrated its usage in fermented meat producs (Erkkilä et al. 2001; Arihara, 2006; De Vuyst et al. 2008). In 1998, Germany was the first country to produce salami containing three probiotic strains of intestinal lactic acid bacteria (*Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium* spp.), demonstrating that it is possible to expand the supply of probiotic products (Hammes and Hertel 1998). The development of sausage containing probiotics able to provide health benefits and that ensure the safety of the product is of great interest. Generally, probiotic bacteria of the genus *Lactobacillus* are capable to produce bacteriocins, inhibiting the growth of gram-positive bacteria like *Staphylococcus aureus* and *Listeria monocytogenes* (Incze, 1998). Some species of LAB are used as starter cultures, contributing to the sensory quality and with the microbiological safety of industrial fermented products (Leroy et al. 2006). One way to attach both characteristics is to use the new generation of ‘functional starter cultures' (De Vuyst 2000). Wild strains are

naturally dominant in natural fermentation and show a high capacity for metabolic formation of flavor. Natural selected strains are interesting because frequently they produce antimicrobial compounds, enhancing the competitiveness against pathogens (Ayad et al. 2000; Maldonado et al. 2002).

The study of functional cultures has aroused great attention in recent years (Erkkilä et al. 2001; Arihara, 2006; De Vuyst et al. 2008), either on the addition of free probiotics (Pennachia et al. 2006; Leroy et al. 2006) or in encapsulated forms (Muthukurasami and Holley 2006; Ruiz 2011). Most of the probiotic bacteria are found in the human intestinal tract, milk or vegetables when it would be more consistent the use of meat isolated bacteria that could contribute to a better sensory quality of the product. The aim of this study was to isolate LAB from fermented pork sausages and to characterize potential functional starter cultures for the use in sausage production.

2 Material and methods

2.1 Isolation of LABs

Microorganisms were isolated from fermented pork sausages produced without the addition of starter cultures from three different batches, produced in a processing unit in the State of Santa Catarina, Southern Brazil. Samples of 25 g of pork sausage were diluted in 225 ml of 0.1% peptone water and

homogenized inside sterile plastic bags (Stomacher, AES, France). One aliquot of 100 µl of each dilution was inoculated on MRS agar (Acumedia), using pour-plate method, and plates were incubated at 37 °C for 48 h using anaerobic jars (Permution, Brazil). After incubation, different colonies were randomly selected and checked for its purity and were analyzed after Gram staining. All selected isolates were stored at -20 °C in 30% (v / v) glycerol and propagated into tubes containing 5 ml of MRS broth before next experiments.

2.2 Molecular Identification of Isolates

Genomic DNA of isolates were extracted and the 16s rDNA genes were amplified using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') (Lane 1991). For those samples whose identification by 16S rDNA was not possible or inaccurate, the 16S-23S rDNA intergenic spacer region (ITS) was amplified using bacterium specific universal primers 13BF (5'-GTGAATACGTTCCCGGGCCT-3') and 6R (5'-GGGTTYCCCCRTTCRGAAAT-3') (where Y=C or T, R=A or G). The sequencing reactions were made using the same primers previously used in the PCR (LUDWIG BIOTEC, BRAZIL). The 16S and ITS sequences obtained were submitted to the BLAST search program of the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov) in order to search for homologous sequences. BioEdit was used to edit sequences and for the construction of its contigs.

2.3 CO_2 and exopolysaccharides production

The production of carbon dioxide gas (CO_2) was determined using MRS broth inside glass tubes with inverted Durham tubes. Ammonium citrate was replaced by ammonium sulfate in order to induce glucose use for fermentation. The tubes were incubated during 48 hours at 37 °C and gas production was verified from the glucose fermentation. Exopolysaccharides production was determined checking the growth of the strains on MRS agar, substituting glucose by 5% of sucrose. The plates were incubated for 7 days at 25 °C and checked for exopolysaccharides production by negative staining (Hugas et al. 1993).

2.4 Acidification Capacity

The acidification capability of isolates was determined using tubes containing 10 ml of MRS broth (pH 6.6) without KH_2PO_4, but containing ammonium citrate, 0.3% of sodium citrate (Dinamica) (Hugas et al. 1993). The pH level was evaluated using Tecnal Tec-5 electrode Mettler Toledo (Tecnal, Brazil) after 48h of incubation at 37°C. The mean value of three pH measurements was calculated.

2.5 Tolerance to sodium chloride and sodium nitrite

Isolates were checked according to their tolerance for 5% and 8% NaCl (Vetec), to the tolerance of 3% NaCl associated with $NaNO_2$ (Vetec) and of 200

ppm for check the ability of growing in MRS broth after 72 h at 37 °C incubation (Hugas et al. 1993). Growth was determined by the McFarland scale.

2.6 Growth at different temperatures

The ability to grow at different temperatures is a very important technology in the production of sausages property. The growth temperatures was observed in MRS broth after incubation for 72 hours at 15, 37 and 45 °C and incubated for seven days at 4 °C (Dalla Santa 2008) and checked with MacFarland scale.

2.7 Determination of antagonistic activity

The antagonistic activity of each LAB isolate was determined by the presence of a growth inhibition zone of foodborne indicator strain around the spot of LAB. For assessment of antagonistic activity, 5 ml of each culture was inoculated in the center of a Petri dish containing MRS agar and incubated at 37°C for 24 hours in microaerophilic conditions. After this period, the plates were treated with chloroform in the lids fitted with sterile filter paper, during 30 minutes, and residual chloroform was evaporated, with the lids open for another 30 minutes. Then, 10^8 colony forming units (CFU)/ml of the pathogen in semi-solid BHI (himedia) agar (0.75% p/v) were inoculated using the overlay method. Indicator strains were tested against *Salmonella enterica* serovar Enteritidis SE86 (one of most important foodborne pathogen in the State of Rio Grande do Sul, Southern Brazil) (Tondo and Ritter 2012), *Salmonella* Typhimurium (ATCC

14078), *Staphylococcus aureus* (ATCC 14458), *Escherichia coli* O157:H7 (ATCC 43888), *Bacillus cereus* (ATCC 33010) and *Listeria monocytogenes* (ATCC 6477). After incubation under anaerobic conditions, the diameters of the inhibition zones were determined with a caliper (Martins et al. 2010).

2.8 Tolerance to simulated gastric juice and intestinal juice

The BLS29 strain was checked according to its tolerance to the upper gastrointestinal tract by simulated gastric and intestinal juices according to Meira et al. (2012) with modifications. The simulated intestinal juice consisted of 1 mg/ml of pancreatin (Sigma, USA), 0.5 % NaCl and adjusted to pH 8.0 with or without 5g/l of porcine bile (Sigma, USA). Both solutions were filtered using membrane filter of 0.22 µm. After two propagations, the bacterial cells incubated 24 h on MRS broth were concentrated by centrifugation (12,000 g for 5 min), washed twice in phosphate buffer pH 7.0 and suspended in 0.5% NaCl solution. Two hundred microliters (200 µl) of the cell suspension were incubated at 37 °C in a bath (De Leo, Brazil) in the presence of 1.0 ml of simulated gastric juice. Viable cell counts were carried out at initial time (0h), 3 h and 4h of exposure to simulated gastric juice, simulating intestinal transit tolerance. The simulated gastric juice solution was daily prepared with 3 mg/ml of pepsin (Sigma, USA), 0.5% NaCl and acidified with HCl until pH 4-0, 3-0, 2-5 and 2-0. The surviving BLS29 cells to pH 2.0 (acid-exposed) and not acid-exposed were inoculated in simulated intestinal juice.

2.9 Hemolysis

Lactobacilli strains were cultured on blood agar plates and incubated for 48 hours at 30 ºC (Maragkoudakis et al. 2006; Meira et al. 2012). Strains that did not change the medium (γ-hemolysis) or that produced a clear green halo around the colony (α-hemolysis) were classified as non-hemolytic, while strains that showed a lytic zone around the colony were classified as hemolytic (β-hemolysis).

3. Results

3.1 Isolation and characterization of microorganisms

Twenty-six isolates of LAB were isolated from pork sausage starter-free manufactured in a meat processing unit in the western part of the State of Santa Catarina, Southern Brazil. On the basis of 16S rRNA gene and ITS region sequencing data, isolates were identified as belonging to the genus *Lactobacillus* and *Enterococcus* (Table 1). *L. plantarum* was the most frequently isolated species. The accession numbers were identified and corresponded to the sequences in Genbank where the highest identified scores after submitting the sequence query to the BLAST tool were obtained.

3.2 Exopolysaccharide, CO_2 and hemolysis production

All strains produced exopolysaccharides on MRS plates supplemented with 5% of sucrose. None of the *Lactobacillus* strains were positive for producing CO_2 or hemolytic reaction.

3.3 Acidification capacity, salt tolerance and growth at different temperatures

All the isolated strains were tested for their ability of acidification, tolerance to curing salts and growth at different temperatures (Table 2) in order to select a fermentative strain with the potential to be used in the preparation of sausage with functional culture.

Among the *L. plantarum* strains, BLS29 was chosen and tested for its probiotic potential, based on the high acidification of MRS broth; BLS29 grow well in the presence of curing salts and grow at temperature of 15 °C, what is usually practiced during sausages ripening.

3.4 Antimicrobial activity

Lactobacilli isolated from pork sausages were tested for inhibitory potential against *Salmonella enterica* serovar Enteritidis SE86, *Salmonella* Typhimurium (ATCC 14078), *Staphylococcus aureus* (ATCC 14458), *Escherichia coli* O157:H7 (ATCC 43888), *Bacillus cereus* (ATCC 33010) and

Listeria monocytogenes (ATCC 6477) (Table 3), and demonstrated to be able to inhibit all pathogens.

3.5 Tolerance to gastric simulated and intestinal juice

The BL29 strain was tested for resistance to simulated gastric juice and intestinal juices (Tables 4 and 5), because in previous tests showed the ability to produce exopolysaccharides, but not CO_2, was tolerant to cure salts, was able to acidifiy MRS broth, could grow at different temperatures and demonstrated to be able to inhibit food pathogens.

L. plantarum BLS29 was incubated at 37°C, then subjected to simulate gastric conditions for up to three hours. It was found that although the viable cell count had declined by about 3 log, this strain retained about 5 log viable cells. *L. plantarum* BLS29 and together *L. plantarum* BLS29 survived to pH 2.0 (BLS29 acid exposed) were bile salts exposure for four hours, simulating tolerance passage through the intestinal transit and both strains showed no significant difference microbial counts. In both treatments in the BAL study it was shown able to survive and multiply demonstrating its potential probiotic species.

4. Discussion

In spontaneous fermentation, it is common to find several species of

lactobacilli like *L. sakei and L. curvatus* (Hugas *et al*., 1993), staphylococci, micrococci and enterococci as most of the fermenters, in contrast to the results obtained in this study which reported the prevalence of *L. plantarum* and *E. faecium. L. plantarum* has a wide distribution in nature (fermented vegetables, meats, dairy products and intestine) and present a metabolic versatility, may be an important potential probiotic used in foods. Other researchers also demonstrated *L. plantarum* as an important fermenter in foods, for example, Axelsson et al. (2012) reported that *L. plantarum* NC8 isolated in 1980s from grass silage and identified as naturally plasmid free.

The flavor of the sausage can be attributed to *Lactobacillus* and other LAB, such as enterococci that has several metabolic pathways, such as proteolytic, lipolytic and esterolytic (Sarantinopoulos 2001), which contribute to the flavor both of the Mediterranean cheeses and the fermentation of sausages (Hugas et al. 1993). *Enterococci* are associated with the fermentation of meat products, being detected in low numbers as 10^2 – 10^5 UFC g^{-1} in the final product (Samelis et al. 1998, Franz et al. 2003). These bacteria are especially found in handmade products of Southern Europe, and can contaminate meat at the time of slaughter.

Only *Lactobacillus* were tested once despite being characterized as probiotics because enterococci may possess virulence factors not being as secure as *Lactobacillus* (Franz et al. 2003). Although some *Enterococci* strains are characterized as functional, as this bacterium has plasmids including some

that may be related to resistance factors to antibiotic and pathogenicity, it is not recommended to use this type of microorganism in food as starter (Franz et al. 2003). In the production of sausages, it is important that the lactic acid bacteria be homofermentative to prevent the production of voids caused by the production of carbon dioxide, which would result in a displeasing appearance (Ordoñez et al. 2005). Furthermore, heterolactic bacteria can produce acetic acid, which in high quantities can produce astringent taste.

Production of exopolysaccharides by the isolates of this study can be considered positive, due to its thickener and emulsifier feature, which gives origin to the product (Ballús et al. 2010). Furthermore, production of exopolysaccharides may contribute to the adhesion of probiotics to the intestine, contributing to the colonization of this surface (Sanders and Klaenhammer 2001).

Although bacteriocins of LAB are inefficient to inhibit Gram-negative bacteria, our results demonstrated similar effects at few exceptions have seen described Hu et al. (2013) where plantaricin 163 of *L. plantarum* showed broad inhibitory activity against foodborne pathogens including *L. monocytogenes, S. aureus, S.* Typhimurium and *E. coli,* indicating the potential to be used as a biopreservative in the food industry. Gong et al. (2010) have reported the action of plantaricin MG against *L. monocytogenes* and *S.* Typhimurium. Lee et al. (2014) checking protects of *L. plantarum* HY7712 and found good results against the impairment of NK-Cells activity caused by y-irradiation in mice. This

study showed the benefic action of *L. plantarum* in functional food, highlighting the possible use of this functional starter.

In order to provide beneficial effect to the hosts, the microorganisms used as probiotics must reach and colonize the intestine, and for that they must survive the conditions of the gastric acidic fluid and bile salts. These abilities were demonstrated by BLS29. It is remarkable that BLS29 demonstrated a population increase in the presence of pancreatin with or without bile salts and similar results were obtained by Pennacchia et al. (2004) studying Lactobacillus strains from fermented sausages in Italy.

Some factors may negatively impact on the development of functional cultures in meat environment, as the concentration of curing salts, low pH and water activity (De Vuyst et al. 2008), so it is appropriate to use strains adapted to these conditions (Hugas et al. 1993; Pennacchia et al. 2006). Therefore, the BLS29 strain was chosen in order to follow the study, since the strain survived well in this kind of conditions, growing at temperatures prevailing during maturation product and developed well in the presence of curing salts.

Besides the good manufacturing practices that should be adopted in the production of meat products, a quick acidification carried out by LAB and the production of bacteriocins may contributes to the inhibition of food pathogens such as *Salmonella*, *E. coli, S. aureus* and *L. monocytogenes.* The flavor of fermented foods depends on several factors, mainly by the quantity and quality

of the source, amount and type of ingredients (eg meat, salt and spices), temperature, processing time, smoking, and type of starter culture used (Leroy et al. 2006).

The involvement of proteolytic enzymes also have their contribution to the liberation of peptides that together with free fatty acids, resulting in the action of lipases, lead to volatile compounds, giving the characteristic odor and flavor characterized of each fermented sausage (Leroy et al. 2006). These enzymes may be originate in parts of the raw material itself, and most often involved in the starter culture fermentation. With the exception of the post-mortem glycolysis, the catabolism of carbohydrates is carried mostly by LAB which produces compounds such as lactic acid, acetic acid, ethanol, acetoin, and other complex compounds which contribute for the flavor in the sausages (Viallon et al. 1996; Leroy et al. 2006).

In Conclusion, this study demonstrated that BLS29 *L. plantarum* presents probiotic potential, and can be used in the production of pork sausages. However, in order to confirm its functional and sensory properties more studies are necessary.

5. Acknowledgment

The authors acknowledge the financial support from the Fundação de

Amparo à Pesquisa de Santa Catarina (FAPESC - Universal No. 04/2012, TO nºTR 2012000055) and Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação (PRPPGI – Fortalecimento de Grupos de Pesquisa No. 13/2012) of the Federal Institute Santa Catarina - IFSC.

6. References

1. Arihara K (2006) Strategies for designing novel functional meat products. Meat Sci 74:219–229.
2. Ayad EHE, Verheul A, Wouters JTM, Smit G (2000) Application of wild starter cultures for flavour development in pilot plant cheese making. Int Dairy J 10:169–179.
3. Axelsson L, Rud I, Naterstad K, Blom H, Renckens B, Boekhorst J, Kleerebezem M, van Hijum S, Siezen RJ (2012) Genome Sequence of the Naturally Plasmid-Free *Lactobacillus plantarum* Strain NC8 (CCUG 61730). J Bacteriol 2391-2392.
4. Ballús CA, Klajn VM, Cunha MF, Oliveira ML, Fiorentini AM (2010) Aspectos científicos e tecnológicos do emprego de culturas probióticas na elaboração de produtos lácteos fermentados: revisão. B. CEPPA 28:85-96.

5. BRASIL. Ministério da Agricultura, Pecuária e do Abastecimento . Secretaria de Defesa Agropecuária. Instrução Normativa no. 22 de 31 de julho de 2000. Aprova o Regulamentos Técnicos de Identidade de Qualidade de Salames. Diário Oficial da União, Brasília, p. 15-28, 03 de agosto de 2000.
6. Dalla Santa OR (2008) Avaliação da qualidade de salames artesanais e seleção de culturas starter para a produção de salame tipo italiano. Thesis, Federal University Paraná).
7. De Vuyst L (2000) Technology aspects related to the application of functional starter cultures. Food Technol Biotech 38:105-112.
8. De Vuyst L, Falony G, Leroy F (2008) Probiotics in fermented sausages. Meat Sci 80:75-78.
9. El-Ghaish S, Ahmadova A, HadjiSfaxi I, Mecherfi KEE, Bazukyan I, Choiset Y, Rabesona H, Sitohy M, Popov YG, Kuliev AA, Mozzi F, Chobert J-M, Haertl T (2011) Potential use of lactic acid bacteria for reduction of allergenicity and for longer conservation of fermented foods. Trends Food Sci Tech 22:509-516.
10. Erkkilä S, Suihko ML, Eerola S, Pestäjä E, Mattila-Shandholm T (2001) Dry sausage fermented by *Lactobacillus rhamnosus* strains. Int J Food Microbiol 64:205-210.
11. Fiorentini AM, Sawitzki MC, Bertol TM, Cunha Júnior A, Sant'Anna ES (2010) Influence of a Native Strain of *Staphylococcus xylosus* on the

Microbiological, Physicochemical and Sensorial Characteristics on Milano Salami Type. Braz Arch Biol Tech 53:961-974.

12. Food and Agriculture Organization of United Nations; World Health Organization – FAO/WHO (2001) Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Report of a joint FAO/WHO expert consultation, Córdoba, Argentina. ftp://ftp.fao.org/es/esn/food/probioreport_en.pdf. Accessed 12 April 2014.
13. Franz CMAP, Stiles ME, Schleifer KL, Holzapfel WH (2003) Enterococci in foods – a conundrum for food safety. Int J Food Microbiol 88:105-122.
14. Gong H-S, Meng X-C, Wang H (2010) Mode of action of plantaricin MG, a bacteriocin active against *Salmonella typhimurium.* J Basic Microb 50:S37-S45.
15. HAMMES WP, HERTEL C (1998) New developments in meat starter cultures. Meat Sci 49:125-138.
16. Hu M, Zhao H, Zhang C, Yu J, Lu Z (2013) Purification and Characterization of Plantaricin 163, a Novel Bacteriocin Produced by *Lactobacillus plantarum*163 Isolated from Traditional Chinese Fermented Vegetables. J Agr Food Chem 61:11676-11682.
17. Hugas M, Garriga M, Aimerich T, Monfort JM (1993) Biochemical characterization of lactobacilli from dry fermented sausages. Int J Food Microbiol 18:107-113.

18. Incze K (1998). Dry fermented sausage. Meat Sci 49:169-177.
19. Lane DJ (1991) 16S/23S rRNA sequencing. In: E Stackebrandt, MN Goodfellow (Eds.). Nucleic Acid Techniques in Bacterial Systematics, pp. 115-147, Wiley, Chichester.
20. Lee H, Ahn Y-T, Park S-H, Park D-Y, Jin Y-W, Kim SC, Sung SH, Huh C-S, Kim D-H (2014) *Lactobacillus plantarum* HY7712 Protects Against the Impairment of NK-Cell Activity Caused by Whole-Body γ-Irradiation in Mice. J Microbiol Biothecnol 24:127-131.
21. Leroy F, Verluyten J, De Vuyst L (2006) Functional meat starter cultures for improved sausage fermentation. Int J Food Microbiol 106:270-285.
22. Macedo, R.E.F., Pflanzer, P.S., & Gomes, C.L., 2012. Probiotic Meat Products. In: Rigobelo IC, (Org.) Probiotic in animals. http://www.intechopen.com/books/probiotic-in-animals
23. Maldonado A, Ruiz-Barba JL, Floriano B, Jime´nez-Dı´az R (2002) The locus responsible for production of plantaricin S, a class IIb bacteriocin produced by *Lactobacillus plantarum* LPCO10, is widely distributed among wild-type *Lact. plantarum* strains isolated from olive fermentations. Int J Food Microbiol 77:117-124.
24. Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E (2006) Probiotic potential of *Lactobacillus* strains isolated from dairy products. Int Dairy J 16: 189–199.

25. Martins AKS, Martins FS, Gomes DA, Elian DAS, Vieira AT, Teixeira MM, Cara DC, Nardi RMD, Nicoli JR (2010). Evaluation of *in vitro* antagonism and of *in vivo* immune modulation and protection against pathogenic experimental challenge of two probiotic strains of *Bifidobacteriumanimalis* var. *lactis*. Arch Microbiol 192:995-1003.

26. Meira SMM, Helfer VE, Velho RV, Lopes FC, Brandelli A, (2012) Probiotic potential of *Lactobacillus* spp. isolated from Brazilian regional ovine cheese. J Dairy Res 79:119-127.

27. Metchnikoff E (1907) Lactic acid as inhibiting intestinal putrefaction. In: The prolongation of life: Optimistic studies. W. Heinemann, London. 161-183.

28. Muthukurasami P, Holley RA (2006) Microbiological and sensory quality of dry fermented sausages containing alginate-microencapsulated *Lactobacillus reuteri*. Int J Food Microbiol 111:164-169.

29. Ordóñez Pereda JÁ (2005) Tecnologia de Alimentos – Alimentos de origem animal. Vol. 2. Tradução: Murad F. Artmed, Porto Alegre.

30. Pennacchia C, Ercolini D, Blaiotta G, Pepe O, Mauriello G, Villani F (2004) Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics. Meat Sci 67:309-317.

31. Pennacchia C, Vaughan EE, Villani F (2006). Potential probiotic *Lactobacillus* strains from fermented sausages: Further investigations on their probiotic properties. Meat Sci 73:90-101.

32. Raigorodsky B (2011) Embutidos: da sobrevivência à gastronomia. Senac, São Paulo.

33. Ruiz JN, (2011) Aplicação de microrganismos probióticos na forma livre e encapsulada em salame tipo italiano. Dissertation, Escola Superior de Agricultura 'Luiz de Queiroz'.

34. SANDERS ME, KLAENHAMMER TR (2001) The scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. J Dairy Sci 84:319-331.

35. Samelis J, Metaxopoulos J, Vlassi M, Pappa A (1998) Stability and safety of traditional Greek salami—a microbiological ecology study. Int J Food Microbiol 44:69-82.

36. Sarantinopoulos P, Andrighetto C, Georgalaki MD, Rea MC, Lombardi A, Cogan TM, Kalantzopoulos G, Tsakalidou E (2001) Biochemical properties of enterococci relevant to their technological performance. Int Dairy J 11:621-647.

37. Terra, N., Terra, A.B. de M., Terra, L. de M., 2004. Defeitos nos produtos cárneos: origens e soluções. Varela, São Paulo.

38. Tondo EC, Ritter AC (2012) *Salmonella* and Salmonellosis in Southern Brazil: A review of the last decade. *In*: Monte AS, De Santos PE (eds). Salmonella: Classification, Genetics and Disease Outbreakes. Nova Science Publishers, NewYork, USA, 175-191.

39. Viallon C, Berdague´ JL, Montel MC, Talon R, Martin JF, Kondjoyan N, Denoyer C (1996) The effect of stage of ripening and packaging on volatile content and flavour of dry sausage. Food Res Int 29:667- 674.

40. Yoon H-S, Ju J-H, Lee J-E, Park H-J, Lee J-M, Shin H-K, Holzapfel W, Park K-Y, Do M-S (2013) The probiotic *Lactobacillus rhamnosus* BFE5264 and *Lactobacillus plantarum* NR74 promote cholesterol efflux and suppress inflammation in THP-1 cells. J Sci Food Agr 93:781-787.

7. Legend of the tables

Table 1. 16S rDNA gene and 16S-23S ITS sequence identification of Lactic Acid Bacteria isolated from starter-free pork sausage produced in Southern Brazil.

Table 2. Tolerance to NaCl 3% + NaNO2, Nacl and different temperatures of lactic bacteria isolated from fermented pork sausage produced in Southern Brazil.

Table 3. Antimicrobial activity of *Lactobacillus* bacteria isolated from pork sausages in Southern Brazil against foodborne pathogens.

Table 4. *Lactobacillus plantarum* BLS29 isolated from a pork sausage survival at different pH, simulating gastric conditions .

Table 5. *L. plantarum* BLS29 strain tolerance nonacid-adapted and acid-adapted (BLS29aa) in simulated intestinal juice. NC (negative control), PC (intestinal juice containing pancreatin), PC+SB (intestinal juice containing pancreatin and bile salts).

8. Tables

(Tabela 2.1) **Table 1.** 16S rDNA gene and 16S-23S ITS sequence identification of Lactic Acid Bacteria isolated from starter-free pork sausage produced in Southern Brazil.

Isolate	Sequence Identity	Sequence length	% similarity	Corresponding GenBank strain accession number
BLS02	*Lactobacillus plantarum*[2]	438	100%	AB083123
BLS03	*Lactobacillus plantarum*[2]	441	100%	AB362387
BLS04	*Lactobacillus plantarum*[1]	720	100%	KF472174
BLS05	*Enterococcus durans*[1]	847	100%	KF317897
BLS06	*Enterococcus hirae*[1]	1148	100%	AB841310
BLS07	*Enterococcus faecium*[1]	658	100%	KF012874
BLS08	*Enterococcus faecium*[1]	1145	100%	JN792505
BLS09	*Lactobacillus plantarum*[1]	1161	100%	KF472174
BLS10	*Lactobacillus plantarum*[2]	471	100%	AB362387
BLS11	*Enterococcus faecium*[1]	1148	100%	EU717954
BLS12	*Enterococcus faecium*[1]	858	99%	HQ293078
BLS13	*Enterococcus faecium*[1]	1166	100%	KF318400
BLS14	*Enterococcus faecium*[1]	1162	100%	KF149621
BLS15	*Enterococcus faecium*[1]	988	99%	KF318400
BLS16	*Enterococcus faecium*[1]	1149	99%	KF149621
BLS17	*Lactobacillus plantarum*[2]	467	100%	AB102857
BLS19	*Lactobacillus plantarum*[2]	481	100%	AB083123

BLS20	*Lactobacillus plantarum*[2]	464	100%	AB102857
BLS21	*Lactobacillus plantarum*[1]	1153	100%	KF472174
BLS23	*Lactobacillus plantarum*[2]	760	100%	JN587506
BLS26	*Lactobacillus plantarum*[2]	817	100%	HE646365
BL27	*Lactobacillus brevis*[1]	1126	100%	KF307784
BLS29	*Lactobacillus plantarum*[1]	1363	100%	KF472174
BLS30	*Lactobacillus plantarum*[2]	466	100%	AB102857
BLS31	*Lactobacillus plantarum*[2]	481	100%	AB083119
BLS32	*Lactobacillus plantarum*[2]	468	100%	AB102857

[1] Identification based on the 16S rDNA sequence data

[2] Identification based on the ITS sequence data

(Tabela 2.2) **Table 2.** Tolerance to NaCl 3%+$NaNO_2$, NaCl and different temperatures of lactic bacteria isolated from fermented pork sausage produced in Southern Brazil.

Strain	Salts tolerance			Growth at different temperatures				pH
	NaCl 3% + $NaNO_2$ 200 ppm	5% NaCl	8% NaCl	4ºC	15ºC	37ºC	45ºC	
BLS02	+	+	+	-	+	+	-	3,89 ± 0,03 [a]
BLS03	+	+	+	-	+	+	-	3,87 ± 0,00 [a]
BLS04	+	++	+	-	+	+	-	3,92 ± 0,02 [a]
BLS05	+	+	-	-	+	+	-	4,54 ± 0,05 [b]
BLS06	+	+	-	-	+	+	-	4,67 ± 0,01 [b]
BLS07	+	++	-	-	+	+	+	4,56 ± 0,00 [b]
BLS08	+	++	-	-	+	+	+	4,53 ± 0,01 [b]
BLS09	+	++	+	-	+	+	-	3,90 ± 0,00 [a]
BLS10	+	++	+	-	+	+	-	3,96 ± 0,00 [a]
BLS11	+	++	-	-	+	+	+	4,58 ± 0,00 [b]
BLS12	+	+	-	-	+	+	+	4,58 ± 0,00 [b]
BLS13	+	+	-	-	+	+	+	4,58 ± 0,00 [b]
BLS14	+	+	-	-	+	+	+	4,59 ± 0,01 [b]
BLS15	+	++	-	-	+	+	+	4,59 ± 0,01 [b]
BLS16	+	++	-	-	+	+	+	4,61 ± 0,01 [b]
BLS17	+	++	+	-	+	+	-	4,00 ± 0,01 [c]
BLS19	++	+	++	-	+	+	-	4,00 ± 0,01 [c]
BLS20	+	++	+	-	+	+	-	3,90 ± 0,01 [a]
BLS21	+	++	+	-	+	+	-	3,83 ± 0,06 [a]
BLS23	+	+	+	-	+	+	-	3,81 ± 0,00 [a]
BLS26	+	+	+	-	+	+	-	3,90 ± 0,01 [a]
BLS27	+	+	+	-	+	+	-	4,61 ± 0,01 [b]
BLS29	++	++	++	-	+	+	-	3,89 ± 0,01 [a]
BLS30	+	++	+	-	+	+	-	3,91 ± 0,07 [a]
BLS31	+	+	+	-	+	+	-	3,90 ± 0,01 [a]
BLS32	+	+	+	-	+	+	-	3,82 ± 0,01 [a]

- No growth; + Growth; ++ Strong growth

[a,b,c] Different letters indicate significant difference (P<0,05) between pH values by Tukey test.

(Tabela 2.3) **Table 3.** Antimicrobial activity of *Lactobacillus* bacteria isolated from pork sausages in Southern Brazil against foodborne pathogens.

Strains	*S.*Enteritidis SE86	*S.*Typhimurium ATCC 14028	*S.aureus ATCC 14458*	*E.coli O157:H7 ATCC 43888*	*B.cereus ATCC 33010*	*L.monocytogenes ATCC 6477*
L. plantarum BLS2	54,95 ^{aA}	51,20 ^{abABC}	48,45 ^{bA}	55,80 ^{aACE}	32,55 ^{cACD}	54,60 ^{aACE}
L. plantarum BLS3	53,00 ^{aA}	49,25 ^{Babc}	59,05 ^{cC}	53,00 ^{cC}	44,05 ^{dB}	58,75 ^{cAC}
L. plantarum BLS4	44,50 ^{aB}	47,4 ^{aABC}	39,00 ^{abBD}	45,15 ^{aBCD}	33,35 ^{bACD}	45,80 ^{aBDE}
L. plantarum BLS9	56,65 ^{aA}	43,70 ^{abAB}	49,15 ^{abA}	46,05 ^{abABCD}	36,30 ^{bACD}	55,95 ^{aAC}
L. plantarum BLS10	51,30 ^{aAB}	49,35 ^{aAB}	52,10 ^{aA}	50,10 ^{aABC}	42,50 ^{aBC}	42,75 ^{aBD}
L. plantarum BLS17	44,40 ^{aB}	42,25 ^{aABC}	51,75 ^{bA}	43,65 ^{aBCD}	34,00 ^{cABCD}	49,20 ^{bABE}
L. plantarum BLS19	43,05 ^{aB}	38,00 ^{bABC}	40,05 ^{abA}	48,40 ^{cABCD}	34,65 ^{bACD}	44,55 ^{acBDE}
L. plantarum BLS20	42,00 ^{aBC}	39,60 ^{abAB}	38,05 ^{bdBDE}	55,30 ^{cACE}	35,15 ^{dACD}	51,55 ^{cABCE}
L. plantarum BLS21	51,70 ^{aAB}	37,85 ^{bB}	37,05 ^{bdBDE}	52,90 ^{aABC}	35,80 ^{bACD}	48,60 ^{aBDE}
L. plantarum BLS23	48,30 ^{acAB}	44,95 ^{acB}	33,70 ^{bBDE}	43,65 ^{acBCD}	34,05 ^{bACD}	52,40 ^{adBDE}
L. plantarum BLS26	43,50 ^{aBC}	37,75 ^{bB}	43,45 ^{aAB}	39,15 ^{abBD}	34,85 ^{bcACD}	40,55 ^{abBD}
L. brevis BLS27	45,85 ^{abBC}	44,65 ^{abB}	41,85 ^{abAB}	56,40 ^{cBD}	37,10 ^{bACD}	55,60 ^{cBD}
L. plantarum BLS29	57,10 ^{acA}	57,55 ^{acAC}	60,80 ^{acC}	64,00 ^{aAE}	37,05 ^{bABCD}	53,20 ^{cACE}
L. plantarum BLS30	44,85 ^{aB}	41,00 ^{aAB}	37,70 ^{bDE}	47,90 ^{aABCD}	34,80 ^{abACD}	45,30 ^{aBDE}
L. plantarum BLS31	49,50 ^{aAB}	42,15 ^{abAB}	39,70 ^{bBD}	47,00 ^{abABCD}	30,50 ^{cACD}	45,40 ^{abBDE}
L. plantarum BLS32	51,50 ^{aAB}	48,15 ^{aABC}	35,10 ^{bBDE}	47,60 ^{aABCD}	38,70 ^{bABC}	49,55 ^{aABE}

^{aA} Different letters indicate significant difference (P<0,05) between halos values.

(Tabela 2.4) **Table 4.** *Lactobacillus plantarum* BLS29 isolated from a pork sausage survival at different pH, simulating gastric conditions.

Strain	Time (hours)	pH			
		2	2,5	3	4
Lactobacillus plantarum BLS29	0	8,23 ± 0,17^{aA}	8,48 ± 0,08^{aA}	8,23 ± 0,17^{aA}	8,23 ± 0,17^{aA}
	3	5,44 ± 0,13^{bB}	6,28 ± 0,25^{bB}	8,71 ± 0,13^{aA}	8,31 ± 0,0^{aA}

^{aA} Different letters in the same column or same line differ significantly (*P*>0.05) by Tukey test between the different pH values.

(Tabela 2.5) **Table 5.** *L. plantarum* BLS29 strain tolerance nonacid-exposed and acid- exposed (BLS29ae) in simulated intestinal juice. NC (negative control), PC (intestinal juice containing pancreatin), PC+SB (intestinal juice containing pancreatin and bile salts).

Strain	Time (hours)	Log_{10} (CFU/ml^{-1})		
		CN	PC	PC+SB
BLS29	0	8,78± 0,09^{a}	7,99 ± 0,14^{a}	8,21 ± 0,05^{b}
BLS29ae	0	8,03 ± 0,25^{b}	8,18± 0,07^{a}	8,07± 0,01^{b}
BLS29	4	7,92 ± 0,11^{b}	8,17 ± 0,10^{a}	8,5 ± 0,08^{a}
BLS29ae	4	8,02 ± 0,06^{b}	8,27± 0,11^{a}	8,49± 0,03^{a}

^{a} Different letters in the same column differ significantly (*P*>0.05) by Tukey test.

CAPÍTULO 3 – Artigo 2

Submetido à revista científica Anaerobe (ANAEROBE-S-15-00078).

Assessment of the antagonistic effect of *Lactobacillus plantarum* BLS29 on *Salmonella enterica se*rovar Enteritidis SE86 *in vitro* and in gnotobiotic mice

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Abstract

The present study was aimed to assess the antagonistic effect of *Lactobacillus plantarum* BLS29 against *Salmonella enterica sero*var Enteritidis SE86 *in vitro,* as well the ability of the lactic acid bacteria to colonize intestine in gnotobiotic mice. Therefore, BLS29 and SE86 were co-cultured for 168 hours in Brain

Heart Infusion (BHI) broth, with monitoring of change in pH values, count of viable cells and assessment of the diameter of inhibition zones (mm) in MRS agar. For the *in vivo* assay, the animals (n=18) were divided into three groups: 1) inoculated only with BLS29; 2) treated with BLS29 on day 0 and challenged with SE86 on day 10; 3) inoculated with sterile saline on day 0 and challenged with SE86 on day 10 of the experiment. Counts of viable cells of BLS29 on stool in MRS agar and SE86 on MacConkey agar were performed. In the *ex-vivo* experiment, animal feces were inoculated in MRS agar and kept refrigerated for 24 hours and then inoculation with the pathogen was performed using overlay plate technique. After incubation the sizes of the inhibition zones were determined when present. The *in vitro* trial showed that BLS29 was able to inhibit SE86, producing inhibition zones in isolated culture and in co-culture. However, BLS29 was not able to produce antimicrobial activity in the *ex vivo* experiment. BLS29 showed high population levels in the digestive tract of gnotobiotic mice, reducing the counts of SE86 compared to the control group. It is possible to conclude that BLS29 has important properties for using as probiotic in food production.

Keywords: Salmonella Enteritidis SE86, probiotic, gnotobiotic mice, *Lactobacillus plantarum* BLS29.

1 Introduction

Vertebrates and invertebrates maintain a permanent association with microorganisms acquired from their mothers and/or the environment soon after birth, and most of them can provide benefits to the host [1]. Despite the differences in the composition of the microbiota of each individual to the level of strains, Firmicutes (*Lactobacillus*), Actinobacteria (*Bifidobacterium*), Bacteroidetes (*Bacteroides*) and Proteobacteria (enterobacteria) are the constant and predominant phila in the human gastrointestinal tract. In adults, this intestinal indigenous microbiota composition is very stable over time. Fluctuations, however, may occur due to environmental factors such as dietary change, stress and use of antibiotics, among others [2].

Many microorganisms from the intestinal microbiota of animal or human hosts have been used as probiotics. These are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [3]. Thus, foods or pharmaceutical preparations containing probiotics may promote health, facilitate bowel movements, improve food digestion, modulate immune response and protect against inflammatory or infectious bowel disorders [4].

Studies with mice have been conducted to demonstrate the antagonistic effect of probiotic bacteria against food pathogens. For example, Vasconcelos et al. [1] investigated the protective and antagonistic effect of *Lactobacillus*

murinus against *Salmonella* Typhimurium on the digestive tract of gnotobiotic animals. Martins et al. [5] also observed antagonistic effects against the same pathogen when gnotobiotic mice were treated with *Bifidobacterium animalis* subsp. *Lactis.* Stimulation of the immune system of the host was also observed. Fayol-Messaoudi et al. [6] reported that the strain *Lactobacillus plantarum* ACA-DC287 had anti-*Salmonella* action similar to the one produced by commercial probiotic strains *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shirota YIT9029 e *Lactobacillus johnsonii* La1, both in conventional and gnotobiotic mice. In their study, Mahoney and Henriksson [7] observed that when mice were fed salami produced with starter culture and challenged with *Listeria monocytogenes*, there was a reduction of food-borne pathogens in the feces of these animals. Tests involving probiotic strains and *Salmonella* Enteritidis SE86 carried out by Scapin et al. [8] found that *Lactobacillus acidophilus* LA10 had antagonistic effect against this pathogen, especially when the probiotic was administered after the infection. Finally, Bambirra et al. [9] observed that the strain *Lactobacillus sakei* 2a was able to antagonize *Listeria monocytogenes* both in a processed product (sausage) and in the digestive tract of gnotobiotic mice.

The strain *Lactobacillus plantarum* BLS29 was isolated from a naturally fermented pork sausages [10] produced in Santa Catarina, Brazil (Casa do Salame, Xaxim). This strain has important technological features regarding its role in the production of processed foods, such as fast acidification and

absence of CO_2 production, as well as some features that are desirable for probiotic purposes, being able to inhibit various food-borne pathogens (*Listeria monocytogenes, Salmonella* Typhimurium, *Salmonella* Enteritidis and *Escherichia coli* O157:H7) and tolerating acid pH and bile salts (data not showed).

Salmonella enterica serovar Enteritidis SE86 has been identified as the main food-borne pathogen in the State of Rio Grande do Sul, Brazil, being resistant to biocides, antibiotics, high temperatures and sub-lethal pH [11]. Since 1999, this microorganism has caused salmonellosis outbreaks in southern Brazil [12] being considered a major food-borne pathogen.

The present study aimed to test the antagonist activity of the strain *Lactobacillus plantarum* BLS29 against food-borne pathogen *Salmonella* Enteritidis SE86 *in vitro* and *in vivo*.

2 Materials and methods

2.1 Bacterial strains

Lactobacillus plantarum BLS29 was isolated from pork sausage produced in an industry of the West of the State of Santa Catarina, in southern Brazil, without the addition of starter cultures for fermentation. This lactic acid bacterium (LAB) was stocked at -18ºC in a 30% (v/v) solution of glycerol and

propagated twice before each experiment in tubes containing 5 ml of Man, Rogosa and Sharp (MRS, ACUMEDIA) broth and incubated at 37ºC for 24 hours using anaerobic jars (Permution, Brazil). *Salmonella* Enteritidis SE86 was isolated from cabbage involved in an outbreak occurred in 1999, in the State of Rio Grande do Sul, Brazil. This strain is predominant in 90% of salmonellosis outbreaks occurred in Rio Grande do Sul from 1999 to 2006 ([11,12]). This pathogen was stocked at -18ºC in 80% (v/v) glycerol solution, with the cultures kept at 4ºC in Brain Heart Infusion (BHI, Himedia) agar plates and transferred to tubes containing BHI broth, incubated at 37ºC for 24 hours before each experiment.

2.2 *In vitro* antagonism assay

The experiment was conducted in 500 ml Scott bottles containing 100 ml of BHI broth, with the following cultures: a) only *Lactobacillus plantarum* BLS29; b) *Lactobacillus plantarum* BLS29 and *Salmonella* Enteritidis SE86; and c) only *Salmonella* Enteritidis SE86. Each experiment was performed in triplicate.

Lactobacillus plantarum BLS29 and *Salmonella* Enteritidis SE86 were co-cultured for 168 hours in BHI broth, with monitoring of change in pH value, count of viable cells and assessment of the diameter of inhibition zones (mm) in MRS agar (Acumedia). For pH measurement, two milliliters of each culture were removed at each one of the times and pH value was measured in digital potentiometer (Tecnal). For assessment of antagonistic activity, every three

hours of incubation, five µl of each culture containing BLS29 were inoculated in the center of Petri dishes containing MRS agar and incubated at 37°C for 24 hours in microaerophilic conditions. After this period, the plates were treated with chloroform in the lids fitted with sterile filter paper, during 30 minutes, and residual chloroform was evaporated, with the lids open for another 30 minutes. Then, 10^8 colony forming units (CFU)/ml of the pathogen in semi-solid BHI (himedia) agar (0.75% p/v) were inoculated using the overlay method. After incubation under anaerobic conditions the diameters of the inhibition zones were determined with a caliper [5].

2.3 Gnotobiotic mice

NIH Swiss mice, under germ-free conditions, aged 21 days (Taconic Farms, Germantown, NY, USA) were used in this study. The animals were kept in flexible plastic isolators (Standard Safety Equipment Co.,McHenry, IL, USA) and handled according to procedures established by Pleasants [13]. The experiments were performed in micro-isolators (Alesco Ltda., Campinas, SP, Brazil) and both the water and the commercial diet (Nuvital, Curitiba, PR, Brazil) were sterilized by autoclaving and administered *ad libitum*. The animals were kept in cages with controlled lighting (12h light, 12 h dark). The experimental protocol was previously approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais nº 96/2011 (CETEA/UFMG).

2.4 Experimental association

The germ-free mice were divided into three experimental groups, with six animals each. Group 1 was inoculated on the first day of the experiment by gavage with a 0.1 ml dose containing 10^8 cells of *Lactobacillus plantarum* BLS29. Group 2 was initially inoculated with the same dose of group 1 and challenged on the tenth day with 0.1 ml containing 10^2 cells/ml of *Salmonella* Enteritidis SE86. Group 3 was inoculated only with sterile saline, and after 10 days was also challenged with SE86. All animals were sacrificed after the 13th day by cervical dislocation [14].

2.5 Bacterial count in the stool

Fresh feces of gnotobiotic mice were aseptically collected on days 0, 3, 5, 7, 10, 11, 12 and 13 after inoculation with the microorganisms. The feces were weighted and used for decimal dilutions, with 0.1 ml aliquots of each dilution plated on the surface of MRS agar plates (Acumedia) for the count of viable cells of BLS29 and in MacConkey agar (Acumedia) for the count of SE86. The plates were incubated at 37ºC, for 24 hours under anaerobic and aerobic conditions, respectively.

2.6 E*x-vivo* antagonism assay

Samples of feces (~50mg) from three animals of each experimental group were centrally included in plates containing MRS agar and incubated at

4ºC, for 24 hours. Then, after exposure to chloroform for 30 minutes, residual chloroform was allowed to evaporate. For the determination of the antagonist activity, the plates were recovered with 3.5 mL of semi-slide BHI agar 0.75% (p/v), previously inoculated with 10^7 UFC/mL of the strain SE86. The plates were incubated at 37ºC, for 24 hours, under aerobic condition [5]. Antimicrobial activity was assessed for the presence of an inhibition zone of SE86 around the feces. The diameter of the inhibition zone was measured with a digital caliper (Mitutoyo, SP, Brazil).

2.7 Histopathological analysis

After being sacrificed by cervical dislocation, six animals of group 1 had their intestinal tissue (ileum and colon), spleen and liver removed and fixed in 4% buffered formaldehyde. The histological cuts (3–5 µm) were stained with hematoxylin and eosin (H&E). The slides were coded and analyzed by optical microscopy by a single pathologist who was blinded for the experimental conditions of each sample [5].

2.8 Statistical analysis

Data analysis was performed using STATISTICA 7.0 software (StatSoft Inc., Tulsa, USA). Analysis of variance (ANOVA) was used to determine significant differences ($P<0.05$) between population rates, and regarding changes in pH value and inhibition zones. The differences between the means were detected by Tukey test.

3 Results and discussion

3.1 *In vitro* antagonism assay

The results of the count of viable cells, change in pH value and antagonism of BLS29 against SE86, when associated in the same environment (simulating a situation that might occur in the bodies of germfree animals) are shown in Figure 1. *L. plantarum* BLS29 was able to inhibit *Salmonella* Enteritidis SE86 at all the times analyzed and even when pH value was above 7.0 as demonstrated by the inhibition zones of the pathogen. This inhibition may have occurred due to bacteriocin production, since the pH value remained unchanged.

In fact, several strains of *L. plantarum* are able to produce this type of substances, which can inhibit both Gram-negative [15] and Gram-positive bacteria [16]. However, since the investigation of the chemical nature of inhibitory compounds was not the purpose of this study, we cannot make such statement here.

It was found that, although the count of *L. plantarum* BLS29 was lower compared to the pathogen in co-culture, it was more stable than in the control group on the last five days of culture, suggesting that the LAB may have been triggered by some mechanism to better survive under that condition, causing

pathogen inhibition to be greater than that in the isolated culture of BLS29, on the last collection points. However, there was no significant difference between the points according to Tukey test.

3.2 Intestinal colonization

Lactobacillus plantarum BLS29 was able to colonize the intestine of the animals, as shown in Figure 2 (group 1). After challenge with *S.* Enteritidis SE86, BLS29 had a one $\log_{10}$ reduction in the population compared to the pathogenic bacteria, as it was expected according to the *in vitro* study (Fig. 1A). However, BLS29 maintained high population rates on the feces until the 13rd day of the experiment in group 2, and only the animals of group 3 began to die at that time. This ability demonstrated by *Lactobacillus plantarum* BLS29 of colonizing the intestine of the animals and reducing the count of cells of *S.* Enteritidis SE86 in group 2 compared to data obtained in group 3, is similar to data obtained in the study of Fayol-Messaoudi et al. [5] who tested the probiotic potential of the strain *Lactobacillus plantarum* ACA-DC287 against *S*. Typhimurium SL1344 in conventional and gnotobiotic mice. These authors observed that the lactobacillus had an antagonist effect against the pathogen tested, both *in vitro* and *in vivo*. The same authors also found that *Lactobacillus plantarum* ACA-DC287 was more abundant in the intestine of the animals than *L. casei* Shirota YIT9029. Jensen et al. [17] tested various commercial strains of probiotic LAB and found that *Lactobacillus plantarum* MF1298, isolated from mutton salami, besides being more tolerant to the intestinal gastric juice, had

greater success in intestinal colonization, overcoming strains of *Lactobacillus sakei*. As shown in the present study, *Salmonella* Enteritidis SE86 also suffered antagonistic effect and reduction of the population in conventional mice treated with the commercial strain *Lactobacillus acidophilus* LA10 [8].

3.3 *Ex-vivo* antagonism assay

The feces collected from germ-free mice inoculated with sterile saline did not produce antagonist action against SE86, as expected, because there were no microorganisms colonizing these animals (Fig. 3A). The monoassociated group with *Lactobacillus plantarum* BLS29 produced antagonistic action against SE86, according to the results shown in Figure 3B. However, unlike the results obtained *in vitro*, BLS29 did not produce antagonistic action against the pathogen when both were associated in the intestinal tissue of the animal (Fig. 3C). This phenomenon has not yet explained, and it is possible that interactions of the immune system of the animal, or a mechanism of *quorum sensing*, or another mechanism associated to the inhibition of the production of antimicrobial compound by *Lactobacillus plantarum* BLS29.

3.4 Histopathological analysis

Histopahological analysis of samples from 06 animals of group 1 (monoassociated with BLS29) were analyzed in order to evaluate potential *Lactobacillus plantarum* BLS29 induced damage to intestinal, spleen and liver

tissues. No abnormality was observed in the analyzed tissues, as observed in Figure 4, indicating its safety for probiotic use.

4 Conclusions

It can be concluded that *L. plantarum* BLS29 had *in vitro* ability to reduce the multiplication of *S*. Enteritidis SE86. *L. plantarum* BLS29 has also shown to be a safe strain to be used as probiotic in foods, since it colonized high population rates of animals without causing damage to the intestine, spleen and liver. Nevertheless, further studies are needed to investigate the mechanism of inhibition of *S*. Enteritidis SE86 by *L. plantarum* BLS29 in animals.

5 Acknowledgments

The authors acknowledge the financial support from *Fundação de Amparo à Pesquisa de Santa Catarina* (FAPESC - Universal No. 04/2012, TO nºTR 2012000055) and *Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação* (PRPPGI – Fortalecimento de Grupos de Pesquisa No. 16/2013) of the Federal Institute Santa Catarina - IFSC.

6 References

1. ALS Vasconcelos, JR Nicoli, RMD Nardi. Antagonistic and protective effects against *Salmonella enterica*serovarTyphimurium by *Lactobacillus murinus* in the digestive tract of gnotobiotic mice. Braz J Microbiol. 34 (2003) 21–24.

2. F. Guarner. The Enteric Microbiota. *In:* Colloquium series on integrated systems physiology: from molecule to function to disease. 88p. 2011. http://www.morganclaypool.com/doi/abs/10.4199/C00047ED1V01Y201110ISP029. DOI: 10.4199/C00047ED1V01Y201110ISP029.

3. Food and Agriculture Organization of United Nations; World Health Organization – FAO/WHO. Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Report of a joint FAO/WHO expert consultation, Córdoba, Argentina. (2001). <ftp://ftp.fao.org/es/esn/food/probioreport_en.pdf>.

4. SMI Saad, AG da Cruz, JAF Faria. Probióticos e Prebióticos em Alimentos – Fundamentos e Aplicações Tecnológicas. Varela: São Paulo; 2011.

5. AKS Martins, FS Martins, DA Gomes, SAD Elian, AT Vieira, MM Teixeira, DC Cara, RND Nardi, JR Nicoli. Evaluation of *in vitro* antagonism and of *in vivo* immune modulation and protection against pathogenic experimental challenge of two probiotic strains of *Bifidobacterium animalis* var. *lactis*. Arch Microbiol 192 (2010) 995-1003.

6. D Fayol-Messaoudi, M-H Coconnier-Polter, L Lievin, V Moal, F Atassi, CN Berger, AL Servin. The *Lactobacillus plantarum* strain ACA-DC287 isolated from a Greek cheese demonstrates antagonistic activity *in vitro* and *in vivo* against *Salmonella enterica* serovar Typhimurium. J Appl Microbiol 103 (2007) 657-665.

7. M Mahoney, A Henriksson.The effect of processed meat and meat starter cultures on gastrointestinal colonization and virulence of *Listeria monocytogenes* in mice. International J Food Microbiol. 84 (2003) 255-261.

8. D Scapin, WF Grando, EM Rossi, KJ Perez, PS Malheiros, EC Tondo. Antagonistic activity of *Lactobacillus acidophilus* LA10 against *Salmonella enterica* serovar Enteritidis SE86 in mice. Braz J Microbiol 44 (2013) 57–61.

9. FHS Bambirra, KGC Lima, BDGM Franco, DC Cara, RMD Nardi, FHF Barbosa, JR Nicoli. Protective effect of *Lactobacillus sakei* 2a against experimental challenge with *Listeria monocytogenes* in gnotobiotic mice. Lett Appl Microbiol 45 (2007) 663–667.

10. L SENTER, LP CAMARGO, E MOREIRA, EC TONDO. Caracterização de *Lactobacillus plantarum* BLS29 isolado de linguiça suína colonial, um novo potencial probiótico em embutidos fermentados. Anais SEPEI 2014. *In:* 4º Seminário de Pesquisa, Extensão e Inovação do IFSC. http://arquivos.ifsc.edu.br/comunicacao/anais_sepei_2014.pdf

11. EC Tondo, AC Ritter. *Salmonella* and Salmonellosis in Southern Brazil: A review of the last decade. *In*: Monte AS, De Santos PE (eds). Salmonella: Classification, Genetics and Disease Outbreakes. Nova Science Publishers: NewYork; 2012, 175-191.

12. MP Geimba, EC Tondo, FA Oliveira, CW Canal, A Brandelli. Serological characterization and prevalence of spvR genes in *Salmonella* isolated from foods involved in outbreaks in Brazil. J Food Prot 67 (2004) 1229-1233.

13. JR Pleasants. Gnotobiotics. In: Handbook of Laboratory Animal Science. EC Melby, Jr and NHAltmann, (eds) pp. 119–174, CRC Press, Cleveland, OH. 1974.

14. KJ Perez, FS Martins, DC Cara, JR Nicoli, EC Tondo. Evaluation of intestinal invasion in germ-free mice challenged with acid-adapted and nonacid-adapted *Salmonella* Enteritidis SE86 and *Salmonella* Typhimurium ST99. J Food Safety 32 (2011)108-114.

15. LMT Dicks, FD Mellett, LR Hoffman. Use of bacteriocin-producing starter cultures of *Lactobacillus plantarum* and *Lactobacillus curvatus* in production of ostrich meat salami. Meat Sci 66 (2004) 703-708.

16. Campanini, M., Pedrazzoni, I., Barbuti, S., Baldini, P., 1993. Behaviour of *Listeria monocytogenes* during the maturation of naturally and artificially contaminated salami: effect of lactic-acid bacteria starter cultures. Int J Food Microbiol 76 (1993) 138-146.

17. Jensen, H., Grimmer, S., Naterstad, K., Axelsson, L., 2012. *In vitro* testing of commercial and potential probiotic lactic acid bacteria. Int J Food Microbiol 153 (2012) 216-222.

7 Legend of the figures

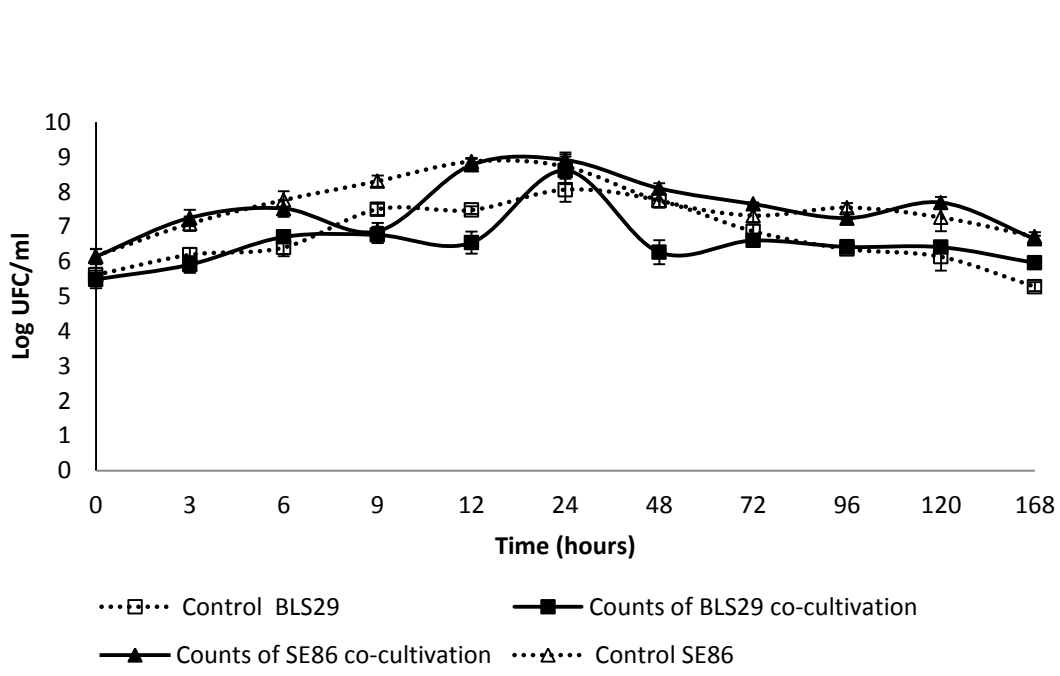
Figure 1. Count of viable cells (A), evolution of pH(B) and diameter of the inhibition zone produced by *L. plantarum* BLS29 against *S.* Enteritidis SE86 (C) in monoculture of *L. plantarum* BLS29, co-culture of *L. plantarum* BLS29 and *S.* Enteritidis SE86, and monoculture of *S.* Enteritidis SE86 during 168 hours of culture in BHI broth.

Figure 2. Population rates in the feaces of gnotobiotic mice associated with *L. plantarum* BLS29 (Group 1 and 2) or *S.*Enteritidis SE86 (Groups 2 and 3) during 13 days of association.

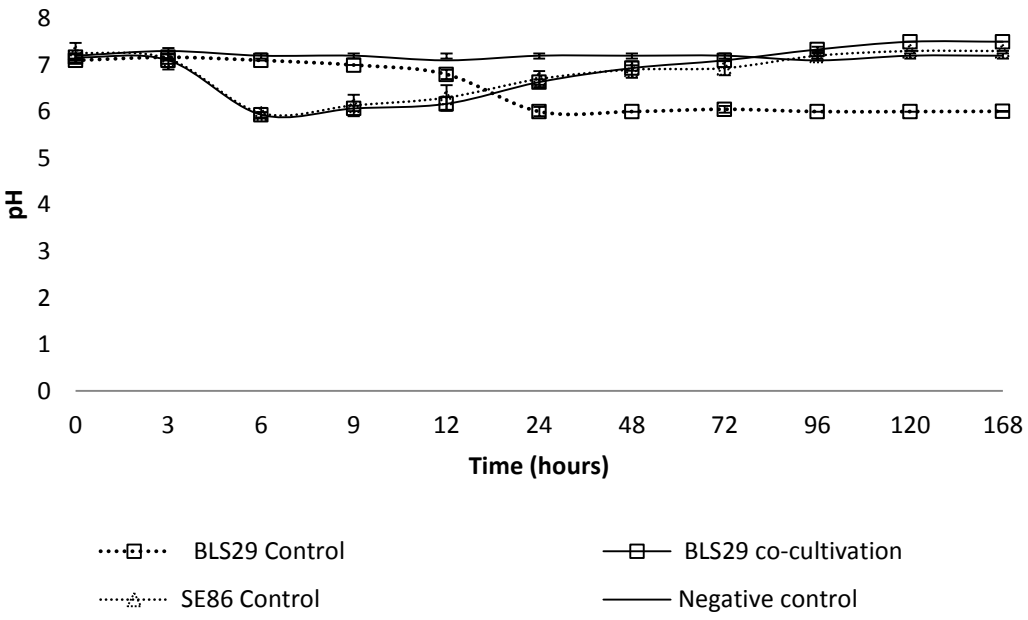
Figure 3. *Ex-vivo* antagonist activity against *S*. Enteritidis SE86 using feces of gnotobiotic mice inoculated with sterile saline (A), monoassociated with *L. plantarum* BLS29 (B) or diassociated with *L. plantarum* BLS29 and *S*. Enteritidis SE86 (C).

Figure 4. Histological aspects of the ileum (A and B, 10 and 20 x, respectively), spleen and liver (E and F, 10 and 4x, respectively) and colon (C and D, 10 and 20x, respectively) of gnotobiotic mice monoassociated with *Lactobacillus plantarum* BLS29 for 13 days.

(Figura 3.1) **Figure 1.** Count of viable cells (A), evolution of pH(B) and diameter of the inhibition zone produced by L. plantarum BLS29 against S. Enteritidis SE86 (C) in monoculture of L. plantarum BLS29, co-culture of L. plantarum BLS29 and S. Enteritidis SE86, and monoculture of S. Enteritidis SE86 during 168 hours of culture in BHI broth.



B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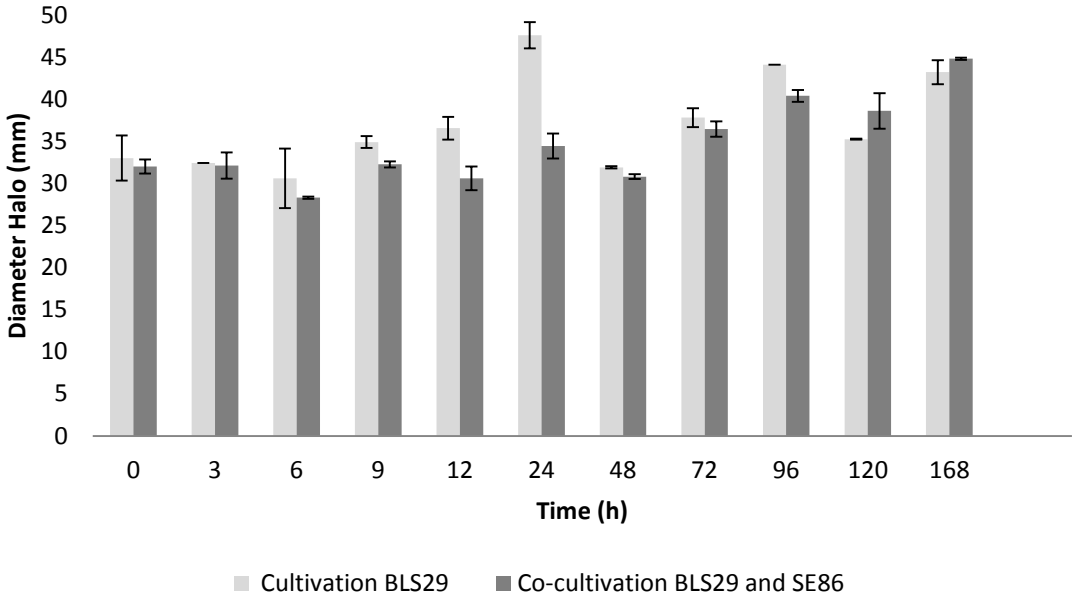
Time (hours)
BLS29 Control
BLS29 co-cultivation
SE86 Control
Negative control

C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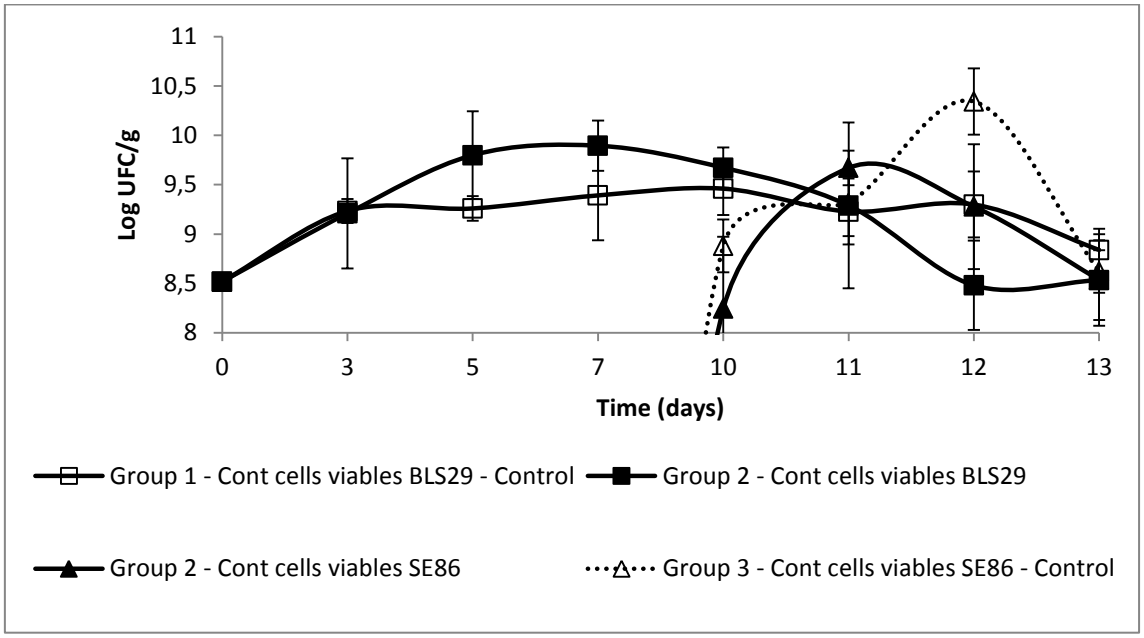
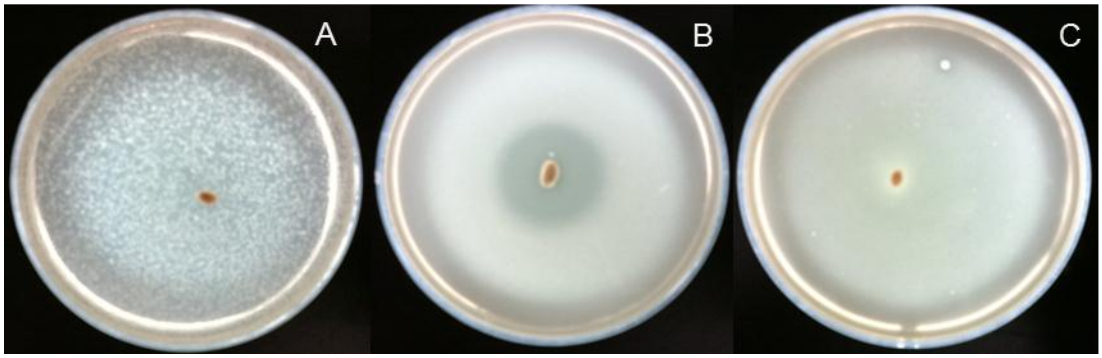
Time (h)
Cultivation BLS29
Co-cultivation BLS29 and SE86

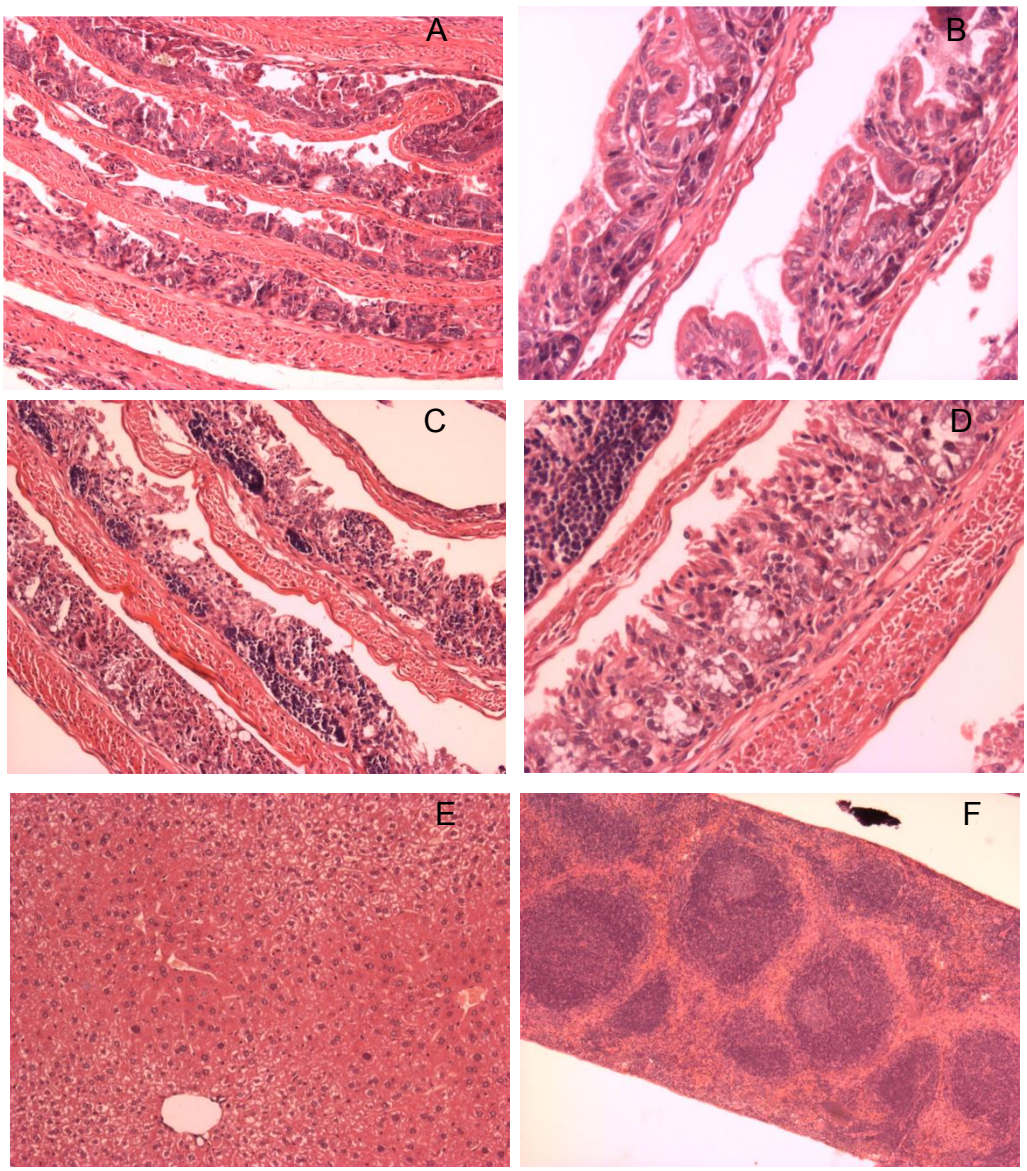
(Figura 3.2) **Figure 2.** Population rates in the feaces of gnotobiotic mice associated with L. plantarum BLS29 (Group 1 and 2) or S.Enteritidis SE86 (Groups 2 and 3) during 13 days of association.



(Figura 3.3) **Figure 3.** *Ex-vivo* antagonist activity against *S*. Enteritidis SE86 using feces of gnotobiotic mice inoculated with sterile saline (A), monoassociated with *L. plantarum* BLS29 (B) or diassociated with *L. plantarum* BLS29 and *S*. Enteritidis SE86 (C).



(Figura 3.4) **Figure 4.** Histological aspects of the ileum (A and B, 10 and 20 x, respectively), colon (C and D, 10 and 20x, respectively) and liver and spleen (E and F, 10 and 4x, respectively) of gnotobiotic mice monoassociated with *Lactobacillus plantarum* BLS29 for 13 days.



CAPÍTULO 4 – Artigo 3

Submetido à revista científica Meat Science (MEATSCI-S-15-00285)

Fermented pork sausage using *Lactobacillus plantarum* BLS29 as potentially functional starter culture

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Abstract

The growing demand for probiotic products has stimulated the development of some foods, such as meat products. Since most probiotics are dairy-based products, which cannot be consumed by people with lactose intolerance. *Lactobacillus plantarum* BLS29, isolated from naturally fermented pork sausage produced in the western region of Santa Catarina, southern Brazil, was inoculated as potentially functional starter culture in the sausage formulation of the company where the bacterium was isolated. The traditional (naturally fermented) and functional (containing *L. plantarum* BLS29) sausage batches were processed and cured, and measurements of pH, moisture, ash, lipid, protein, bacteria count. No differences were found in pH, moisture, weight loss, ashes, proteins and lipids between the products. However, the total lactic acid bacteria counts were higher in the functional product than in the traditional product. The results demonstrated that *L. plantarum* BLS29 can be used as a potentially functional starter culture in the sausage fermentation, without changing the original properties of the product.

Keywords: probiotics, *Lactobacillus plantarum,* sausages, pork sausage.

1 Introduction

Small industries use the traditional method for manufacturing colonial sausages, without the addition of starter cultures (Sawitzki et al. 2008). Besides, part of the population produces and eats their handmade sausages. The lactic acid bacteria present as contaminants of meat and ingredients are responsible for the natural fermentation of the product. However, the absence of starter cultures may impair the quality of the sausage, facilitating the development of contaminant/pathogenic microorganisms. Hammes and Hertel (1998) reported that the formation of flavor of sausages is caused by the action of microbial metabolism together with the spices added, and claimed that the addition of starter cultures accelerated the maturation of sausages thanks to the action of microbial proteinases.

The essential ingredients of colonial sausage are bacon and pork, salt, nitrite and/or sodium nitrate and/or potassium. Some optional ingredients include powder milk, sugars, maltodextrins, milk proteins, intentional additives, wine, seasonings, flavorings and spices and substances that provide a protective coating when applied to the external surface of a food (external coating). Starter cultures are also allowed (BRASIL, 2000).

The search for products with functional properties has stimulated the search for microbial cultures that provide health benefits to consumers and, at the same time, technological characteristics applicable to the development of

new products. In addition to the ability to survive in the food matrix, it is desirable that microorganisms could survive low pH and bile salts of the stomach, colonize the gut, compete with the normal microbiota, especially inhibiting the pathogens that can cause infection to the host (Hammes and Hertel 1998; Ammor and Mayo 2007).

Most probiotic food items available in the market are composed of dairy products, and there are few meat products with these characteristics. In 1998, Japan and Germany were the first countries to produce salami from probiotic bacteria of intestinal origin (Arihara 2006). However, it is important to be attentive to the technological characteristics of the functional bacteria used. Samelis*et al*. (1998) noticed that the sausages prepared by natural fermentation in the south of Europe had better sensory quality than those prepared with starter cultures from northern Europe. This was due to the poor ability of these later cultures to compete with the local microbiota. Considering the need to find new strains with probiotic characteristics that can be used as functional starter cultures, while preserving the technological and sensory characteristics of the sausages, the present study aimed to develop a probiotic pork sausage inoculated with *Lactobacillus plantarum* BLS29, which was isolated from naturally fermented pork sausage and showed *in vitro* and *in vivo* probiobic properties (data not showed).

2 Materials and methods

2.1 Functional starter culture

L. plantarum BLS29 was previously isolated from naturally fermented pork sausage and was checked for its probiotic potential (Senter et al. 2014). This strain was selected because it was not able to produce $CO_{2,}$ demonstrated to be homolactic and could multiply at temperatures ranging from 15° C to 37° C, usually practiced during the sausage cure process. *L. plantarum* BLS29 showed better growth than other LABs in curing salts and antimicrobial activity against foodborne pathogens. A pure *L. plantarum* BLS29 colony was cultivated in MRS broth (Acumedia, EUA), for 24 h, under microaerophilic conditions, at 37ºC. One hundred and fifty milliliters (8.5 $\log_{10}$ UFC/ml) of the culture was concentrated by centrifugation (3000 x g) for 15 minutes and inoculated in 15 kg of fresh raw material (Erkkilä et al. 2001a) and homogenized.

2.2 Preparation of sausages

The sausages were prepared in a meat processing plant from southern Brazil, according to the formulation of the company. Thirty kilograms of meat mass formulated by the company were divided into two parts. The control batch (natural fermentation) was processed after 24 hours of rest period in cold chamber, while the functional batch was inoculated with *L. plantarum*BLS29and homogenized in the meat, after the same rest period. Each piece (n=43) weighing about 350 g was fermented for one day at 25°C and cured on the

following day at a temperature up to 35°C, for approximately 10 hours, and then hold at room temperature for up to 45 days for maturation.

2.3 Microbiological analyzes

On days 0, 3, 10, 17 total lactic acid bacteria counts, coliforms at 45°C, coagulase-positive staphylococci, clostridium sulfite reducers and presence of *Salmonella* sp. (BRASIL, 2001) and *Listeria monocytogenes* were verified. Concomitantly to the microbiological analyses, the pH and moisture of sausages were monitored. In order to carry out the microbiological analyses, twenty-five grams of the sausages were weighed in sterile plastic bags, diluted with 0.1% sterile peptone water and homogenized using a Stomacher (AES, France). For lactic acid bacteria counts, 1 ml of the dilutions were inoculated by pour-plate method in MRS agar (Acumedia, USA) and incubated at 37°C, for 48 hours, in anaerobic jars (Permution, Brazil). For pathogen detection, the methodologies described by Normative Regulation n^{o}62, MAPA (BRASIL, 2003) and APHA (1992) were used.

2.4 Physical and chemical analyzes

All physical and chemical analyzes were conducted according to the methods described by the IAL (2008). The pH values were obtained from mean values of three measurements (Tecnal, modelo TEC-5, Brazil) carry outon 10g-samples, homogenized with 100 ml of distilled water. Moisture analysis was determined by direct drying in oven at 105°C. Protein concentration was

determined by classical Kjeldahl method. The lipid content was measured via direct extraction in Soxhlet, and the ash content was determined by incineration in an oven at 550-570°C.

2.5 Weight loss

Weight loss of sausages was calculated in percentage by subtracting the values obtained on days 3, 5, 10, 17, 25, 31 and 45 of maturation from the initial weight (day 0) (Erkkilä et al. 2001a).

2.5 Statistical analyses

Differences between the results of the physical, chemical and microbiological analyzes of the traditional and functional (probiotic) sausages were assessed by analysis of variance (ANOVA, Statistica, version 7.0). Differences with $p < 0.05$ values were considered significant by Tukey test. All analyzes were performed in triplicate, except for weight loss that was obtained by the mean value of 13 pieces.

3 Results and discussion

The sausages were processed, identified and packed in the laboratory of meat technology of IFSC, campus of Xanxerê, at a temperature of around 25 ºC. Each piece was weighed and the weight was monitored for approximately

45 days, when the product is usually consumed. Regardless of the treatment, the sausages demonstrated the same pattern of weight low throughout the assessed period, requiring around 25 days to lose approximately 40% of the weight, as it can be seen in Figure 1, which is consistent with the findings obtained by Erkkilä et al. (2001a). The determination of weight loss usually concerns the elimination of water during drying and depends on temperature, environmental moisture and processing time (Garcia et al. 2000). The percentage of water loss and water activity is related to a product, and the availability of water in the food has direct influence on microbial development (Jay 2005). Therefore, the lower the humidity, the greater the microbiological safety of the product, which can be confirmed as early as at 17 days of maturation of a product where no pathogens were detected (Table 1).

The sugars present in the formulation of the product provide reducing conditions during the curing process, preventing the development of oxidized flavors. On the other hand, they facilitate reduction of nitrites into nitrates and of nitrates into nitric oxide, reactions that contribute to the formation of the red pigment characteristic of cured sausages. (Ordoñez et al. 2005). LAB (lactic acid bacteria) are responsible for the lactic fermentation of matured meat products. The sugars also provide a source of energy for fermenting bacteria such as *Lactobacillus* that produce lactic acid, lowering the pH of the product and, associated to sodium chloride, reduce water retention in meat fibers, dehydrating the product and making it difficult for pathogenic microorganisms

such as *Pseudomonas* e *Salmonella* to survive (Terra et al. 2004; Fiorentini et al. 2010). These combined factors are crucial for the survival of most pathogens. However, according to some studies, the occurrence of lactic bacteria in naturally fermented salami is relatively low, so that the time needed to reach a suitable pH allows pathogens that may be present survive and multiply in the food (Fiorentini et al. 2010; Saad et al. 2011). Dalla Santa (2012), obtained counts between 6 and 8 $\log_{10}$ (UFC/g) for lactic bacteria in commercialized products, and 24% of the analyzed samples of salami did not meet the requirements of the current Brazilian legislation (Brasil 2001) regarding contamination by coliforms at 45°C or *Staphylococcus aureus*. In our study, we observed that both in natural and functional fermentations, the count of food-borne pathogens was reduced, in accordance with the current legislation (BRASIL 2001), as shown in Table 1. It can be affirmed that preparing the product with meat inspected and handled within hygiene standards has contributed to the achievement of this result.

On the third day of sausage production, soon after curing, the difference in the lactic-acid bacteria (BAL) counts between the functional and traditional treatments was already noticeable although no significant differences were detected in pH and moisture values (Table 1). Erkkilä et al. (2001b) tested different strains of *Lactobacillus rhamnosus* (GG, LC-705, E-97800) and *L. plantarum* (E-98098) in the production of fermented pork sausages in order to check for differences in the flavor profile produced by the strains. The authors

found that LC-705 and E-98098 increased the number of cells in the product, compared to the other strains, and this increase did not interfere with the change in the pH value of the product in 7 days of maturation, compared to the control. These findings are similar to those obtained in our study. A decrease in pH value to around 5.0 in the fermented sausages in the early days of fermentation makes the environment unsuitable to the development of pathogens such as coliforms and *Salmonella*, increasing the microbiological safety of the product (Erkkilä et al. 2001b; Macedo et al. 2008). Both treatments complied with the microbiological standards required under RDC no 12/2001 (BRASIL 2001), effective in Brazil.

The pH values remained stable from the third until the seventeenth day of production, regardless of the treatments, until the end of the analysis, in contrast with the results obtained by Macedo et al. (2008), who reported that salamis prepared with probiotic cultures showed pH values lower than those of the control treatment with starter culture (*Lactobacillus casei, L. paracasei e L. rhamnosus*). According to Terra et al. (2004), from the seventh day of maturation on, pH values are increased due to decarboxylation and deamination reactions of aminoacids, releasing ammonia in the environment, which makes it alkaline. However, pH may suffer further reduction due to lipolysis reactions that release free fatty acids in the environment.

The use of starter cultures with functional properties was boosted with the growing probiotics market, combining fermentation characteristics with the

ability to produce antimicrobial compounds capable of inhibiting most pathogens contained in processed meat (Leroy et al. 2006). Arihara (2006) claims that is difficult to maintain live probiotic cultures in the salami, due to the low water activity of the product, which ultimately harms the development of probiotics. However, Erkkilä et al. (2001a) succeeded in preparing probiotic salami with commercial strains of *Lactobacillus rhamnosus* GG and E-97800 for the production of fermented salami, as well as Macedo et al. (2008) used strains of *Lactobacillus casei* and *Lactobacillus paracasei* in Italian salami production, corroborating the results shown in Table 1.

According to the Brazilian legislation (Brasil 2000), colonial sausages should contain no more than 30% fat, at least 18% protein and total carbohydrates should not exceed 1.5%. The data obtained in our study characterize a product with high protein content and low fat content. This can be seen in Table 2 that shows the ash, lipid and protein contents of the samples determined after the 17th day of maturation. The ash content obtained in this study is similar to the one in Milano-type salami made in southern Brazil by Sawitzki et al. (2008), inoculated with *L. plantarum* AJ2. However, the protein and lipid values in the same study are higher than those obtained in our product, possibly because of the addition of beef and the higher lipid content of that processed meat product.

4 Conclusions

The analyzed parameters demonstrate that the addition of *L. plantarum* BLS29 did not change the original physical and chemical composition of the product. Since the strain was isolated from pork sausages, it has adapted very well to the product, and can be used in sausages with high counts of acid lactic bacteria that act as functional starter cultures.

5. Acknowledgments

The authors acknowledge the financial support from *Fundação de Amparo à Pesquisa de Santa Catarina* (FAPESC - Universal No. 04/2012, TO nºTR 2012000055) and *Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação* (PRPPGI – Fortalecimento de Grupos de Pesquisa No. 19/2014 and Universal No 14/2014) of the Federal Institute Santa Catarina - IFSC.

6. References

1. Ammor, M.S., Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science*, 76, 138-146.

2. APHA - American Public Health Association (1992). *Compendium of methods for microbiological examination of food.* 3 ed. Washington: APHA.
3. Arihara, K. (2006). Strategies for designing novel functional meat products. *Meat Science,* 74, 219-229.
4. Brasil, Ministério da Agricultura, Pecuária e do Abastecimento (2000). Secretaria de Defesa Agropecuária. *Instrução Normativa no. 22 de 31 de julho de 2000. Aprova os Regulamentos Técnicos de Identidade de Qualidade de Salames*. Diário Oficial da União, Brasília, p. 15-28, 03 de agosto de 2000.
5. Brasil, Ministério da Saúde. Agência Nacional de Vigilância Sanitária (2001). *Resolução – RDC nº 12 de 02 de janeiro de 2001. Aprova o regulamento técnico sobre Padrões Microbiológicos para alimentos e seus anexos I e II.* Diário oficial da União, Brasília, DF, 10 de janeiro de 2001. Pg 45 – 51. Disponível em<http://www.anvisa.gov.br>.
6. Brasil, Ministério da Agricultura, Pecuária e Abastecimento (2003). Departamento de Inspeção de Produtos de Origem Animal. *Instrução Normativa n. 62, de 26 de agosto de 2003. Oficializa os Métodos Analíticos Oficiais para Análises Microbiológicas para Controle de Produtos de Origem Animal e Água*. Diário Oficial da República Federativa do Brasil, p. 14, 18 set.2003. Seção I.

7. Dalla Santa, O.R., Alvarez, D.C., Dalla Santa, H.S., Zanette, C.M., Freitas , R.J.S., Macedo, R.E.F. & Terra, N.N. (2012). Microbiota of sausages obtained by spontaneous fermentation produced in the South of Brazil. *Food Science Technolology*, 32(4), 653-660.
8. Erkkilä, S., Suihko, M.L., Eerola, S., Pestäjä, E. & Mattila-Shandholm, T. (2001a) Dry sausage fermented by *Lactobacillus rhamnosus* strains. *International Journal of Food Microbiology,* 64, 205-210.
9. Erkkilä, S., Pestäjä, E., Eerola, S., Lilleberg, L., Mattila-Shandholm, T. & Suihko, M.L. (2001b). Flavour profiles of dry sausages fermented by selected novel meat starter cultures. *Meat Science,* 58, 111-116.
10. Fiorentini, A.M., Sawitzki, M.C., Bertol, T.M., Júnior, A.C. & Sant'anna, E.S. (2010). Influence of a native strain of *Staphylococcus xylosus* on the microbial, physicochemical and sensorial characteristics on Milano salami type. *Brazilian Archives of Biology and Technology* 53(4), 961-974.
11. Garcia, F.T., Gagleazzi, U. A. & Sobral, P.J.A. (2000). Variação das propriedades físicas e químicas do salame tipo italiano durante secagem e fermentação. *Brazilian Journal of Food Technology,* 3(48), 151-158.
12. Hammes, W.P. & Hertel, C. (1998). New developments in meat starter cultures. *Meat Science*, 49, 125-138.
13. Instituto Adolfo Lutz - IAL (2008). *Métodos físico-químicos para análise de alimentos* /coordenadores Odair Zenebon, Neus Sadocco Pascuet e Paulo Tiglea - São Paulo: Instituto Adolfo Lutz.

14. Jay, M.J. (2005). *Microbiologia de Alimentos.* (6 ed). Porto Alegre: Artmed.
15. Leroy, F., Verluyten, J. & De Vuyst, L. (2006). Functional meat starter cultures for improved sausage fermentation. *International Journal of Food Microbiology*, 106, 270-285.
16. Macedo, R.E.F., Pflanzer, S.B., Terra, N.N. & Freitas, R.J.S. (2008). Desenvolvimento de embutido fermentado por *Lactobacillus* probióticos: características de qualidade. *Ciência e Tecnologia de Alimentos*, 28(3), 509-519.
17. Ordóñez Pereda, J.A. (2005). *Tecnologia de Alimentos – Alimentos de origem animal.* Vol. 2. Tradução: Fátima Murad. Porto Alegre: Artmed.
18. Saad, S.M.I.; Da Cruz, A.G. & Faria, J.A.F. (2011). *Probióticos e prebióticos em alimentos: fundamentos e aplicações tecnológicas*. (1. Ed). São Paulo: Varela.
19. Samelis, J., Metaxopoulus, J., Vlassi, M. & Pappa, A. (1998). Stability and safety of traditional Greek salami — a microbiological ecology study. *International Journal of Food Microbiology,* 44, 69-82.
20. Sawitzki, M.C., Fiorentini, A.M., Júnior, A.C., Bertol, T.M. & Sant'anna, E.S. (2008). *Lactobacillus plantarum* AJ2 isolated from naturally fermented sausage and its effects on the technological proprieties of Milano-type salami. *Food Science Technology,* 28(3), 709-717.
21. Senter, L., Camargo, L.P., Moreira, E. & Tondo, E.C. (2004). Caracterização de *Lactobacillus plantarum* BLS29 isolado de linguiça

suína colonial, um novo potencial probiótico em embutidos fermentados. Anais SEPEI 2014. *In:* **4º Seminário de Pesquisa, Extensão e Inovação do IFSC.** Disponível em http://arquivos.ifsc.edu.br/comunicacao/anais_sepei_2014.pdf

22. TERRA, A.B de M., FRIES, L.L. & TERRA, N.N. (2004). *Particularidades na fabricação de salame.* São Paulo: Varela.

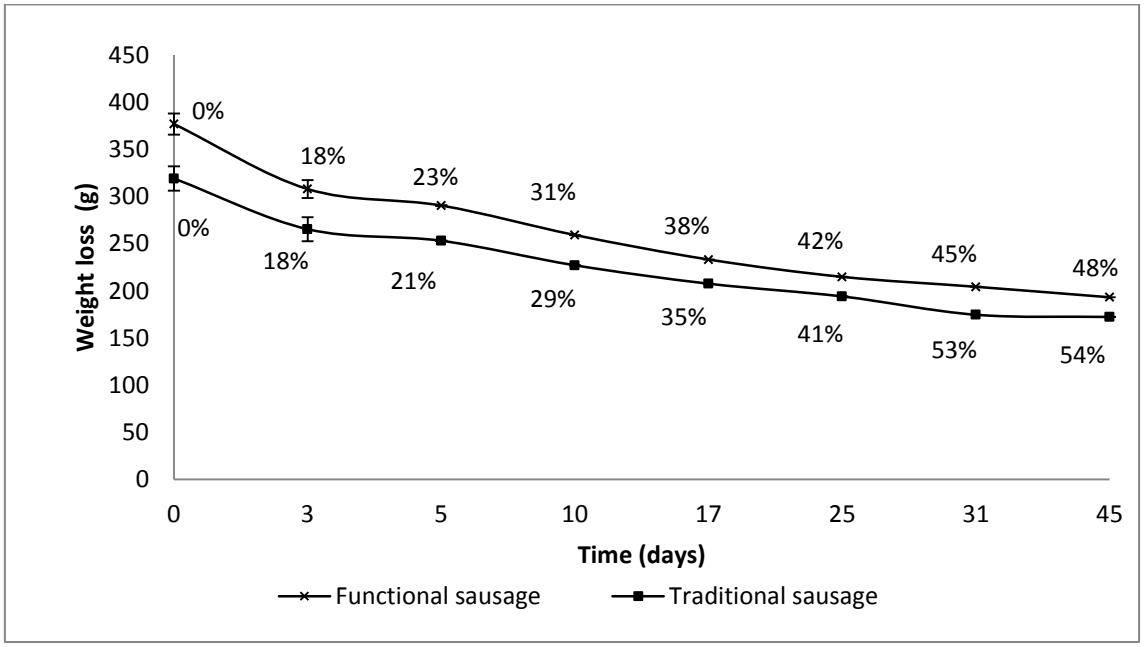
7. Legends of figure and tables

Figure 1. Weight loss in grams and in percentage of pork sausages in the traditional (natural fermentation) and functional (L. plantarum BLS29) during 45 days after the production of the pieces.

Table 1. Microbial population (Log_{10} UFC/g^{-1}) in the Functional (inoculated with *L. plantarum* BLS29) and Traditional (natural fermentation) treatments and pH and moisture values during 17 days of maturation of sausages.

Table 2. Chemical composition of samples of pork sausage in the Functional (inoculated with *L. plantarum* BLS29) and Traditional (natural fermentation) treatments after 17 days of maturation of the sausages.

(Figura 4.1) **Figure 1.** Weight loss in grams and in percentage of pork sausages in the traditional (natural fermentation) and functional (L. plantarum BLS29) during 45 days after the production of the pieces.



(Tabela 4.1) Table 1. Microbial population (Log_{10} UFC/g^{-1}) in the Functional (inoculated with *L. plantarum* BLS29) and Traditional (natural fermentation) treatments and pH and moisture values during 17 days of maturation of sausages.

Maturation time	Type of sausage	pH	Moisture	Microbial population					
				BAL	Coliforms at 45°C*	Coagulase-positive staphylococci*	Clostridium sulfite reducers*	*Salmonella* sp.*	*Listeria monocytogenes*
0 days	Functional	5.60 ± 0.06 [a]	62.34 ± 1.06 [a]	8.00 ± 0.3 [a]	$<10^3$	$<5x10^3$	0	Absent	Absent
	Traditional	5.70 ± 0.10 [a]	60.23 ± 1.38 [a]	7.60 ± 0.2 [a]	$<10^3$	$<5x10^3$	0	Absent	Absent
3 days	Functional	5.15 ± 0.01 [b]	59.14 ±0.29 [a]	8.99 ± 0.1 [b]	-	-	-	-	-
	Traditional	5.22 ± 0.02 [b]	58.26 ± 0.40 [a]	7.47 ± 0.4 [a]	-	-	-	-	-
10 days	Functional	5.07 ± 0.10 [b]	54.65 ± 0.79 [b]	12.59 ± 0.3 [c]	-	-	-	-	-
	Traditional	5.08 ± 0.03 [b]	53.33 ± 2.20 [b]	10.36 ± 0.2 [d]	-	-	-	-	-
17 days	Functional	5.27 ± 0.11 [b]	47.13 ± 2.60 [c]	12.86 ± 0.2 [c]	0	0	0	Absent	Absent
	Traditional	5.27 ± 0.01 [b]	48.29 ± 1.37 [c]	10.62 ± 0.1 [d]	0	0	0	Absent	Absent

[a,b,c,d] Different letters in the same column differ significantly (*P*>0.05) between the functional and traditional treatments by Tukey test.

* BRASIL, (2001);

- analysis not done

(Tabela 4.2) Table 2. Chemical composition of samples of pork sausage in the Functional (inoculated with *L. plantarum* BLS29) and Traditional (natural fermentation) treatments after 17 days of maturation of the sausages.

Type of sausage	Ashes (%)	Protein (%)	Lipids (%)
Functional	6.62 ± 0.33[a]	21.58 ± 2.68[a]	10.67 ± 0.01[a]
Traditional	6.23 ± 0.32[a]	20.49 ± 3.58[a]	11.05 ± 0.03[a]

[a]Equal letters in the same column demonstrate that there is no significant difference ($p<0.05$) between the treatments, according to Tukey test.

CAPÍTULO 5 – DISCUSSÃO GERAL

De acordo com a BBC Research (2013), o mercado global de micro-organismos e produtos microbianos, no mundo, foi de mais de 117 bilhões de dólares, em 2012, e para 2018, há previsão de 175 bilhões com um crescimento anual de 6%. Entre os exemplos mais comuns de micro-organismos comercializados, estão o tradicional fermento de pão, culturas lácticas para iogurtes e os famosos leites fermentados com lactobacilos vivos. Lima *et al*. (2001) descreve a importância do emprego de micro-organismos na biotecnologia para produção de alimentos fermentados, proteínas, lipídeos, antibióticos, aminoácidos, esteróides, vitaminas, ácido orgânicos e inoculantes agrícolas entre muitos outros produtos.

O desenvolvimento de produtos probióticos tem desafiado tanto a indústria quanto a ciência, que buscam atender um mercado que busca alimentos atrativos e saudáveis (KOMATSU *et al.,* 2008), tornando o ato de se alimentar principalmente prazeroso. Por outro lado, o mercado de probióticos ainda deve expandir, uma vez que muitos consumidores não estão convencidos de que a ingestão desses microrganismos não desencadeia reações adversas à saúde, após o consumo (PIMENTEL, 2012).

O iogurte Activia, da Danone, foi lançado com êxito em diversos países europeus, em meados da década de 1990, e chegou no mercado brasileiro apenas em 2004, levando a uma considerável revitalização do mercado dos

produtos lácteos (RAUD, 2008). Apesar da venda de produtos cárneos probióticos ocorrer desde 1998, em países como a Alemanha e no Japão, são necessários mais estudos de base humana para estabelecer provas documentadas do efeito benéfico destes produtos, com relação principalmente na promoção da saúde em humanos (MACEDO *et al*., 2012). Só depois desses estudos será possível confirmar o valor intrínseco da carne fermentada e contribuir para o reconhecimento de produtos como alimentos saudáveis.

Incze (1998) relatou que desde 1935 foram demonstrados tratamentos de terapia envolvendo *Lactobacillus acidophilus* de origem intestinal. Os primeiros embutidos cárneos probióticos foram produzidos com cepas intestinais e ainda hoje são encontradas na forma de cepas comerciais para aplicação em fermentados lácteos. Desde então, concentrou-se um grande esforço na busca de cepas de origem alimentar com características probióticas para aplicação em embutidos fermentados (PARENTE *et al*., 2001; PENNACCHIA *et al*., 2004; PENNACCHIA *et al*., 2006; DICKS *et al*., 2007).

O presente estudo é pioneiro no Brasil no que se refere ao uso de bactéria láctica isolada de linguiças fermentadas naturalmente, testando seu potencial probiótico em animais e aplicando no desenvolvimento de embutido funcional. No Brasil, é desconhecida a produção e comercialização de embutidos fermentados probióticos, embora já existam alguns poucos estudos desenvolvidos, nos últimos anos (MACEDO *et al*., 2008; DALLA SANTA, 2008; RUIZ, 2011), envolvendo cepas probióticas comerciais de aplicação láctea de

origem intestinal. No mês de fevereiro de 2014, jornais de diferentes países mundo (http://www.dailymail.co.uk; http://saude.terra.com.br; http://www.livescience.com/43465-baby-poop-sausage-probiotic.html) divulgaram o desenvolvimento de embutido suíno fermentado espanhol denominado '*fuet*', elaborado com bactérias probióticas isoladas de fezes de bebês. Apesar de serem bactérias probióticas conhecidas (*Lactobacillus* e *Bifidobacterium),* a ideia da origem fecal dessas bactérias não causa uma boa impressão ao público consumidor. Por esse motivo é importante escolher cepas que tenham características tecnológicas adequadas, como é o caso deste estudo que aplica a cepa de *L. plantarum* BLS29 no desenvolvimento de linguiça suína fermentada. Komatsu *et al.* (2008) lembram que o veículo usado para a incorporação das cepas probióticas deve ser cuidadosamente estudado, principalmente em alimentos fermentados quando a multiplicação dos micro-organismos pode resultar em características não peculiares ou mesmo indesejáveis no produto.

No presente estudo, os isolados mais frequentes foram de *L. plantarum* e *Enterococcus faecium* (Tabela 1 da sessão Capítulo II). Sawitzki *et al*. (2007) também isolaram predominantemente *L. plantarum* de salames artesanais no noroeste do Rio Grande do Sul, Brasil, isso se deve possivelmente à proximidade das regiões de estudo, onde a espécie é predominante. Na Europa, Hugas *et al.* (1993) isolaram principalmente *Lactobacillus sake* (55%) e apenas 8% dos isolados corresponderam à *L. plantarum* de salames na

Espanha. Cepas de *Enterococcus* sp. não são recomendadas para uso em alimentos, apesar de algumas cepas serem caracterizadas como probióticas, pois possuem plasmídeos que podem transmitir genes de resistência à antibióticos à microbiota do hospedeiro (FRANZ *et al.*, 2003) por isso não foram avaliadas quanto ao potencial probiótico *in vitro*.

A capacidade de inibir patógenos alimentares nos alimentos e no intestino é uma característica desejável aos probióticos. Todas as cepas de *Lactobacillus* testadas em nosso estudo apresentaram efeito antagonista sobre todos os patógenos testados *in vitro*. Uma das cepas com melhor potencial probiótico (*L. plantarum* BLS29) foi testada quanto à tolerância às condições ácidas do estômago e aos sais biliares intestinais simuladas em laboratório, demonstrando boa funcionalidade (Capítulo 2).

O mecanismo de ação antagonista de *L. plantarum* BLS29 ainda não está confirmado, mas além do ácido láctico, é possível que exista a produção de bacteriocina(s). Tal fato foi demonstrado no artigo científico apresentado no Capítulo III, onde, em condições de co-cultivo de *L. plantarum* BLS29 e *S.* Enteritidis SE86, com pH próximo à 7,0, houve inibição do patógeno alimentar. Outra possibilidade para a inibição da *Salmonella* Enteritidis SE86 é a produção de peróxido de hidrogênio por *L. plantarum*, relatado por Sawitzki *et al.*, (2007). A atividade antagonista de bactérias ácido lácticas tem sido demonstrada por vários mecanismos de ação, incluindo a produção de peróxido de hidrogênio, de metabólitos como ácido láctico e compostos

antimicrobianos como bacteriocinas ativas também contra bactérias gram-negativas. Fayol-Messaoudi *et al.* (2012) verificaram que a ação antagonista de *L. plantarum* ACA-DC287 contra *Salmonella enterica* sorovar Typhimurium SL1344 foi causada por substância antagonista não-acido láctica.

A segurança da cepa estudada pôde ser confirmada no mesmo artigo, através de sua incapacidade de translocação para o fígado e baço e não haverem focos inflamatórios no intestino (cólon e íleo), embora as contagens da bactéria láctica tenham ficado entre 8,5 e 10 $\log_{10}$ UFC/g de fezes dos camundongos gnotobióticos. Um fato intrigante ainda é a ausência do antagonismo no teste *ex-vivo* dos animais di-associados, ao contrário do que ocorreu em condições *in vitro*. Esse fato demonstra a necessidade de testes adicionais em animais para comprovar a eficácia do probiótico, já que as interações do sistema imunológico e da microbiota intestinal podem afetar o comportamento dos micro-organismos. Além disso, é preciso considerar a alta patogenicidade de *S.* Enteritidis SE86.

Bambirra *et al*., (2007) demonstraram que a cepa de *Lactobacillus sakei* 2a foi capaz de proteger o trato digestivo de camundongos *germ-free* contra infecção causada por *L. monocytogenes*, apresentando potencial para utilização como cultura *starter* em produtos fermentados. Vasconcelos *et al*. (2003) detectaram atividade antagonista *ex-vivo* produzida por *Lactobacillus murinus* contra *S.* Typhimurium também em animais gnotobióticos.

Na elaboração das linguiças suínas fermentadas a contagem de bactérias lácticas no tratamento inoculado dom *L. plantarum* BLS29 teve um decréscimo no dia do embutimento, possivelmente devido à mudança de ambiente e à baixa temperatura em que foi submetida a massa cárnea para ocorrer o processo de cura, recuperando as contagens (total) a partir do terceiro dia, mantendo-se com cerca de 12 $\log_{10}$ UFC/g de amostra até o 17º dia de fabricação, quando geralmente o produto é consumido na região onde ele foi fabricado. Estes resultados obtidos foram semelhantes aos encontrados por Sawitzki *et al*. (2007) que inocularam *L. plantarum* AJ2 em salame tipo Milano.

Na Figura 5.1 está representado o produto desenvolvido antes da defumação, quando este ficou fermentando à temperatura ambiente, por cerca de 24 horas.

Figura 5.1 Linguiças funcional (acima) e tradicional (abaixo) no primeiro dia de fabricação, antes do processo de defumação.

Após a defumação o produto adquiriu coloração característica, apresentando uma leve diferença visual na coloração, conforme pode ser percebido na figura 5.2. Embora não tenha ocorrido diferença no pH nem na umidade, a contagem de bactérias lácticas na linguiça funcional foi maior do que na linguiça tradicional, conforme a Tabela 1 do Capítulo 4.

Figura 5.2 Linguiças funcional (abaixo) e tradicional (acima) no terceiro dia de fabricação após o processo de defumação.

No décimo sétimo dia, quando o produto geralmente está pronto para consumo, foram avaliadas as contagens de bactérias ácido lácticas, pH e umidade, havendo diferença apenas na contagem total de bactérias lácticas. Apesar de o produto apresentar diferença visual após a defumação, a coloração do produto estabilizou tornando-se praticamente imperceptível, tanto externa quanto internamente, conforme é possível perceber a seguir, nas Figuras 5.3 e 5.4.

Figura 5.3. Linguiças funcional (à direita) e tradicional (à esquerda) no décimo sétimo dia de fabricação, pronto para consumo.

Figura 5.4. Corte das linguiças funcional (à esquerda) e tradicional (à direita) no décimo sétimo dia de fabricação, evidenciando a coloração muito similar dos produtos.

Os parâmetros de pH, umidade, teor de proteínas, lipídeos e cinzas não diferiram entre os tratamentos controle e funcional, o que pode ser um bom resultado já que os consumidores preferem produtos que mantenham suas características sensoriais originais.

CONCLUSÕES

Foram isoladas vinte e seis bactérias ácido-lácticas de linguiças suínas fermentadas naturalmente, das quais a espécie predominante foi *L. plantarum*. Das dezesseis bactérias ácido-lácticas isoladas, todas apresentaram atividade antagonista contra todos os patógenos testados. *L. plantarum* BLS29 apresentou as melhores características tecnológicas, apresentando crescimento melhor que as demais na presença de sais de cura, não produzindo gás e acidificando o meio rapidamente. Entre as características probióticas da cepa selecionada estão a capacidade de produção de exopolissacarídeo (importante para a aderência da bactéria, facilitando a colonização intestinal), sobrevivência ao pH ácido e aos sais biliares, simulando condições digestivas.

L. plantarum BLS29 mostrou capacidade de inibição do patógeno alimentar *S.* Enteritidis SE86 *in vitro* e *in vivo* demonstrando sua segurança para aplicação em alimentos, como era esperado por ocorrer naturalmente em um embutido fermentado, além características tecnológicas aceitáveis para a elaboração de embutidos cárneos fermentados podendo ser aplicado em testes posteriores envolvendo análise sensorial do produto.

Recomenda-se a investigação da natureza do composto antimicrobiano e testar sua estabilidade na matriz cárnea, já que nem todos compostos se mantém estáveis nesse tipo de alimento, como é o caso da nisina, uma

importante bacteriocina utilizada em queijos mas é degradada em carnes. Recomenda-se também como perspectivas de trabalhos futuros testar o potencial inibitório de *L. plantarum* BLS29 contra *S.* Enteritidis SE86 no produto para verificar se há inibição do patógeno nesta condição, o que contribuiria para o controle de patógenos em embutidos cárneos fermentados, não dispensando á claro as boas práticas de fabricação.

REFERÊNCIAS

1. AB-BIOTICS-SA. Castellana Jordi Cuñé. ***Lactobacillus plantarum* strains as hypocholesterolemic agents**. WO2011042333 A1, 09 Out. 2009, 14 Abr. 2011
2. ABIA – ASSOCIAÇÃO BRASILEIRA DAS INDÚSTRIAS DA ALIMENTAÇÃO. **O Setor em números**. Disponível em http://abia.org.br/vst/o_setor_em_numeros. html. Acessado em 30 de março de 2014.
3. ALBANO, H.; VAN REENEN, C.A.; TODOROV, S.D.; CRUZ, D.; FRAGA, L.; HOGG, T.; DICKS, L.M.T.; TEIXEIRA, P.; Phenotypic and genetic heterogeneity of lactic acid bacteria isolated from ‘‘Alheira”, a traditional fermented sausage produced in Portugal. **Meat Science**. v. 82, p. 389–398, 2009.
4. AMMOR, M.S.; MAYO, B. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage pruduction: An update. **Meat Science**. n.76, p. 138-146, 2007.
5. ARIHARA, K. Strategies for designing novel functional meat products. **Meat Science**. n.74, p. 219-229, 2006.
6. ASSOCIAÇÃO BRASILEIRA DA INDÚSTRIA PRODUTORA E EXPORTADORA DE CARNE SUÍNA (ABIPECS). Disponível em: http://www.abipecs.org.br/news/724/100/Conquistas-da-carne-suina-brasileira.html . Acessado em 31 de março de 2014.

7. ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (AOAC). **Official methods of the AOAC International**, 18th ed. Maryland: AOAC, 2005.
8. BBC RESEARCH. **Microbial Products: Technologies, Applications and Global Markets**, Report code: BIO086B , BCC Research, Wellesley, MA, USA (2013) (http://www.bccresearch.com/market-research/biotechnology/microbial-products-applications-bio086b.html) Acessado em: 05 de abril de 2014.
9. BAMBIRRA, F.H.S.; LIMA, K.C.G.; FRANCO, B.D.G.M.; CARA, D.C.; NARDI, R.M.D.; BARBOSA, F.H.F.; NICOLI, J.R. Protective effect of *Lactobacillus sakei* 2a against experimental challenge with *Listeria monocytogenes* in gnotobiotic mice. **Letters in Applied Microbiology**. V.45, p.663-667, 2007.
10. BERSOT, L.S.; LANDGRAF, M.; FRANCO, B.D.G.M.; DESTRO, M.T. Production of mortadela: behavior of *Listeria monocytogenes* during processing and storage conditions. **Meat Science**. v.57, p.13-17, 2001.
11. BIONDO, A.; WOLLHEIM, C.; SIVIERO, J.; PETRINI, L.A.; RICALDE, S.F. Higiene dos manipuladores de alimentos de uma empresa de refeições coletivas em Caxias do Sul, RS. **Revista Higiene Alimentar**. v. 25, p. 198-199, 2011.
12. BRASIL. Ministério da Agricultura, Pecuária e do Abastecimento . Secretaria de Defesa Agropecuária. Instrução Normativa no. 22 de 31 de julho de 2000. Aprova o Regulamentos Técnicos de Identidade de

Qualidade de Salames. **Diário Oficial da União**, Brasília, p. 15-28, 03 de agosto de 2000.

13. BRASIL, Ministério da Saúde. Agência Nacional de Vigilância Sanitária. Resolução – RDC nº 12 de 02 de janeiro de 2001. Aprova o regulamento técnico sobre Padrões Microbiológicos para alimentos e seus anexos I e II. **Diário oficial da União**, Brasília, DF, 10 de janeiro de 2001. Pg 45 – 51. Disponível em <http://www.anvisa.gov.br>.

14. BRASIL, Ministério da Saúde. Agência Nacional de Vigilância Sanitária. Resolução – RDC nº 2 de 07 de janeiro de 2002. Aprova o regulamento técnico de Substâncias Bioativas e Probióticos Isolados com Alegação de Propriedades Funcional e ou de Saúde. **Diário oficial da União**, Brasília, DF, 09 de janeiro de 2002. Disponível em <http://www.anvisa.gov.br>.

15. DALLA SANTA, O.R. **Avaliação da qualidade de salames artesanais e seleção de culturas *starter* para produção de salame tipo italiano.** 2008, 147 f. Tese (Doutorado em Tecnologia de Alimentos) – Universidade Federal do Paraná, Curitiba, Brasil, 2008.

16. DALLA SANTA, O.R.; ALVAREZ, D.C.; DALLA SANTA, H.S.; ZANETTE, C.M.; FREITAS , R.J.S.; MACEDO, R.E.F.; TERRA, N.N. Microbiota of sausages obtained by spontaneous fermentation produced in the South of Brazil. **Food Science and Technology**. v.32, n.4, p.653-660, 2012.

17. DORTU, C.; HUCH, M.; HOLZAPFEL, W.H.; FRANZ, C.M.A.P.; THONART, P. Anti-listerial activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat. **Letters in Applied Microbiology**. v.47, p.581-586, 2008.

18. ERKKILÄ, S.; SUIHKO, M.L.; EEROLA, S.; PESTÄJÄ, E.; MATTILA-SHANDHOLM, T. Dry sausage fermented by *Lactobacillus rhamnosus*

strains. **International Journal of Food Microbiology**, v.64, p.205-210, 2001.

19. FAO. FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS; WORLD HEALTH ORGANIZATION. Evaluation of health and nutritional proprieties of probiotics in food including powder milk with live lactic acid bacteria: Report of Joint Food and Agriculture Organization of the United Nations, World Health Organization Expert Consultation, Córdoba, Argentina, 2001. Disponível em: ftp://ftp.fao.org/es/esn/food/probio_report_en.pdf. Acessado em 23 de outubro de 2012.
20. FIORENTINI, A.M.; SAWITZKI, M.C.; BERTOL, T.M.; JÚNIOR, A.C.; SANT'ANNA, E.S. Influence of a native strain of *Staphylococcus xylosus* on the microbial, physicochemical and sensorial characteristics on Milano salami type. **Brazilian Archives of Biology and Technology**. v.53, n.4, p.961-974, 2010.
21. FRANZ, C.M.A.P.; STILES, M.E.; SCHLEIFER, K.L.; HOLZAPFEL, W.H.; Enterococci in foods – a conundrum for food safety. **International Journal of Food Microbiology**. v.88, p.105-122, 2003.
22. GEIMBA, M.P.; TONDO, E.C.; OLIVEIRA, F.A.; CANAL, C.W.; BRANDELLI, A. Serological characterization and prevalence of *spvR* genes in *Salmonella* isolated from foods involved in outbreaks in Brazil. **Journal of Food Protect**. v.6, n.67, p.1229-1233, 2004.
23. HAMMES, W.P.; HERTEL, C. New developments in meat starter cultures. **Meat Science**. V.49, S125-S138, 1998.
24. HUGAS, M.; GARRIGA, M.; AIMERICH, T.; MONFORT, J.M. Biochemical characterization of lactobacilli from dry fermented sausages. **International Journal of Food Microbiology.** v.18, p.107-113, 1993.

25. HUGAS, M.; MANFORT, J.M. Bacterial starter cultures for meat fermentation. **Food Chemistry**. V.59, p.547-554, 1997.

26. INCZE, K. Dry fermented sausage. **Meat Science**, 49:169 -177, 1998.

27. IBGE. Instituto Brasileiro de Geografia e Estatística. Disponível em: http://www.ibge.gov.br/home/estatistica/economia/ppm/2010/ppm2010.pdf. Acessado em: 23 de outubro de 2012.

28. INSTYTUT BIOTECHNOLOGI PRZEMYSLU ROLNO-SPOZYWCZEGO. ZIELINSKA, KRYSTYNA.; STECKA, KRYSTYNA.; MIECZNIKOWSKI, ANTONI.; KAPTUROWSKA, AGATA.; KUPRYS, MARTA. New strain of *Lactobacillus plantarum* s, the use of the strain of *Lactobacillus plantarum* s and the preparation for roughages ensiling. EP2582851 A1, 16 jun. 2010, 24 Abr. 2013.

29. JAY, M.J. **Microbiologia de Alimentos**. 6 ed. Porto Alegre: Artmed. 2005. 711p.

30. KOMATSU, T.R.; BURITI, F.C.A.; SAAD, S.M.I. Inovação, persistência e criatividade superando barreiras no desenvolvimento de alimentos probióticos. **Braz Journal of Pharmaceutical Science.** vol. 44, n. 3, jul./set., 2008.

31. LEROY, F.; VERLUYTEN, J.; DE VUYST, L. Functional meat starter cultures for improved sausage fermentation. **International Journal of Food Microbiology**, v.106, p.270-285, 2006.

32. LIMA, U. de A.; AQUARONE, E.; BORZANI, W.; SCHMIDELL, W. **Biotecnologia industrial – processos fermentativos e enzimáticos.** Vol. 3. São Paulo: Blucher, 2011. 593p.

33. MACEDO, R.E.F.; PFLANZER, P.S.; GOMES, C.L. Probiotic Meat Products. *In*: Rigobelo IC, (Org.) **Probiotic in animals**. 2012. http://www.intechopen.com/books/probiotic-in-animals

34. MACEDO, R.E.F.; PFLANZER, S.B.; TERRA, N.N.; FREITAS, R.J.S. Características de culturas láticas probióticas para uso em produtos cárneos fermentados: sensibilidade aos sais de cura e uso dos antibióticos para contagem seletiva. **Boletim do CEPPA**. v.33, n.1, p.123-134, 2005.

35. MACEDO, R.E.F.; PFLANZER, S.B.; TERRA, N.N.; FREITAS, R.J.S. Desenvolvimento de embutido fermentado por *Lactobacillus* probióticos: características de qualidade. **Ciência e Tecnologia de Alimentos**. v.28, n.3, p.509-519, 2008.

36. MACEDO, R.E.F.; PFLANZER, S.B.; GOMES, C.L. Produtos cárneos probióticos. In: **Probióticos e Prebióticos em Alimentos – Fundamentos e Aplicações Tecnológicas.** São Paulo: Varela. 2011. 669p.

37. MAHONEY, M.; HENRIKSSON. A. The effect of processed meat and meat starter cultures on gastrointestinal colonization and virulence of *Listeria monocytogenes* in mice. **International Journal of Food Microbiology**. v.84, p.255-261, 2003.

38. MENDES, C.M.; dos SANTOS, K.C.; de OLIVEIRA, N.L.C; INACIO, J. Pesquisa de coliformes totais e termotolerantes em alimentos minimamente processados, comercializados na cidade de Uberlândia, MG. **Rev. Higiene Alimentar**. v.25, n.198/199, p.58-61, 2011.

39. NASCIMENTO, A.R.; MOUCHREK FILHO, J.E.; MARTINS, A.G.L de A.; SERRA, J.L.; EVERTON, P.C. Ocorrência de *Listeria monocytogenes*

em queijos comercializados no varej de São Luis, MA. **Revista Higiene Alimentar**. v.25, n.198/199, p.113-117, 2011.

40. OLIVEIRA, F.A.; FRAZZON, A.P.G.; BRANDELLI, A.; TONDO, E.C. Use of PCR ribotyping, RAPD, and antimicrobial resistance for typing of *Salmonella* Enteritidis involved in foodborne outbreaks in Southern Brazil. **Journal of Infect Developing Countries**. v.2, n.1, p.170-176, 2007.
41. ORDÓÑEZ PEREDA, J.A. **Tecnologia de Alimentos – Alimentos de origem animal.** Vol. 2. Tradução: Fátima Murad. Porto Alegre: Artmed. 2005. 279p.
42. PENNACCHIA, C.; ERCOLINI, D.; BLAIOTTA, G.; PEPE, O.; MAURIELLO, G.; VILLANI, F. Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics. **Meat science**, v.67, p.309-317, 2004.
43. PENNACCHIA, C.; VAUGHAN, E.E.; VILLANI, F. Potential probiotic *Lactobacillus* strains from fermented sausages: Further investigations on their probiotic properties. **Meat Science**, v.73, p.90-101, 2006.
44. PIMENTEL, T.C. Atitude do consumidor em relação a culturas probióticas e alimentos probióticos. **Revista Brasileira de Tecnologia Agroindustrial**. v.6, n.2, p.796-808, 2012.
45. PORTAL DO AGRONEGÓCIO. http://www.portaldoagronegocio.com.br/conteudo.php?id=45644. Acessado em: 23 de outubro de 2012.
46. PROBI AB. Molin, Göran; Ahrné, Siv; Jeppsson, Bengt. **Compositions comprising *Lactobacillus plantarum* strains in combination with**

tannin and new *Lactobacillus plantarum* strains. EP 2 163 162 A1, 02 abr. 2004, 17 Mar. 2010.

47. RAMOS, E.M.; GOMIDE, L.A. de M. **Avaliação da qualidade de carnes – fundamentos e metodologias.** Viçosa, MG: UFV, 2012. 599p.
48. RAUD, C. Os alimentos funcionais: a nova fronteira da indústria alimentar análise das estratégias da Danone e da Nestlé no mercado brasileiro de iogurtes. **Revista Sociologia Política**, Curitiba, v.16, n.31, p. 85-100, nov. 2008.
49. REVISTA CARNETEC BRASIL. http://www.carnetec.com.br/Industry/News/Details/44229?loginSuccess. Acessado em 25 de agosto de 2013.
50. Ruiz, J.N. **Aplicação de microrganismos probióticos na forma livre e encapsulada em salame tipo italiano.** 2011. 123 f. Dissertação (Mestrado em Ciências) – Escola Superior de Agricultura 'Luiz de Queiroz', Piracicaba, Brasil, 2011.
51. SAAD, S.M.I.; da CRUZ, A.G.; FARIA, J.A.F.; **Probióticos e prebióticos em alimentos**: fundamentos e aplicações tecnológicas. 1. Ed, São Paulo: Varela, 2011. 669p.
52. SANTA CATARINA, SECRETARIA DE ESTADO DA FAZENDA. Caderno de indicadores /Diretoria de Planejamento Orçamentário. Florianópolis, 2013. 189p. Disponível em http://www.sef.sc.gov.br/sites/default/files/Caderno%20de%20Indicadores%20de%202013_site_marcadores%2008.10.2013.pdf.
53. SAMELIS, J.; METAXOPOULOS, J.; VLASSI, M.; PAPPA, A. Stability and safety of traditional Greek salami – a microbiological ecology study. **International Journal of Food Microbiology**, v.44, p.69-82, 1998.

54. SAMESHIMA, T.; MAGOME, C.; TAKESHITA, K.; ARIHARA, K.; ITOH, M.; KONDO, Y. Effect of intestinal *Lactobacillus* starter cultures on the behavior of *Staphylococcus aureus* in fermented sausage. **International Journal of Food Microbiology**. v.41, p.1-7, 1998.

55. SENTER, L.; ROSSI, E.M.; SARDIGLIA, C. U. Avaliação da qualidade microbiológica de salames artesanais e implantação de Boas Práticas de Fabricação em uma mini-indústria. **Revista Higiene Alimentar**., v. 24, n.186/187, 2010.

56. SOCCOL, C.R.; VANDENBERGHE, L.P. de S.; SPIER, M.R.; MEDEIROS, A.B.P.; YAMAGUISHI, C.T.; LINDNER, J.D.D.; PANDEY, A.; THOMAZ-SOCCOL, V. The Potential of Probiotics: A Review. **Food Technology and Biotechnology**. v.48, n.4, p.413-434, 2010.

57. TERRA, A.B de M.; FRIES, L.L.; TERRA, N.N. **Particularidades na fabricação de salame.** São Paulo: Varela, 2004. 152 p.

58. TONDO, E.C.; BARTZ, S. **Microbiologia e Sistemas de Gestão da Segurança de Alimentos.** Porto Alegre: Sulina, 2011. 263 p.

59. VELAZCO, J. Formulando produtos saudáveis de carne suína. **Revista CarneTec**. Julho-Setembro, p.26-30, 2013.

60. WATT, B.; MERRILL, A. L., **Composition of Foods**: Raw, Processed, Prepared; USDA Nutrient Data Laboratory: Bethesda, MD, 1999.

61. WORLD GASTROENTEROLOGY ORGANISATION. **Guia Práticas**: Probióticos e Prebióticos, 2008. Disponível em: http://www.worldgastroenterology.org/assets/downloads/pt/pdf/guidelines/19_probiotics_prebiotics_pt.pdf. Acessado em 04 de abril de 2014.

62. YOON, H-S.; JU, J-H.; LEE, J-E.; PARK, H-J.; LEE, J-M.; SHIN, H-K.; HOLZAPFEL, W.; PARK, K-Y.; DO, M-S. The probiotic *Lactobacillus rhamnosus* BFE5264 and *Lactobacillus plantarum* NR74 promote

cholesterol efflux and suppress inflammation in THP-1 cells. **Journal of the Science of Food and Agriculture,** v. 93, p.781–787, 2013.