FRANCESCA SILVA DIAS NOBRE

IDENTIFICAÇÃO DE MICRORGANISMOS PATÓGENOS, DETERIORANTES E BACTÉRIAS LÁTICAS EM LINGUIÇAS SUÍNAS E AVALIAÇÃO DO POTENCIAL EFEITO PROBIÓTICO

LAVRAS – MG

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

Orientadora

Dra. Rosane Freitas Schwan

Coorientador

Dr. Eduardo Mendes Ramos

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APROVADA em 12 de julho de 2011.

Dr. Disney Ribeiro Dias	UFLA
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LAVRAS – MG

2011

Ao meu esposo, Fernando,

pelo amor, apoio e compreensão.

A minha mãe, Etelvira, por fornecer toda a base do meu sucesso, a educação.

A minha irmã, Mariana, pelo carinho e amor.

DEDICO

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pela confiança e conhecimentos transmitidos.

OFEREÇO

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“Há um tempo em que é preciso
abandonar as roupas usadas...
Que já têm a forma do nosso corpo...
E esquecer os nossos caminhos que
nos levam sempre aos mesmos
lugares...

É o tempo da travessia...
E se não ousarmos fazê-la...
Teremos ficado... para sempre...
À margem de nós mesmos...”

Fernando Pessoa

RESUMO

A qualidade e a inocuidade de linguiças suínas devem ser asseguradas, pois este é o produto de carne suína de maior demanda no mercado brasileiro. Ao longo deste trabalho, linguiças suínas foram avaliadas quanto à detecção de patógenos e deteriorantes e métodos alternativos para controle e inibição da microbiota contaminante foram propostos. No primeiro estudo, "Inibição *in situ* de *Escherichia coli* isolada de linguiça suína frescal por ácidos orgânicos", foi avaliado o efeito inibitório de diferentes concentrações dos ácidos orgânicos cítrico, lático, acético e propiônico em *Escherichia coli* isoladas de linguiça suína. Para a determinação dos melhores ácidos e concentrações na inibição do microrganismo, dois experimentos foram realizados *in vitro*, nos quais os ácidos cítrico e lático foram selecionados para aplicação em linguiças inoculadas com *E. coli*. O ácido cítrico foi o mais eficaz em reduzir a população do microrganismo nas linguiças. No segundo estudo, "Avaliação da resistência térmica e a antimicrobianos de estirpes de *Salmonella* isoladas de linguiça suína", sorovares de *Salmonella* foram identificados e avaliados quanto à resistência térmica e a antimicrobianos. Os isolados foram resistentes a três ou mais antimicrobianos e apresentaram alta resistência térmica em linguiça suína, com valores de D58, D62 e D65, em 10 min 99 seg, 5 min 29 seg e 2 min 16 seg, respectivamente, e um valor de z de 10,1°C. No terceiro estudo, "Análise por PCR-DGGE para a caracterização de bactérias deteriorantes em linguiças suínas refrigeradas", objetivou-se identificar as comunidades bacterianas deteriorantes em linguiça suína frescal armazenada a 4ºC, nos tempos de 0, 14, 28 e 42 dias. Conjuntamente, o método dependente de cultivo (plaqueamento), pH e a_w foram realizados. Pelo método dependente de cultivo, as populações de bactérias mesófilas e bactérias do ácido lático (BAL) aumentaram linearmente ao longo do tempo analisado. No método independente de cultura, as bactérias deteriorantes predominantes foram *Lactobacillus sakei* e *Brochothrix thermosphacta*. No quarto e último estudo, "Triagem de *Lactobacillus* isolados de linguiças suínas para uso como potencial probiótico e avaliação da segurança microbiológica do produto fermentado", estirpes de *Lactobacillus* isoladas de linguiças suínas foram avaliadas em uma série de testes para evidenciar suas características probióticas e atividade antimicrobiana. Um coquetel de estirpes de *Lactobacillus plantarum* selecionadas foi realizado e inoculado em linguiças suínas para avaliar a capacidade de inibir o crescimento de patógenos. Foi realizada a determinação de pH e a_w nas linguiças. O coquetel de *Lactobacillus* foi eficiente em inibir *Listeria monocytogenes* e apresentou potencial para ser utilizado como cultura iniciadora em linguiças suínas.

Palavras-chave: Linguiça suína. *Escherichia coli. Salmonella.* PCR-DGGE. Bactérias deteriorantes. *Lactobacillus plantarum.* Cultura iniciadora.

ABSTRACT

The quality and safety of pork sausages should be ensured, because the product represents the largest demand in pork meat in the Brazilian market. Along this work, pork sausages were evaluated for pathogens and spoilage bacteria detection and alternative methods of control and inhibition of microbial contaminants were proposed. In the first study, "*In situ* inhibition of *Escherichia coli* isolated from fresh pork sausage by organic acids", the effects of different organic acids was evaluated on inhibition of growth of *E. coli* strains isolated from pork sausage. To determine the best acid and concentration to inhibit the microorganism, two experiments were performed *in vitro,* where the citric and lactic acids were selected for application in sausages inoculated with with *E. coli*. Citric acid was the most effective in reducing the microbial population in the sausages. In the second study, 'Evaluation of thermal and antimicrobial resistance of *Salmonella* strains isolated from pork sausages", *Salmonella* serovars were identified and evaluated for heat resistance and antimicrobial agents. The isolates were resistant to three or more antimicrobials and showed high heat resistance in pork sausage with values of D58, D62 and D65 at 10.99, 5.29 and 2.16 min, respectively, and a z value of 10.1 °C. In the third study, "PCR–DGGE analysis for the characterization of spoilage bacteria in fresh pork sausages refrigerated", PCR–DGGE analysis was used to identify spoilage bacterial communities in fresh pork sausage stored at 4°C for 0, 14, 28 and 42 days. Simultaneously, culture dependent methods, pH and a_w measurements were performed. By culture dependent method, the population of mesophilic bacteria and LAB increased linearly over storage time. In culture independent method, the predominant spoilage bacteria were *Lactobacillus sake*i and *Brochothrix thermosphacta.* In the fourth and last study, "Screening of *Lactobacillus* isolated from pork sausages for potential probiotic use and evaluation of the microbiological safety in fermented product", *Lactobacillus* strains isolated of pork sausage were evaluated in a series of tests to demonstrate probiotic characteristics and antimicrobial activity. A cocktail of selected strains of *Lactobacillus plantarum* was carried out and inoculated in pork sausages to evaluate the ability to inhibit the growth of pathogens. In sausages was also conducted to determination the pH and a_w. The cocktail of *Lactobacillus* was effective in inhibiting *Listeria monocytogenes* and showed a good potential to be used as starter culture in pork sausages.

Keywords: Pork sausage. *Escherichia coli. Salmonella.* PCR-DGGE. Spoilage bacteria. *Lactobacillus plantarum.* Starter culture.

SUMÁRIO

PRIMEIRA PARTE

EMBASAMENTO BIBLIOGRÁFICO ABORDANDO OS PRINCIPAIS TEMAS ENVOLVIDOS NO TRABALHO: IDENTIFICAÇÃO DE MICRORGANISMOS PATÓGENOS, DETERIORANTES E BACTÉRIAS LÁTICAS EM LINGUIÇAS SUÍNAS E AVALIAÇÃO DO POTENCIAL EFEITO PROBIÓTICO

1 INTRODUÇÃO GERAL

O Brasil é o quarto maior produtor mundial de carne suína. Uma forma de aumentar a demanda do produto no mercado interno é por meio do processamento, como a produção de embutidos (ASSOCIAÇÃO BRASILEIRA DA INDÚSTRIA PRODUTORA E EXPORTADORA DE CARNE SUÍNA - ABIPECS, 2008). Porém, os principais problemas são a qualidade e a inocuidade dos embutidos. Atualmente, o Brasil ainda possui uma tecnologia insipiente para a produção de embutidos com alta segurança microbiológica (CORTEZ et al., 2004; MÜRMANN; SANTOS; CARDOSO, 2009).

A detecção de patógenos em embutidos é de fundamental importância para caracterizar a qualidade higiênico-sanitária do produto, uma vez que, no Brasil, dois terços da carne suína consumida são representados por produtos processados, principalmente a linguiça (ABIPECS, 2008).

Por outro lado, os principais microrganismos deteriorantes e a ecologia microbiana do produto durante o armazenamento devem também ser investigados. Mas, para uma eficiente caracterização da diversidade microbiana no embutido, o método utilizado para a detecção de microrganismos deve ser considerado. Muitas vezes o isolamento tradicional não detecta microrganismos presentes na amostra, seja pelo estado de injúria deste, pela falha do meio de

cultivo em proporcionar as condições que os microrganismos requerem para crescer ou, ainda, microrganismos de diferentes espécies que compartilham características fenotípicas em comum sendo difícil sua identificação. Para evitar as falhas do isolamento tradicional, a técnica de PCR-DGGE vem sendo empregada como alternativa para estudo da diversidade microbiana. Esta pesquisa contribui para a aplicação de novos métodos preservativos ou processos mais eficazes na qualidade do produto (AMANN; LUDWIG; SCHLEIFER, 1995; HANSEN; HUSS, 1998; HOLLEY, 1997; MUYZER; WALL; UITTERLINDEN, 1993).

Uma alternativa para a elaboração de embutido de melhor qualidade é a aplicação de inóculo de bactérias do ácido lático (BAL). O inóculo, após seleção, pode ser capaz de inativar patógenos e microrganismos deteriorantes via produção de ácido lático e bacteriocinas, além de contribuir para atributos sensoriais no embutido e apresentar, ainda, características probióticas (AMMOR; MAYO, 2007; BONOMO et al., 2008; LEROY; VERLUYTEN; VUYST, 2006; LÜCKE, 2000; PENNACCHIA et al., 2004; RUIZ-MOYANO et al., 2011; TALON; LEROY; LEBERT, 2007; TYÖPPÖNEN; PETÄJÄ; MATTILA-SANDHOLM, 2003; URSO et al., 2006).

Assim, visando estimular o grande potencial do mercado brasileiro ao consumo de carne e processados suínos de qualidade, este trabalho foi realizado com os seguintes objetivos: i) detectar e avaliar a resistência de microrganismos patogênicos (*E. coli* e *Salmonella* spp.) presentes em linguiça de carne suína industriais, ii) caracterizar a microbiota deteriorante durante a estocagem de linguiça suína industrial, utilizando a técnica PCR-DGGE e iii) selecionar inóculo de bactérias do ácido lático (BAL) com características probióticas para aplicação no produto fermentado e avaliação da sua segurança microbiológica, em função do uso deste inóculo.

2 REVISÃO BIBLIOGRÁFICA

2.1 Mercado da carne suína

A carne suína é a fonte de proteína animal mais importante no mundo, com produção de 115 milhões de toneladas, sendo quase a metade produzida na China e o restante na União Europeia (UE) e nos Estados Unidos da América. A participação do Brasil tem crescido em importância no mercado mundial. O país é o quarto maior produtor, com produção, em 2010, de 3,24 milhões de toneladas, detendo aproximadamente 3% da produção e 11% das exportações (ABIPECS, 2010).

O comércio internacional de carne suína movimenta 5,4 milhões de toneladas, gera uma receita anual aproximada de 11,9 bilhões de dólares e está concentrado em cinco países importadores (Japão, Federação Russa, México, Coreia do Sul e Hong Kong). Os Estados Unidos, a União Europeia, o Canadá, o Brasil e a China são responsáveis por 96% das exportações mundiais. O principal destaque dos últimos anos é o desempenho das vendas externas brasileiras que, em dez anos, ampliaram sua participação nas exportações mundiais de 4% para 11%. Mesmo com as barreiras sanitárias, o aumento dos subsídios europeus e o crescimento da concorrência internacional, as exportações brasileiras cresceram acima da média dos competidores (ABIPECS, 2010).

Segundo Camargo Neto (2007) a posição de destaque obtida pelo Brasil no mercado externo criou uma importante responsabilidade. O país deve continuamente adequar-se às demandas crescentes por qualidade e, para que a cadeia da carne suína obtenha maior desempenho ao que vem apresentando, deverá, ainda, enfrentar muitos desafios que envolvam, principalmente, inovação tecnológica.

Em relação ao consumo interno, o Brasil é o sexto país consumidor de carne suína, em termos absolutos (2,2% do total). No ano de 2010, o potencial de consumo no país, ainda baixo, foi estimado em 15 kg por habitante/ano (ABIPECS, 2010). O consumo interno de carne suína ocorre, em 70% dos casos, na forma de produtos industrializados, sobretudo linguiças (ABIPECS, 2008).

2.2 Linguiças suínas

As linguiças são definidas como produtos obtidos de carnes de animais de açougue, adicionadas ou não de tecidos adiposos, ingredientes, embutidas em envoltórios naturais ou artificiais e submetidas a processo tecnológico adequado. Podem ser classificadas segundo a tecnologia de fabricação ou de acordo com a composição da matéria-prima. Têm como ingredientes obrigatórios as carnes de diferentes espécies de animais de açougue, sal e água e, como ingredientes opcionais, gordura, proteínas vegetais ou animais, açúcares, plasma, aditivos intencionais, aromas, especiarias e condimentos (BRASIL, 2000).

A atribuição de função de aditivos, aditivos e seus limites máximos de uso para carne e produtos cárneos (industrializados, industrializados frescos embutidos ou não embutidos) está disposta no Regulamento Técnico nº 51, de 29/12/2006, do Ministério da Agricultura Pecuária e Abastecimento (MAPA) (BRASIL, 2007).

A classificação das linguiças é variável de acordo com a tecnologia de fabricação (produto fresco, seco, curado e ou maturado), a composição da matéria-prima e das técnicas de fabricação (calabresa, portuguesa, toscana e paio) e designação (denominação de venda - linguiça de carne bovina, carne suína, lombo suíno, lombo e pernil suíno, carne suína defumada, calabresa, portuguesa, toscana, carne de peru, carne de frango, mista, tipo calabresa, tipo

portuguesa, paio e outros). Assim, o produto será designado de linguiça, seguido de denominação ou expressões que o caracterizem (BRASIL, 2000).

Por sua composição, a linguiça apresenta alto risco microbiológico. Como passa por grande manuseio durante o processamento, pode apresentar condições propícias para a manutenção e a multiplicação de grande número de microrganismos, muitos dos quais capazes de provocar doenças nos humanos (MANHOSO, 1998). Ritter et al. (2003) e Sartz et al. (2008) destacam, ainda, que o embutido tem alta atividade de água (a_w) e não passa por processamento térmico durante a fabricação. Por isso é importante a preocupação com os vários aspectos relacionados à segurança do produto, tais como hábitos higiênicos dos manipuladores, qualidade de ingredientes e matérias-primas utilizados, bem como a correta sanitização de equipamentos empregados na elaboração do embutido.

Outro agravante para o risco microbiológico no embutido é que, para a sua elaboração, não há a necessidade de equipamentos caros ou de grandes tecnologias, podendo ser produzida tanto por grandes como por pequenas empresas. Neste último caso, particularmente, nem sempre as regras das Boas Práticas de Fabricação (BPF) são seguidas, mesmo porque muitos desses pequenos estabelecimentos não são registrados junto ao Serviço de Inspeção, desconhecendo princípios de higiene de produção e, muitas vezes, adquirindo matéria-prima de origem clandestina (RITTER et al., 2003; SABIONI; MAIA; LEAL, 1999).

Assim, devido a falhas no processamento e à qualidade da matéria prima, em diversos estudos há o relato da presença de patógenos em carnes e embutidos, principalmente *Escherichia coli*, *Salmonella* spp. e *Listeria monocytogenes* (BARBUTI; PAROLARI, 2002; SCHLUNDT, 2002).

2.3 Detecção de *Escherichia coli* em linguiças suína

A detecção de *E. coli* em linguiças suínas no Brasil foi descrita por Cortez et al. (2004), Magnani et al. (2000), Marques et al. (2006), Silva et al. (2002) e Tanaka et al. (1997). No cenário internacional, houve relatos no Reino Unido (SMITH et al., 1991), nos Estados Unidos (DUFFY et al., 2001), na Itália (VILLANI et al., 2005) e na Suécia (SARTZ et al., 2008).

A presença do microrganismo no embutido é indicadora de contaminação fecal direta ou indiretamente e de possível presença de outros patógenos entéricos. Altas contagens de *E. coli* e coliformes em alimentos, geralmente, indicam a falta de higiene no manuseio e em operações de produção, armazenamento inadequado e contaminação pós-processo, sendo a enumeração dos microrganismos referente a parâmetro de qualidade microbiológica no produto (GÓNZALES et al., 2003; SOUSA et al., 2002). No Brasil, a legislação vigente (BRASIL, 2001) estabelece limites de coliformes termotolerantes de $5x10^3$ NMP/g para linguiças.

2.4 Detecção de *Salmonella* em linguiças suínas

No Brasil, o consumo de produtos processados a partir da carne suína pode conduzir a surtos de salmonelose humana, sendo a linguiça o produto de maior risco (SPRICIGO et al., 2008). Segundo Silva et al. (2011), em 52 linguiças suínas analisadas em Londrina, PR, 5 amostras estavam contaminadas com *Salmonella*. Mürmann, Santos e Cardoso (2009) pesquisaram a presença de *Salmonella* em um total de 336 amostras de linguiças suínas em Porto Alegre, RS, e a encontraram em 82 (24,4%) amostras. Spricigo et al. (2008) relataram a prevalência de *Salmonella* spp. em 12,8% das 125 amostras analisadas em Lages, SC. Prevalências semelhantes foram encontradas no Rio de Janeiro e no

Rio Grande do Sul, 10,0% e 11,8%, respectivamente (CHAVES et al., 2000; LOGUERCIO et al., 2002). No Brasil, a legislação vigente (BRASIL, 2001) estabelece ausência de *Salmonella* spp. em 25 g de linguiças.

A detecção de *Salmonella* também foi relatada na Dinamarca (ALBAN et al., 2002), na Irlanda (BOUGHTON et al., 2004), na Itália (BIANCHI et al., 2007) e na Espanha (FONTÁN et al., 2007). Segundo Pielaat (2011), são necessárias medidas para rastreamento de *Salmonella* na cadeia suína. Em concordância, Rostagno e Callaway (2011) também julgam a necessidade de maiores estudos para a associação entre os fatores de risco relatados na literatura e infecção por *Salmonella* em populações suínas. Alguns fatores, como limitações metodológicas e a epidemiologia complexa e dinâmica de Salmonelas nos animais, limitam conclusões definitivas. Portanto, o controle de *Salmonella* na exploração de rebanhos suínos implica, ainda, em dificuldades, o que constitui um desafio persistente para a produção industrial mundial de carne suína.

2.5 Detecção de *Listeria monocytogenes* em linguiças suínas

No Brasil, a incidência de *Listeria monocytogenes* em linguiças suína vem sendo reportada em muitos estudos. Rossi et al. (2011) detectaram, em 80 amostras de linguiças frescais, 12 amostras positivas para *Listeria* spp. Destas, 3 amostras corresponderam a *L. monocytogenes*. De quatro diferentes marcas de linguiça industrial suína pesquisadas, em duas foi detectado o referido microrganismo. Miyasaki et al. (2009) analisaram 100 amostras de linguiças suína em diferentes pontos de venda em São Paulo e a positividade para *Listeria* spp. nas amostras foi de 90%, sendo 29% identificadas como *L. monocytogenes*. Lima et al. (2005), em uma planta de processamento de linguiça mista frescal, em Pelotas, RS, coletaram amostras da matéria-prima utilizada no

processamento de linguiça mista (carne bovina e suína), do ambiente de processamento, dos equipamentos, dos manipuladores, da massa pronta para o embutimento e do produto final. *L. monocytogenes* foi isolada em 25% das amostras, incluindo as linguiças embaladas para comercialização. No estudo de Silva et al. (2004) foi encontrada *L. monocytogenes* em 33,3% das amostras de carne suína e em 20% das amostras de gordura suína utilizadas para a fabricação das linguiças, além de 16,6% no produto final, em três frigoríficos em Pelotas, RS.

Também em outros países, embutidos de origem suína veiculam *Listeria monocytogenes*. Segundo Thévenot, Dernburg e Vernozy-Rozand (2006), os produtos derivados de carne suína foram fontes de surtos de listeriose na França e em outros países da Europa, durante a última década. Em Portugal, López et al. (2008) detectaram a bactéria em 10% dos produtos finais em uma planta processadora de carne suína. Karakolev (2009) relatou a incidência de *L. monocytogenes* em 11,3% de 141 amostras de linguiças na Bulgária. Cesare, Mioni e Manfreda (2007) verificaram, na análise de 288 linguiças frescais italianas, que 38,9% dos embutidos foram positivos para *Listeria monocytogenes*. A presença do microrganismo no embutido também foi reportada na Bélgica (UYTTENDAELE; TROYB; DEBEVEREA, 1999), na Suíça (JEMMI; PAK; SALMAN, 2002), na Turquia (COLAK et al., 2007) e na Espanha (CABEDO et al., 2008).

Segundo Karakolev (2009), há a necessidade de controle de *Listeria monocytogenes* ao longo de todo o processo de produção de linguiças suínas, ou, de acordo com Thévenot, Dernburg e Vernozy-Rozand (2006), a adição de tecnologias alternativas que contribuam para a inibição do microrganismo no embutido, como o emprego de bactérias do ácido lático (BAL) na elaboração do produto.

2.6 Deterioração de produto cárneo e caracterização da diversidade microbiana

Devido ao seu alto teor de água e à abundância de importantes nutrientes disponíveis em sua superfície, a carne é reconhecida como um dos alimentos mais perecíveis. A deterioração pode ser definida como qualquer mudança em um produto alimentar que o torna inaceitável para o consumidor a partir do ponto de vista sensorial (GRAM et al., 2002). No caso da carne, a deterioração microbiana conduz ao desenvolvimento de odores indesejáveis e, muitas vezes, à formação de "limo", o que torna o produto indesejável para consumo humano (ERCOLINI et al., 2006; HUIS-VELD, 1996). As mudanças organolépticas podem variar de acordo com a associação microbiana, a contaminação da carne e as condições em que a carne ou seus produtos são armazenados (ERCOLINI et al., 2006).

Para a indústria cárnea, o conhecimento sobre microbiota do produto e organismo específico de deterioração ("specific spoilage organisms", ou SSO) podem auxiliar nas inspeções microbiológicas, nas predições da vida útil e nas novas concepções de preservação ou métodos de produção (HANSEN; HUSS, 1998). Porém, a investigação de bactérias deteriorantes em produtos cárneos, quando dependente de métodos microbiológicos tradicionais, baseados na contagem microbiana em placas, isolamento e identificação bioquímica, pode apresentar falhas. Por exemplo, na identificação bioquímica, características fenotípicas podem ser compartilhadas entre espécies e no cultivo, meios de enriquecimento podem não imitar as condições particulares que os microrganismos exigem para a proliferação em seu habitat. Além disso, muitos microrganismos são ligados a partículas de sedimento na matriz e não são, portanto, detectados por microscopia convencional (AMANN; LUDWIG;

SCHLEIFER, 1995; HOLLEY, 1997; MUYZER; WALL; UITTERLINDEN, 1993).

Nos últimos anos, o desenvolvimento de métodos de tipagem molecular oferecem a possibilidade de avançar mais rápido e eficientemente em identificação bacteriana. A técnica eletroforese em gel em gradiende desnaturante, ou DGGE, permite o estudo de populações microbianas e sua diversidade, bem como a análise simultânea de múltiplas amostras e a comparação de comunidades microbianas com base em diferenças temporais e geográficas (AMPE; MIAMBI, 2000; MUYZER; SMALLA, 1998). Na última década, PCR-DGGE vem sendo aplicada com sucesso para caracterizar bactérias deteriorantes dominantes no setor de carne refrigerada durante o seu armazenamento (ERCOLINI et al., 2006; FONTANA; COCCONCELLI; VIGNOLO, 2006; LI et al., 2006; RUSSO et al., 2006) e contribuindo com as indústrias cárneas, que têm a necessidade de métodos analíticos mais precisos e rápidos para predizer a qualidade higiênico-sanitária e a vida útil dos seus produtos.

2.7 Aplicação de bactérias do ácido lático em linguiça suína

O potencial de consumo da carne suína e seus derivados, no Brasil, ainda é baixo (ABIPECS, 2010). Uma das principais causas é o fato de que consumidores consideraram moderadamente ruim o nível de segurança da carne suína (FONSECA; SALAY, 2008). Como forma de produzir produtos cárneos de origem suína com maior segurança microbiológica, o emprego de cultivos "starters" vem sendo estudado como uma solução, já que bactérias ácido láticas são capazes de inibir os microrganismos naturais competidores, incluindo bactérias deteriorantes e patógenos, como *Listeria monocytogenes* (JAY, 2005; PAPAMANOLI et al., 2002).

As principais espécies de BAL que vêm sendo utilizada em produtos cárneos são: *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus casei*, *Pediococcus pentosaceus* e *Pediococcus acidilactici* (HUGAS; MONFORT, 1997; LEROY; VERLUYTEN; VUYST, 2006).

Vários trabalhos relatam o emprego de culturas iniciadoras selecionadas e seus benefícios em produtos cárneos, como o controle da produção de aminas biogênicas (GARDINI et al., 2002; KOMPRDA et al., 2004; LATORRE-MORATALLA et al., 2007), significativa melhora no atributo coloração (CASABURI et al., 2007), inibição de microrganismos patogênicos (AYMERICH et al., 2002; DABOUR et al., 2009; MESSI et al., 2001; NOONPAKDEE et al., 2003; TODOROV et al., 2009), produção de catalase, enzima que decompõe o peróxido de hidrogênio, liberando oxigênio que contribui para a oxidação lipídica e a descoloração do pigmento nitrosomioglobina (ABRIOUEL et al., 2004; AMMOR; MAYO, 2007; MARES; NEYTS; DEBEVERE, 1994).

BAL são catalase negativas, porém, algumas estirpes envolvidas na fermentação de carnes, tais como *L. sakei*, *L. plantarum*, *L. pentosus* e *Pediococcus acidilactici*, possuem atividade heme-dependente catalase, ativa em produtos cárneos, uma vez que o substrato contém mioglobina em abundância (AMMOR; MAYO, 2007; SALMINEN; WRIGHT; OUWEHAND, 2004).

Ao selecionar BAL para exercer controle microbiológico e o aumento da vida útil nas linguiças, a produção de ácidos orgânicos é fator primordial. A inibição da microbiota patogênica e deteriorante é dependente da rápida e adequada formação destes ácidos orgânicos (VUYST; FALONY; LEROY, 2008). Foi comprovada a inativação de patógenos como *E. coli* O157:H7, presente na linguiça pela estirpe de *Lactobacillus reuteri* por produção de ácidos orgânicos e bacteriocina (MUTHUKUMARASAMY; HOLLEY, 2007).

Além da produção de ácidos orgânicos, BAL podem restringir o crescimento de outros microrganismos por meio de competição e geração de bacteriocinas e hipotiocianato (CHAILLOU et al., 2005; CHEN; HOOVER, 2003; HOLZAPFEL; GEISEN; SCHILLINGER, 1995; JONES, 2004).

Para a intensificação do aroma e do sabor no produto, estirpes de *L. sakei*, *L. curvatus* e *L. plantarum* possuem leucina e valina amino-peptidases que geram aminoácidos livres, precursores de 'flavor' agradável no produto final, como 3-metil-1-butanol, diacetil, 2-butanona, acetoína, benzaldeído, acetofenonas e metil-cetonas (AMMOR; MAYO, 2007; LEROY; VERLUYTEN; VUYST, 2006).

Ainda é intensamente estudada a atividade probiótica de BAL, com potencial aplicação probiótica em carne (PENNACCHIA et al., 2004; PENNACCHIA; VAUGHAN; VILLANI, 2006; RUIZ-MOYANO et al., 2011; VUYST; FALONY; LEROY, 2008). A benéfica ação probiótica de BAL foi descrita na prevenção e no tratamento de doenças do trato gastrintestinal, respiratório e urogenital (GARDINER et al., 2002), manutenção da microbiota intestinal (AIMMO; MODESTO; BIAVATI, 2007; LOURENS-HATTINGH; VILJOEN, 2001), modulação do sistema imune, redução de intolerância à lactose (GILLILAND, 1990; KIM; GILLILAND, 1983), redução dos níveis de colesterol sérico e pressão sanguínea (RASIC, 2003), atividade anticarcinogênica (OUWEHAND et al., 1999; RASIC, 2003) e melhor aproveitamento dos nutrientes e valor nutricional aos alimentos (LOURENS-HATTINGH; VILJOEN, 2001).

A utilização de estirpes iniciadoras e probióticas em produtos cárneos ainda é muito rara; apenas dois países vêm investindo na aplicação de BAL neste segmento da indústria: Alemanha e Japão (ARIHARA, 2006).

No Brasil, o uso de microrganismos como probióticos é potencialmente utilizado na indústria láctea. Na indústria cárnea, esta aplicação também é

promissora, pois a cultura pode exercer os inúmeros benefícios probióticos, tecnológicos (sanitários) e sensoriais, como já descrito na literatura científica, no embutido, agregando maior valor e confiabilidade ao produto.

3 CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

Os trabalhos realizados apresentaram como contribuição:

a) detecção de *Escherichia coli* em linguiças suínas, susceptibilidade do microrganismo a antimicrobianos e ácidos orgânicos *in vitro* e definição da mínima concentração inibitória de ácidos orgânicos para controle do patógeno em linguiças suínas;
b) detecção de *Salmonella* em linguiças suínas, caracterização do microrganismo quanto à resistência aos antimicrobianos e resistência térmica *in situ*.;
c) caracterização da microbiota deteriorante presente em linguiças suínas industriais e dinâmica populacional durante o seu armazenamento (4°C) e monitoramento dos parâmetros pH e a_w;
d) foram utilizadas técnicas moleculares (PCR-DGGE e sequenciamento), além de testes de caráter fenotípico (autoagregação, coagregação e hidrofobicidade) com metodologia modificada para leitura da absorvância em leitoras de microplacas, podendo contribuir para futuros trabalhos científicos;
e) linguiças fermentadas suínas foram produzidas adicionadas de cultivo iniciador selecionado a partir do próprio substrato e com ação inibitória sobre *Listeria monocytogenes*. O cultivo iniciador também apresenta potencial características probióticas. Em estudos futuros poderá ser identificada a substância antibacteriana específica produzida pelo cultivo iniciador para a inibição de *Listeria monocytogenes*, assim como ensaios clínicos para a atividade probiótica (ação hipocolesterolêmica, modulações do sistema imune e microbiota intestinal) do cultivo e possível patentemento.

A confirmação clínica (bioensaio com camudongos BALB/c – protocolo nº 25 - Comitê de Bioética em Utilizações de Animais da Universidade Federal de Lavras) da ação probiótica de estirpes de *Lactobacillus* em linguiça suína dará continuidade a este trabalho, com a perspectiva de impulsionar a indústria cárnea, proporcionando o efeito semelhante ocorrido na indústria láctea a partir da inclusão destas estirpes em seus produtos.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

In situ inhibition of *Escherichia coli* isolated from fresh pork sausage by organic acids

Artigo aceito para publicação no periódico *Journal of Food Science*

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RESUMO

Este estudo foi realizado com o principal objetivo de avaliar o efeito inibitório de diferentes concentrações dos ácidos orgânicos cítrico, lático, acético e propiônico, em *Escherichia coli* isoladas de linguiça suína. Dois experimentos foram realizados *in vitro*, respectivamente: difusão em ágar disco e determinação da Concentração Inibitória Mínima (MIC). Na difusão em ágar disco, a concentração mínima de 1,29 M de ácido cítrico inibiu o crescimento bacteriano. Não houve diferença estatistica significativa na MIC de ácidos cítrico e lático. Ambos os ácidos foram mais eficazes que os ácidos acético e propiônico. As estirpes de *E. coli* reagiram de forma diferente para cada ácido. Com base nos resultados *in vitro*, ácido lático e cítrico foram adicionados em linguiça suína inoculada com *E. coli*. A adição de ácido cítrico causou uma redução significativa (P <0,01) no pH das linguiças. Ácido cítrico foi mais eficaz 15 dias após a inoculação; a contagem de *E. coli* foi reduzida em 4,53 unidades logarítmicas (log) comparada ao tempo zero. Devido à ação inibitória tardia de ácido cítrico no estudo, o seu efeito pode ser mais efetivo sobre *E. coli* em linguiças com maior tempo de estocagem, como as fermentadas.

Palavras-chave: *Escherichia coli*, ácidos orgânicos, linguiça

ABSTRACT

The main aim of this study was to evaluate the inhibitory effect of different concentrations of organics acids citric, lactic, acetic and propionic on *Escherichia coli* isolated from pork sausage. Two experiments were performed *in vitro*, respectively: agar disc diffusion and Minimum Inhibitory Concentration (MIC) determination. In agar disc diffusion, the minimum concentration of 1.29 M of citric acid inhibits bacterial growth. There was no statistically significant difference in the MIC of citric and lactic acids; Citric and lactic acids were more effective than acetic and propionic acids. The *E. coli* strains reacted differently to each acid. Based on *in vitro* results, lactic and citric acids were added to pork sausages with *E. coli*. The addition of citric acid caused a significant reduction ($P<0.01$) in the pH of the sausages. Citric acid was most effective 15 days after inoculation; *E. coli* counts were reduced by 4.53 log units compared with time zero. Due to late inhibitory action of citric acid in the study, its effect may be more effective over *E. coli* in sausage that requiring longer storage, such as fermented sausages.

Keywords: *Escherichia coli*, organic acids, sausage

Introduction

E. coli is commonly detected in pork sausage (Normannoa and others 2004; Smith and others 1991). During preparation of the sausage, the quality of the raw material, the pH and the absence or inadequate heat processing directly contribute to the contamination and multiplication of microorganisms (Sartz and others 2008), which might represent a risk to public health. The products manufactured with pork meat also present at greater risk for contamination with *E. coli* resistant to antimicrobials (Lim and others 2007). Therefore, new alternatives should be implemented to auxiliary in better microbiological quality of sausages.

One measure that may prevent bacterial growth could be the inclusion of organic acids in the formulation of the sausage. Some organic acids, such as lactic, citric, acetic and propionic acids, have been used as preservatives in foods (Carpenter and Broadbent 2009) and have an inhibitory effect on pathogenic microbiota present in meat (Nazer and others 2005; Theron and Lues 2007) and *E. coli* resistant to antimicrobials (Samelis and others 2003). Several studies confirm the effective antibacterial action of organic acids and their salts, alone or associated with other methods, in meat and meat products (Schirmer and Langsrud 2010; Dubal and others 2004; Brewer and others 1991).

In the Brazilian legislation, citric, lactic and acetic acids are allowed as acidulants in the preparation of meat products (Brazil 2006), and propionic acid has proven antimicrobial activity in some foods, including bread, cheese, canned vegetables and cakes (Lee and others 2010). This study aimed to evaluate the inhibitory effects of different organic acids on the growth of *E. coli* strains isolated from pork sausage.

Materials and methods

Sample collection

From August to December 2009, 274 sealed packages of raw pork sausage were collected in commercial establishments in different cities located in Minas Gerais State, Brazil. Each sample contained approximately 300 g of sausage. The samples were transported to the laboratory under refrigeration in isothermal boxes and analyzed immediately.

Isolation and identification of *E. coli*

Twenty-five grams of each sample was aseptically transferred to 225 ml of 1% peptone water (Difco Laboratories, Detroit, MI, USA) and homogenized for 2 min in a stomacher (Mayo Homogenius HG 400, São Paulo, Brazil). For detection of *E. coli*, the analytical procedures were carried out as previously described by Kornacki and Johnson (2001). Gram- and oxidase-negative colonies were streaked on Plate Count Agar (PCA, Merck, Damstadt, Germany) slants and again incubated at 37°C for 24 h for the indole-methyl red-Voges-Proskauer-citrate (IMViC) biochemical tests. The API 20E kit (BioMérieux, Marcy l'Étoile, France) was used to complement the biochemical tests, and final identification was performed using the API LAB Plus software (BioMérieux).

To bacterial DNA extraction and PCR analysis, seventeen strains of *E. coli* were selected based on the antimicrobial resistance profile of 45 strains tested for DNA sequence analysis; DNA was extracted using a NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. For PCR analyses, the reaction was carried out in a final volume of 50 µl containing the following: 25 µl of TopTaq Master Mix (Qiagen, Hilden, Germany), 1 µl of each primer (27f /1512r), 2 µl of DNA and 21 µl of free water RNase. The amplification program was: 95 °C for 5 min, 35 cycles of 95 °C for 60 s, 50 °C for 60 s, 72 °C for 60 s and the final elongation step of 72 °C for 7

min. PCR products were sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer, and sequences were compared to those available in the GenBank database using the BLAST algorithm (National Centre for Biotechnology Information, Maryland, USA).

Antimicrobial susceptibility

Based on the previous recommendations of Clinical and Laboratory Standards Institute (CLSI 2011), the following antimicrobials were used in these tests: amikacin (30 µg/disc), tetracycline (30 µg/disc), cephalothin (30 µg/disc), cefotaxime (30 µg/disc), ceftazidime (30 µg/disc), aztreonam (30 µg/disc), cefoxitin (30 µg/disc), ceftriaxone (30 µg/disc), chloramphenicol (30 µg/disc), sulphazotrin (25 µg/disc), gentamicin (10 µg/disc) and ampicillin (10 µg/disc). Strains of *E. coli* biotype I (profile of identification in the kit API 20E in 99.9%) were grown on Case agar (Merck) for 24 h at 37 ºC. The bacteria were inoculated in 4 ml of sterile distilled water to achieve the nº 1 McFarland turbidity standard (Probac, São Paulo, Brazil). A swab was used to spread the inoculum across the surface of Muller Hinton agar (Merck), and antibiotics disks (DME Polisensidisc ® 4x6 Specialized Diagnostic Microbiology, São Paulo, Brazil) were applied to the plate. The resistance profiles of the strains were assessed by a measurement of the inhibition of bacterial growth after incubation for 24 h at 37°C.

Organic acids

E. coli strains were tested for their susceptibility to the following organic acids (Merck): lactic, citric, acetic, and propionic acids at the concentrations of 1, 2, 3 and 4 M. The solutions were prepared with sterile distilled water, adjusted to pH 4 with 5 N NaOH (Sigma-Aldrich, Germany) and

filter-sterilized through membrane filters with 0.22 µm pores (Millipore, Billerica, MA, US).

Experiment 1: Agar disc diffusion

The inhibitory effect of acids was first tested by the agar disc diffusion method. *E. coli* strains were cultured on Tryptcase Soy Agar (TSA, Merck), pH 7 for 24 h at 37°C. Each culture was suspended in 4 ml of sterile water and standardized to approximately 10^8-10^9 CFU/ml, according to the standard turbidity nº1 on the McFarland scale. A sterile swab was soaked in the suspension and spread on the surface of TSA plates. After the inoculum was fully absorbed, sterile 6-mm paper filter (Whatmann nº1) discs, moistened with 20 µl of each acid at each concentration, were added. Tolerance was determined by measuring the inhibition halos (mm diameter) after 24 h incubation at 37°C.

Experiment 2: Minimum Inhibitory Concentration (MIC) determination

E. coli strains were cultured in Brain Heart Infusion Broth (BHI, Himedia) for 24 h at 37ºC. Aliquots (50 µl) were taken from each culture with a cell density of about 10^8-10^9 CFU/ml and added to 96-well microplates (Denmark®) with 50 µl of BHI broth and 20 µl of each acid (citric, lactic, acetic and propionic) at each concentration (1, 2, 3 and 4 M). The microplates were incubated at 37°C. After 24 h, the optical density of cultures was measured at 620 nm (Multiskan FC-ThermoScientific Uniscience, São Paulo, Brazil); samples were blanked against sterile BHI broth. After reading, the viability of the strains was checked by plating on Eosin Methylene Blue (EMB) agar (Merck).

Organic acids in fresh pork sausage

Fresh pork sausages were manufactured to evaluate the efficiency of citric and lactic acids on the inhibition of inoculum *E. coli*. The inoculum contained a mixture of 17 representative strains of *E. coli* suspended in 3 ml of BHI broth grown for 24 h at 37°C to a density of 10^6 CFU/ml (by counting CFU in agar EMB). The solutions of organic acids were tested at concentrations of 1, 2, 3 and 4 M. The sausages were prepared in the laboratory under aseptic conditions using the following formula: 75% lean pork ham, 20% pork fat, 1.5% NaCl, 0.5% Antioxidant Ibracor L600® (IBRAC Additives & Spices, São Paulo, Brazil), 0.5% Cure LF® (IBRAC Additives & Spices, São Paulo, Brazil), 1.0% garlic paste, 0.5% chili pepper and 1% cold water. The total mixture was 1.5 kg, which was divided into ten 150 g batches. Eight batches were inoculated with the *E. coli* mixture and 3 ml of an acid solution. The two remaining batches served as controls: as a positive control, one batch of sausage was inoculated but remained acid free, and as a negative control, the last batch of sausage was not inoculated and remained acid free. Each batch was filled into a natural casing of 26 mm diameter. The sausages were stored at 4°C for 15 days.

Bacterial enumeration

The enumeration of *E. coli* in fresh pork sausages was performed on days 0, 5, 10 and 15 after preparation. At each time, 10 g of the material was aseptically removed from inside the sausage (central part in each sampling), homogenized in the stomacher with 90 ml of 1% peptone water and diluted serially. Samples were plated on EMB agar and incubated at 37°C for 24 h.

pH value analysis

To prepare samples for analysis, sausage (10 g) was homogenized in 100 ml of distilled water. pH values were determined using a pH meter PHS-3B

(Labmeter Model PH2, São Paulo, Brazil) equipped with an electrode (T818-A). The analysis was performed on days 0, 5, 10 and 15 after preparation of sausages.

Statistical analysis

A randomized block design with three replicates was used in all experiments. For the Agar disk diffusion and MIC tests, treatments were arranged in the factorial 4 X 4 X 17: 4 acids were tested at 4 concentrations for 17 strains. For the test of organic acids in fresh pork sausage, treatments were arranged in the factorial 3 x 2 x 4: 3 sausages (without acid, with citric acid and with lactic acid), 2 acids (lactic and citric acid) and 4 time points (0, 5, 10 and 15 days). The parameters were subjected to analysis of variance (ANOVA), and means were compared by the Scott-Knott test and by Scheffé contrasts (P <0.05). Quantitative data were analyzed using regression. The statistical analysis was performed using SISVAR® (Lavras, Brazil) software, version 4.5.

Results and Discussion

A total of 23% of the pork sausage samples analyzed were positive for the presence of coliform above the level recommended by Brazilian Legislation (Brazil, 2001). The samples contained an average of 5.6 x10^4 MPN/g. Of a total of 330 isolates were confirmed as *E. coli* by the IMViC test. Of the 330 *E. coli* isolates, 45 were identified as *E. coli* biotype 1, with a profile of similarity of 99.9% in the kit API 20E.

One option to reduce the occurrence of *E. coli* in pork sausages would be the addition of organic acids. Among the *E. coli* biotype 1 strains isolated, 17 strains (Table 1) were selected based on the proportion and variability of resistance profiles to antimicrobials found in the 45 strains tested.

Table 1 Identification and resistance to antimicrobials of 17 strains of *E. coli* isolated from fresh pork sausage

Strain UFLA SAU	Microorganism Identified	Percentage Identity (%)	Gene bank Accession Number	Resistance to number of antimicrobials
17	*Escherichia coli*	99%	GU811877.1	6
26	*Escherichia coli*	99%	GQ222387.1	5
55	*Escherichia coli*	99%	Z83204.1	1
61	*Escherichia coli*	99%	J01859.1	5
87	*Escherichia coli*	99%	GU811877.1	2
104	*Escherichia coli*	99%	EF191171.1	6
110	*Escherichia coli*	98%	AY319393.1	6
153	*Escherichia coli*	98%	CP001396.1	6
176	*Escherichia coli*	99%	CP001509.3	9
180	*Escherichia coli*	99%	Z83204.1	6
188	*Escherichia coli*	99%	GQ222387.1	3
193	*Escherichia coli*	99%	AB548576.1	6
197	*Escherichia coli*	99%	GU811877.1	0
199	*Escherichia coli*	99%	AB548582.1	4
200	*Escherichia coli*	99%	AB305017.1	6
213	*Escherichia coli*	99%	AB480776.1	6
318	*Escherichia coli*	99%	AB548579.1	6

In the effect of different factors analyzed in experiment 1and 2**,** to agar diffusion test (experiment 1) differences were observed among acid and concentration (P <0.01). To determination of the MIC (experiment 2), there was interaction between all factors studied: strains, acid and concentration.

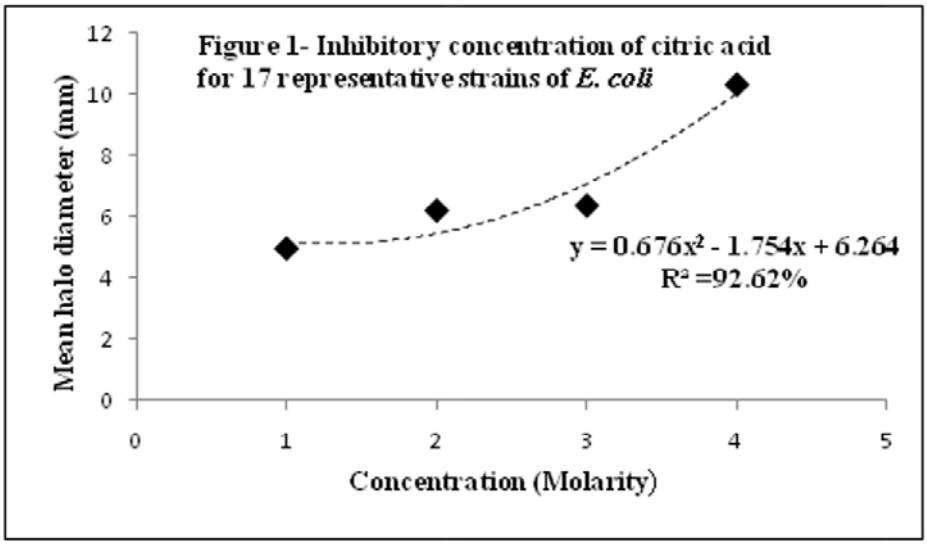
Of the strains analyzed in the agar diffusion test, eight showed a large zone of inhibition (UFLA SAU 26, 200, 176, 17, 55, 110, 193 and 194). For all evaluated concentrations, the greatest halo of inhibition was observed with citric acid (Table 2). With the exception of 3 M, all concentrations of lactic, acetic and propionic acids that were tested resulted in similar inhibition zones. For the four acids tested, the inhibition zone data showed quadratic behavior as a function of concentration (Table 2). The regression equation showed that there was an increasing quadratic of halos in function of the molarity of the acid and the minimum concentration of 1.29 M citric acid inhibits bacterial growth (Figure 1).

Table 2 Inhibition zones (mm) (average of the 17 *E. coli* strains) for different acids at different concentrations

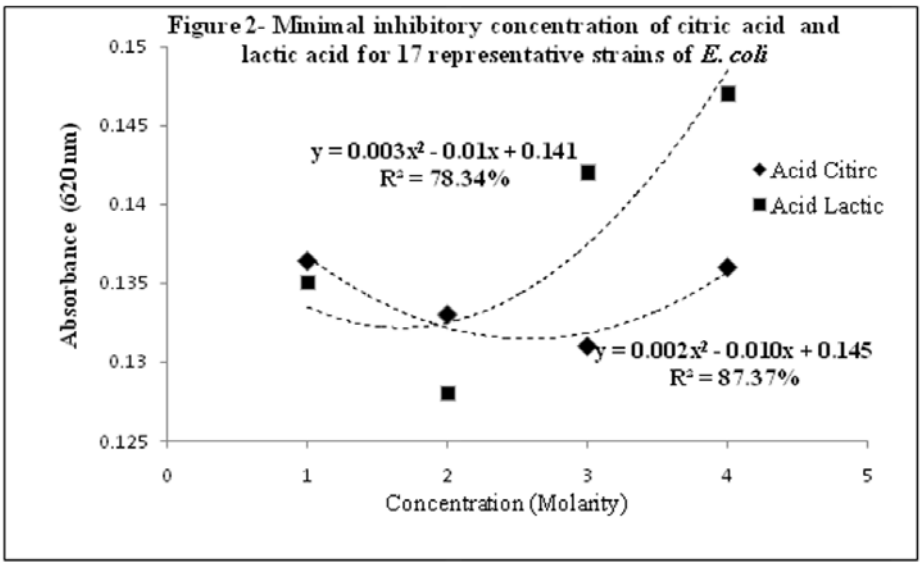
Acids	Concentration (Molarity)				Average	Equation
	1	2	3	4		
Citric	4.941^{A}	6.196^{A}	6.353^{A}	10.313^{A}	6.950^{A}	$0.676x^2$-1.754x+6.264 R^2=92.64%
Lactic	2.680^{B}	3.255^{B}	3.725^{B}	6.509^{B}	4.042^{B}	$0.552x^2$-1.565x+3.814 R^2=96.63%
Acetic	1.686^{B}	3.019^{B}	2.627^{C}	7.137^{B}	3.617^{B}	$0.794x^2$-2.374x +3.598 R^2 = 87.42%
Propionic	2.038^{B}	2.274^{B}	2.000^{C}	7.529^{B}	3.458^{B}	$1.323x^2$-4.996x+ 6.027 R^2 = 90.99%
Average	2.836	3.686	3.676	7.872	4.517	

For each columns, mean values with different letters are significant (P <0.05) by the Scott–Knott test
Standard Error Medium (SEM) = 0.39

With regards to the interaction between acids and concentration of MIC, citric and lactic acids were not statistically different. Both acids caused the same average growth inhibition of *E. coli* (data no shown); both acids were more effective than acetic and propionic acids. According to Hsiao and Siebert (1999), a pKa value close to the pH contributes to higher relative amounts of undissociated acid, a state in which the organic acid crosses the plasma membrane. However, once inside the cell, citric and lactic acids decrease the pH more quickly because the smaller the pKa of an acid, the higher its acidity.



The concentrations of citric and lactic acids that caused maximum inhibition of *E. coli* were 2.5 M and 1.62 M, respectively (data obtained by regression equation). Growth inhibition was proportional to the increase in acid concentration up those concentrations (Figure 2).



In the second experiment, there was interaction between acids and strains. The strains reacted differently to each acid (data no shown); however, based on the average of results from all acids tested, among the strains studied, the strains UFLA SAU 104, 61, 153, 213, 17 and 180 were the most acid-resistant. In accordance with the first experiment, three of these strains (UFLA SAU 153, 180 and 213) also presented lower inhibition zones, indicating that they are more resistant. However, two strains (UFLA SAU 17 and 104) were more sensitive to acids in the diffusion test. The results of contact with acids for 24 h in the 2nd experiment are in agreement with those reported by Bearson and others (1997) may lead to acid adaptation by strains of *E. coli.*

In the experiment with sausages, the acids used were citric and lactic due to better results *in vitro*. In the negative control there was neither microbial growth nor change in pH value. The positive control, the population remained, on average, with 10^6 CFU/g.

There was no significant change (P>0.05) in the pH of sausages treated with 1, 2 and 4 M of acid (CA) over time (Table 3). At concentrations of 2 and 4 M, citric acid more effectively lowered the pH of the sausage than lactic acid; however, at a concentration of 1 M, there was no difference in the use of citric and lactic acids.

Table 3 pH values over time of storage at 4°C for sausages without acid (SA), with citric acid (C) and with lactic acid (L) at concentrations of 1, 2, 3 and 4 M and comparison of groups with acid (CA)/SA and C/L

pH- 1 M acid						
Sausages	Time (days)				Average	Equation
	0	5	10	15		
SA	6.000	5.493	5.370	5.656	5.63 [a]	NS
C	5.210	5.103	5.136	5.166	5.154[b]	NS
L	5.313	5.333	5.210	5.266	5.280 [b]	NS
Average	5.508	5.310	5.238	5.363	5.355	$0.003x^2$-0.058x+5.512 R^2 = 99.41%
Contrast						
CA X SA	***	NS	NS	NS	***	
C X L	NS	NS	NS	NS	NS	
pH- 2 M acid						
Sausages	Time (days)				Average	Equation
	0	5	10	15		
SA	6.000	5.493	5.370	5.656	5.630 [a]	NS
C	5.046	4.873	4.926	4.980	4.956 [c]	NS
L	5.243	5.160	5.093	5.086	5.145[b]	NS
Average	5.431	5.175	5.130	5.241	5.244	$0.003x^2$-0.067x+5.428 R^2 = 99.73%
Contrast						
CA X SA	***	***	***	***	***	
C X L	NS	***	NS	NS	***	
pH- 3 M acid						
Sausages	Time (days)				Average	Equation
	0	5	10	15		
SA	6.00 [a]	5.49 [a]	5.37 [a]	5.65 [a]	5.630 [a]	$0.007X^2$ - 0.142X+6.004 R^2=99.99%
C	4.66 [b]	4.71 [b]	4.85 [b]	4.93 [b]	4.78 [c]	0.018X+4.648 R^2=97.22%

Table 3, continuation

L	4.89 [b]	4.97 [b]	5.02 [b]	5.04 [b]	4.98 [b]	0.010X+4.907 R^2=94.01%
Average	5.18	5.06	5.08	5.20	5.13	***
Contrast						
CA X SA	***	***	***	***	***	
C X L	NS	NS	NS	NS	NS	

pH- 4 M acid

Sausages	Time (days)				Average	Equation
	0	5	10	15		
SA	6.00	5.49	5.37	5.65	5.63 [a]	NS
C	4.56	4.65	4.76	4.85	4.70 [c]	NS
L	5.20	4.74	4.98	5.00	4.98 [b]	NS
Average	5.25	4.96	5.04	5.17	5.106	NS
Contrast						
CA X SA	***	***	***	***	***	
C X L	***	NS	NS	NS	***	

For each row and column, mean values with different letters are significant (P <0.05) according to the Scott–Knott test
NS, not significant; ***, P < 0.05

There was significant change (P<0.05) in the pH of sausages treated with 3 M acid (CA) over time (Table 3). For the sausage without acid (SA) treatment, the pH values changed according to a quadratic equation; the pH values of sausages containing citric (C) and lactic (L) acids increased linearly over time, by 0.018 and 0.010 pH units per day, respectively.

By contrast test there were significant differences between groups SA/CA and C/L (Table 3) at concentrations of 2, 3 and 4 M. At 2, 3 and 4 M, there were significant differences in the pH values at each time point for sausages CA compared with sausages SA. At 0 and 5 days at a concentration of 4 M and 2 M respectively, there were differences between sausages C/L. Treatment with citric acid significantly reduced the pH of the sausage, which is known to prevent the oxidation promoted by metals and contribute to improved durability and color stability of meat products (Ladikos and Lougovois 1990).

The inhibition of *E. coli* growth was not significant when 1 and 3 M citric and lactic acids were used (Table 4). However, there were significant reductions (1.28 and 0.89 log units) in the counts of *E. coli* in sausages containing citric acid compared with the positive control and lactic acid, respectively (Table 4) in the concentration of 4 M. The changes in the *E. coli* population showed a quadratic behavior for the three sausages, with the greatest inhibition of *E. coli* observed on day 15 with citric acid (2.05 log CFU/g). In contrast between groups (C/L), the action of citric acid was reconfirmed, occurred only from day 15 with greater effectiveness in sausages prepared with citric acid. There was not a significant difference in the number of *E.coli* in the sausages without acid and with lactic acid (6.38 and 5.83 log CFU/g, respectively).

The concentration of 2 M of acids in tests *in vitro* and in the food matrix did not show differences using either citric or lactic acid, but there was a different result in relation to citric acid to inhibit the growth of *E. coli.* Based on the results of the MIC test, by regression analysis, 2.5 M citric acid was the ideal concentration, but in sausages, only 4 M acid caused a significant reduction of the microbial population. The high concentration of citric acid required for the inhibition of *E. coli* in the sausage can be explained by its antioxidant action in the sausage.

Table 4 Count log_{10} (CFU/g) of *E. coli* over time during storage at 4 °C in sausage without acid (SA), sausage with citric acid (C) and sausage with lactic acid (L) at concentrations of 2 and 4 M and comparison of groups with acid (CA)/SA and C/L

Count log_{10} (CFU/g) of *E. coli* – 2 M						
Sausages	Time (days)				Average	Equation
	0	5	10	15		
Average	6.63	6.38	6.37	6.05	6.36	-0.035X+6.628 R^2=90.72%
Contrast						
CA X SA	NS	NS	NS	***	***	
C X L	NS	NS	NS	NS	NS	
Count log_{10} (CFU/g) of *E. coli* – 4 M						
Sausages	Time (days)				Average	Equation
	0	5	10	15		
SA	6.79 [a]	6.43 [a]	6.5 [a]	6.38 [a]	6.52 [a]	$0.002x^2$-0.059x+6.763 R^2 = 82.52%
C	6.58 [a]	6.17 [a]	6.16 [a]	2.05 [b]	5.24 [c]	$-0.036x^2$-0.282x+6.359 R^2 = 92.57%
L	5.95 [a]	6.30 [a]	6.43 [a]	5.83 [a]	6.13 [b]	$-0.009x^2$-0.138x+5.933 R^2 = 94.60%
Average	6.44	6.3	6.36	4.75	5.96	***
Contrast						
CA X SA	NS	NS	NS	***	***	
C X L	NS	NS	NS	***	NS	

For each row and column, mean values with different letters are significant (P <0.05) according to the Scott–Knott test
NS, not significant; ***, P <0.05

Lactic acid is generally used in the meat industry with effective action against pathogens (Aymerich and others 2005; Brewer and others 1991), and its use is regulated by laws in the United States and Europe. However, our data did not confirm the inhibitory effect of lactic acid on *E. coli* in sausage. Dubal and others (2004) did not obtain satisfactory results regarding the inhibition of *E. coli* with the exclusive use of lactic acid in meat and meat products

The action of citric acid was most effective at day 15, with a reduction in the count of *E. coli* of 4.53 log units compared with time zero of the sausage treated with the same acid (Table 4). However, this effect is considered late for fresh pork sausages, as they are only considered fresh for a maximum of 15 days. Thus, the *E. coli* remained viable throughout the shelf life of the product. This result is in agreement with that reported by Lindqvist and Lindblad (2009).

Conclusions

Citric and lactic acids inhibited *E. coli* growth during *in vitro* tests more efficiently than acetic and proprionic acids; however, even citric and lactic acids were not effective for the inhibition of *E. coli* in fresh pork sausage under refrigeration. Due to the late inhibitory observed in this study, the citric acid may be more effective for the inhibition of *E. coli* in fermented sausages, which require longer storage. In those circumstances, other stresses, such as lack of glucose, low pH value and activity water, sensitize the microorganism, which inhibits its adaptive response to acid, thereby facilitating the action of acids. Further studies on the effects of organic acids should be undertaken to evaluate to their potential employability in pork sausage to reduce the incidence of *E. coli* in this product and their circulation in the food chain.

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ARTIGO 2

Evaluation of thermal and antimicrobial resistance of *Salmonella* strains isolated from pork sausages.

Normas do periódico *Journal of Food Safety*

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RESUMO

Neste estudo, os sorovares de *Salmonella* foram isolados de linguiças suína e identificados por métodos fenotípico e genotípico combinados. *Salmonella houtenae*, *S. bareilly*, *S. typhimurium*, *S. paratyphi* C e *S. paratyphi* B foram encontradas. Os seis isolados foram resistentes a três ou mais antimicrobianos. As estirpes apresentaram alta resistência térmica em linguiça suína com valores de D58, D62 e D65 em 10 min 99 seg, 5 min 29 seg e 2 min 16 seg, respectivamente, e valor de z de 10,1°C. De acordo com nossos resultados, o efeito binomial de tempo e temperatura pode ser útil para a indústria da carne suína em predizer e estimar processos térmicos específicos para linguiça. Aos consumidores, um maior tempo de aquecimento garante a qualidade microbiológica do produto e reduz o risco de salmonelose.

Palavras-chave: *Salmonella*, linguiça suína, resistência a antimicrobianos, resistência térmica

ABSTRACT

In this study, serovars of *Salmonella* were isolated in the pork sausage and identified by combined phenotypic and genotypic methods. *Salmonella houtenae, S.* Bareilly, *S*. Typhimurium, *S.* Paratyphi C and *S.* Paratyphi B were found. Antimicrobial resistance profiles of these *Salmonella* strains were studied, and the heat resistance of a cocktail of these 6 isolates when challenged in a pork sausage model system was evaluated. All six isolates were resistant to more than three antimicrobials. They also had high heat resistance in pork sausage with values of D_{58}, D_{62} and D_{65} at 10.99, 5.29 and 2.16 min, respectively, and a z-value of 10.1°C. According to our results, the binomial effect of time and temperature can be useful to pork industry in designing and estimating thermal processes specific for sausage. To consumers, a longer heating time ensures the microbiological quality of sausage and reduces the risk of salmonellosis.

Keywords: *Salmonella*, pork sausage, antimicrobial resistance, heat resistance.

INTRODUCTION

Swine is an important source of *Salmonella* (Berends *et al*. 1996; Vieira-Pinto *et al*. 2006). The pork products contamination during the slaughter process is an important vehicle for *Salmonella* spp. dissemination to humans (Oliveira *et al*. 2010). During further processing of the meat, such as cutting and mincing, *Salmonella* from the contaminated pork cuts may be spread into the prepared meat. At the retail and consumer levels, cross-contamination, improper storage and insufficient cooking time can increase the risk to consumers (Gonzales-Barron *et al*. 2010).

In Brazil, there is a high consumption of pork sausage, and the occurrence of *Salmonella* in product is common (Castagna *et al*. 2005; Borowsky *et al*. 2007; Spricigo *et al*.2008; Mürmann *et al*. 2009). These factors increase the population's exposure to the pathogen. Thus, periodic outbreaks of salmonellosis caused by pork clearly demonstrated the need for improved tracking and tracing of *Salmonella* spp. in the pork production chain (Pielaat 2011). In addition to the monitoring of *Salmonella* in pork products, other factors in relation to genus also need be evaluated, such the serovars, antimicrobial resistance profiles and heat resistance. Antimicrobial resistance of *Salmonella* leads to a failure in treatment of salmonellosis; antimicrobial resistance is also a problem in other diseases caused by bacterial pathogens (Travers and Barza 2002). Additionally, few studies referent to heat resistance and the inactivation of *Salmonella* in pork sausage have been carried out.

Studying some features, such as heat and antimicrobial resistance of *Salmonella* circulating in the pork chain may contribute to preventive measures and control the distribution of this pathogen in meat products, lowering the impact on public health. However, the objective of this study was to isolate and identify *Salmonella* strains from pork sausage and evaluate antimicrobial

resistance profiles and heat resistance of these isolates when challenged in a pork sausage model system.

MATERIALS AND METHODS

Sample collection

All possible different trade marks of fresh industrial pork sausage available in four cities of the state of Minas Gerais were collected (total of 14 different trade marks) in sealed packages refrigerated in commercial establishments. The samples were directly transported into the refrigerated isothermal boxes to the Laboratory of Microbiology of the Federal University of Lavras and immediately analyzed.

Isolation and Phenotypic identification of *Salmonella* spp.

To detect *Salmonella* spp., the analytical procedures for isolation were carried out as previously described by Pignato *et al.* (1995). To isolate the strains, an amount of 25 g of each pork sausage was aseptically transferred to 225 ml of pre-enrichment broth base Salmosyst (Merck), homogenized in stomacher for 4 min and incubated at 37 °C for 6 h. For the selective Salmosyst enrichment, 10 ml of pre-enrichment broth base was supplemented with one selective supplement tablet (Merck) and incubated for 18 h at 37 ºC. From each tube, a loopful of broth culture was streaked onto Rambach agar plates (Merck), and the plates were incubated at 37 ºC for 24 h. Five typical colonies on the Rambach agar plates were selected, transferred to tubes containing Agar Triple Sugar Iron (TSI) (Himedia) and Lysine Iron Agar (LIA) and incubated at 37 ºC for 24 h. Colonies were tested for differential staining of Gram, catalase and oxidase. To complement these biochemical tests, the API20E kit (BioMérieux) was used to identify the bacteria; the final identification was performed using the API LAB Plus software (BioMérieux).

Bacterial DNA Extraction and PCR analysis of *Salmonella* strains

DNA was extracted using a QIAamp DNA Mini Kit (Qiagen). The DNA extraction was performed according to the manufacturer's instructions. The 16S rRNA gene was amplified using the primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1512r (5′-CGGCTACCTTGTTACGACT-3′). The reaction was carried out in a final volume of 50 µl containing the following: 25 µl of TopTaq Master Mix (Qiagen), 1 µl of each primer (27f /1512r), 2 µl of DNA and 21 µl of free water RNase. The amplification program was: 95 °C for 5 min, 35 cycles of 95 °C for 60 s, 50 °C for 60 s, 72 °C for 60 s and the final elongation step of 72 °C for 7 min. After amplification, the samples were stored at 4 ºC. Sequencing reactions were performed at Macrogen Inc. (Seoul, Korea). The sequences were then compared to those available in the GenBank database using the BLAST algorithm (National Centre for Biotechnology Information, Maryland, USA).

Antimicrobial susceptibility testing

The antimicrobials used in this test, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2011), were the following: amikacin (30 µg/disc), tetracycline (30 µg/disc), cephalothin (30 µg/disc), cefotaxime (30 µg/disc), ceftazidime (30 µg/disc), aztreonam (30 µg/disc), cefoxitin (30 µg/disc), ceftriaxone (30 µg/disc), chloramphenicol (30 µg/disc), sulphazotrin (25 µg/disc), gentamycin (10 µg/disc) and ampicillin (10 µg/disc). Isolates of *Salmonella* were grown on Case agar (Merck) for 24 h at 37 ºC. The population bacterial was inoculated in 4 ml of sterile distilled water to achieve the nº 0.5 McFarland turbidity standard (Probac, Brazil). A swab was used to spread the inoculum across the surface of Muller Hinton agar (Merck), and antibiotics disks (DME Polisensidisc® 4x6-Specialized Diagnostic Microbiology, São Paulo, Brazil) were applied to the plate. Strain resistance was

assessed by measuring the inhibition of bacterial growth after incubation for 24 h at 37 °C. *Escherichia coli* ATCC 25922 was used for quality control testing.

Heat resistance tests in pork sausage

The thermotolerance of a cocktail of all six *Salmonella* strains isolated and identified in this study was tested in fresh pork sausage. The inoculum was suspended in 2 ml of Tryptone Soy Broth (TSB- Merck) with a density of 10^{10} CFU/ml (by counting in Agar Rambach). The cocktail was added in 80 g of pork sausage. The sausages were prepared in the Laboratory of Microbiology of the Federal University of Lavras under aseptic conditions and composed of the following ingredients: 74% lean pork ham, 20% fat pork, 2.0% NaCl, 0.5% Antioxidant Ibracor L600® (IBRAC Additives & Spices, São Paulo, Brazil), 1.0% Cure LF® (IBRAC Additives & Spices, São Paulo, Brazil), 1.0% garlic paste, 0.5% chili pepper and 1% cold water. The final mixture was filled into a natural casing with a 26 mm diameter. The sausages were packaged in sterile plastic packaging. Heat resistance trials were performed by completely submerging the packaged sausage in a circulating water bath at 58 ºC, 62 ºC and 65 ºC and for 0, 5, 10 and 15 min. Tests were performed in triplicate for each time/temperature. After each test, the sausage was removed and immediately cooled in water and ice. The sausage was then stored at 4 ºC, and survival (cell viability of *Salmonella* strains) counts were performed within 2 h.

Enumeration of *Salmonella* surviving heat-treatment

Survivor counts were performed using the MPN (Most Probable Number) dilution technique (. De Man 1983), using a series of three tubes per dilution in TSB media containing 0.3% yeast extract (Himedia) (TSBP). Bacterial growth was evaluated on the basis of the turbidity of the TSBP broth after 48 h incubation at 37 ºC. The presumptive *Salmonella* survival was

confirmed by counts after isolation of strains on Rambach Agar plates (Merck) and incubation at 37 ºC for 24 h.

Statistical analysis

The number of *Salmonella* strains that were survivors as a function of time was evaluated by regression analysis using SISVAR® (Lavras, Brazil) software, version 4.5. The *D*-values (decimal reduction time) were calculated from the resulting regression equations. The *z*-values were evaluated by the linear regression of $\log_{10}$ *D*-values vs. heating temperatures. The counts were subjected to analysis of variance (ANOVA), and the means were compared by Scott-Knott, with $P < 0.01$. Triplicate thermal inactivation trials were performed at each time/temperature for each sausage sample.

RESULTS AND DISCUSSION

A total of 14 different marks of industrial sausages were investigated, *Salmonella* was detected in five brands (Table 1). From each brand of sausage analyzed, 5 isolates indicating for *Salmonella* spp. in the Rambach agar were stored for biochemical tests (Table 1). Using API 20E kit a total of 70 isolates were analyzed, the results showed that only six isolates were confirmed with profile identification between 95% and 100% for *Salmonella* (Table 1). These isolates were also identified by comparative analysis of 16S rRNA gene sequences using the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) database. The six strains were identified with a similarity of 99% or 100%: *Salmonella enterica* subsp. *houtenae* (AB273733.1) and serovars of *Salmonella enterica* subsp. *enterica:* Bareilly (U92196.1), Typhimurium (AP011957.1), Paratyphi C (EU118097.1) and Paratyphi B (DQ344539.1).

Different serovars of subspecie *Enterica* were detected in four different sausages brands (Table 1). In sample from sausage 7 was detected two

subspecies of *Salmonella*: *houtenae* and *enterica* serovar Typhimurium. *Salmonella houtenae* was detected in two samples of industrial sausage (Table 1). In this study, all *Salmonella* isolates from pork sausage identified are described in scientific literature as human pathogenic. *Salmonella* Paratyphi B and *Salmonella* Paratyphi C cause enteric fever (Parry 2005). *Salmonella houtenae* and *Salmonella* Bareilly are involved in sporadic outbreaks (Cowden *et al.* 2003; Cleary *et al.* 2010). *Salmonella* Typhimurium is one of the major emerging pathogens responsible for salmonellosis in humans (Boughton *et al.* 2004).

Table 1 Detection of *Salmonella* strains in industrial fresh sausages collected in state of Minas Gerais

Industrial sausages (Marks)	Cities of collection	Detection of *Salmonella*	Isolates confirmed by API 20E Kit	Acession number % similarity	Subspecie or Serovars of *Salmonella*
1	Betim	Absence	-		
2	Belo Horizonte	Absence	-		
3	Lavras	Presence	1	DQ34453 9.1 / 99%	Paratyphi B
4	Lavras	Absence	-		
5	Lavras	Absence	-		
6	São João del-Rei	Presence	1	AB27373 3.1 / 99 %	*houtenae*
7	São João del-Rei	Presence	2	AB27373 3.1 / 99 % AP011957 .1 / 99 %	*houtenae* Typhimurium
8	São João del-Rei	Presence	1	U92196.1/ 99 %	Bareilly
9	Lavras	Absence	-		
10	Lavras	Absence	-		
11	Lavras	Absence	-		
12	São João del-Rei	Absence	-		
13	São João del-Rei	Absence	-		
14	São João del-Rei	Presence	1	EU11809 7.1 / 99 %	Paratyphi C

In antimicrobial susceptibility test, all six strains of *Salmonella* isolated and identified in this study were resistant to amikacin (Table 2). Sulfazothrim was the antimicrobial in which all the serovars of *Salmonella enterica* subsp. *enterica* were sensitive. The isolates of *S. houtenae* showed different pattern (resistance or sensitivity) to the following antimicrobial agents: ceftazidime, sulfazothrim, cefoxitin, gentamicin and tetracycline. The serovar Typhimurium was resistant to 9 antimicrobials, including 3rd generation cephalosporins and aztreonam (monobactam). Thus, this serovar may be a producer of Extended Spectrum β-Lactamase (ESBL). ESBLs are enzymes capable of to hydrolyze

penicillins, broad-spectrum cephalosporins and monobactams and also *Enterobacteriaceae* ESBL-producer have been responsible for numerous outbreaks of infection throughout the world and they represent challenging infection control issues (Rupp and Fey 2003.). Relatively high rates of occurrence of strains producing ESBL in animal foods and the high genetic diversity among these strains indicate that there is an established reservoir of these organisms in farm animals (Geser *et al.* 2011). Without good hygienic practices, meats may act as a vehicle of transfer of β-lactamase resistant bacteria to the gastrointestinal tract of consumers (Amador *et al.* 2011).

Table 2 Antimicrobial susceptibility[a] of *Salmonella* strains from pork sausages

Antimicrobial	*Salmonella* strains					
	Paratyphi B	Houtenae	Houtenae	Typhimurium	Bareilly	Paratyphi C
Cefotaxime	R	R	I	R	I	R
Ampicillin	R	R	R	R	R	I
Amikacin	R	R	R	R	R	R
Ceftazidime	I	R	S	R	I	I
Cephalothin	R	R	R	R	I	R
Sulfazothrim	S	R	S	S	S	S
Cefoxitin	R	R	S	R	S	R
Gentamicin	R	S	R	R	R	R
Tetracycline	I	S	R	S	S	S
Ceftriaxone	R	I	S	S	S	I
Chloramphenicol	R	S	I	R	S	S
Aztreonam	I	I	S	R	I	I

[A] R: resistant; I: intermediary; S: susceptible

The isolate *S.* Typhimurium was sensitive to tetracycline, but it did not match the most common resistant phenotype found for the serovar Typhimurium DT 104. In accordance with Beaudin *et al.* (2002) *S.* Typhimurium DT104 strains commonly express resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline.

The large profile of antimicrobial resistance for these pathogenic strains isolated from pork sausage might be a great risk to public health. Mürmann *et al.* (2009) isolated *Salmonella* serovars from fresh pork sausage being *S.* Typhimurium one of them, from those isolates, 85.9% were resistant to more than one antimicrobial. In our studies, 100% of isolates were resistant to more than three antimicrobials.

To determine the heat resistance, a cocktail of the six strains isolated and identified in this study was inoculated into fresh prepared pork sausage. The thermal inactivation curves were linear (Figure 1) in all temperatures evaluated (58, 62 and 65 ºC). The determination of coefficient R^2 of the regression curves was always higher than 0.90 (Table 3). The regression curves of temperatures 58 ºC, 62 ºC and 65 ºC presented a reduction of (CFU/g) 0.091, 0.189 and 0.463 log/min of microorganism, respectively. Thus, the decimal reduction times (D-values) of the *Salmonella* cocktails in the sausage decreased substantially with an increase in temperature (Table 3). The D-values for 58 ºC, 62 ºC and 65 ºC were 10.99, 5.29 and 2.16 min, respectively. The *z*-value (Figure 2) was 10.1 ºC.

Table 3 Heat-resistance (expressed in d-values and *z*-value) for a cocktail of 6 *Salmonella* strains in fresh pork sausage

Temperature (ºC)	Equation	R^2	D-Values (min)	z-Value (ºC)
58	y = -0.091x + 10.29	0.92	10.99	
62	y = -0.189x + 10.49	0.93	5.29	10.1
65	y = -0.463x + 10.99	0.92	2.16	

There is limited information about thermal inactivation of *Salmonella* in pork sausage. Mattick *et al.* (2002) affirmed that the *Salmonella* spp. are present in a significant proportion of sausages, and these strains are not always killed during the cooking process. In pork meat containing curing additives, Quintavalla *et al.* (2001) reported that for six different strains of *Salmonella*

inoculated in pork meat, the D_{58}-value ranged from 2.79 to 4.8 min and the *z*-value ranged from 4.1 to 4.8 ºC. Murphy *et al.* (2004) found that in ground pork, the values of $D_{62.5}$ and D_{65}- were 2.56 and 1.91 min, respectively, and the *z*-value was 5.89 ºC. Juneja *et al.* (2001a) inoculated a cocktail of eight serovars of *Salmonella* in pork meat (8.5% fat) and determined that D_{58}, $D_{62.5}$ and D_{65} values were 6.68, 1.62 and 0.87 min, respectively, and the *z*-value was 7.1 ºC. The D-value and *z*-value determined in this work were higher than the values calculated for pork meat in the previous studies. This difference may be due to the fat content of the substrate. The protective effects of fat may be due to lower heat conductivity or reduced water activity in the fat portion (Senhaji 1977). In general, higher fat content results higher thermal resistance (Ahmed *et al.* 1995; Veeramuthu *et al.* 1998; Juneja *et al.* 2001b; Oteiza *et al.* 2003). Typically, fresh pork sausage contains between 10 to 40% of fat (according to manufacturing industries in Brazil). The comparison of the results between these studies has other sources of variability besides the composition of the substrate, such as strain specificity and the method of enumeration.

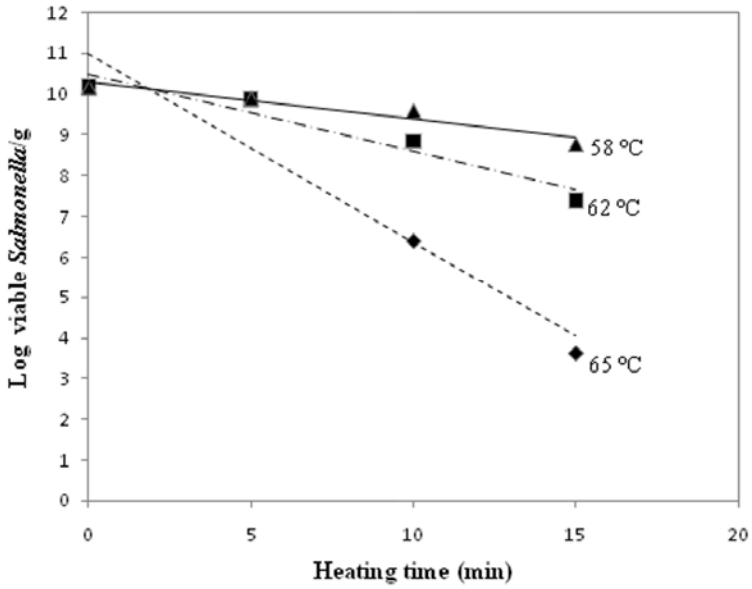

Figure 1 Thermal inactivation curves obtained AT 58 ºC, 62 ºC and 65 ºC for a cocktail of 6 *Salmonella* strains in fresh pork sausage

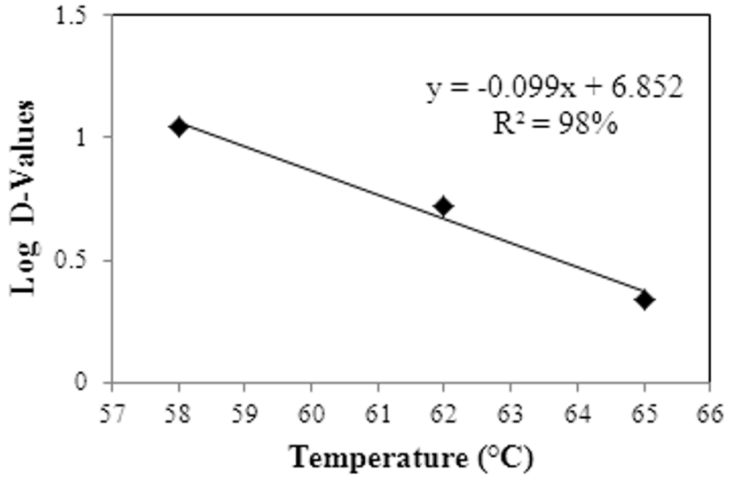

Figure 2 Thermal-death-time curves (z-values) for a cocktail of 6 *Salmonella* strains in fresh pork sausage over a temperature range of 58 ºC to 65 ºC

In the enumeration of cell viability of *Salmonella* strains, there was an interaction ($p<0.01$) between sausages heated at different temperatures and the heating time (Table 4). For each temperature, the reduction (log_{10} CFU/g) of *Salmonella* was significant at each time point. At 5 min, there was no difference between the temperatures of 62 ºC and 65 ºC for inactivation of the microorganism. At this time, the reduction of the microorganism was still low, only 0.32 and 0.33 log units from the initial time for 62 ºC and 65 ºC, respectively. From 10 min, the temperature of 65 ºC was more effective in reducing *Salmonella* strains, with population decline to 3.794 log units in relation to the initial time.

Table 4 Enumeration using log_{10} (CFU/g) at time points (minutes) of a cocktail of 6 *Salmonella* strains in fresh pork sausage heated at 58 ºC, 62 ºC and 65 ºC

Temperature (ºC)	Time (minute)				
	0	5	10	15	Average
58	10.173^{aA}	9.903^{aB}	9.586^{aC}	8.756^{aD}	9.605^{a}
62	10.196^{aA}	9.873^{bB}	8.836^{bC}	7.386^{bD}	9.073^{b}
65	10.190^{aA}	9.860^{bB}	6.396^{cC}	3.623^{cD}	7.517^{c}
Average	10.186	9.878	8.273	6.588	8.731

Mean values bearing the same superscript of uppercase (rows) or lowercase (columns) letters are not significantly different ($P<0.01$) according to the Scott–Knott test
Standard Error Medium (SEM) = 0,007

The specific determination of the time and temperature binomial can eliminate the risk of the product serving as a vehicle for microorganisms. According to Gonzales-Barron *et al.* (2010), cooking pork sausages for an additional half minute can reduce the risk level by ±50%.

In conclusion, it was detected strains of *Salmonella* in Brazilian industrial pork sausage and they were resistant to three or more antimicrobials. According to the results of this study, the time and temperature binomial of the

inactivation of microorganisms in sausages is greater than the binomial typically employed for meat, due to the fat content in the product. Thus, heating times, 11 min to one log cycle reduction of microorganism in internal temperature of 58 ºC or 5.29 min to 62ºC or 2.16 min to 65º C in sausages, ensure a higher level of microbiological quality and offer less risk of salmonellosis to consumers of product. The resulting kinetic parameter can be useful to pork industry in designing and estimating thermal processes specific for sausage.

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ARTIGO 3

PCR–DGGE analysis for the characterization of spoilage bacteria in fresh pork sausages refrigerated

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RESUMO

Linguiças frescais são altamente perecíveis e servem como substrato para vários microrganismos deteriorantes e patogênicos. O conhecimento sobre os metabólitos microbianos e "specific spoilage organisms (SSO), ou organismos deteriorantes específicos, podem eventualmente ser aplicado para predizer a vida útil de um produto. Eletroforese em gel de gradiente desnaturante (DGGE) é um dos métodos mais comumente utilizados para a avaliação de comunidades microbianas, independente de cultivo. Neste estudo, a análise por PCR-DGGE foi aplicada para identificar comunidades bacterianas deteriorantes em linguiça suína frescal armazenada a 4°C, nos tempos de 0, 14, 28 e 42 dias. Simultaneamente, o método dependente de cultivo, mensuração de pH e a_w foram também realizados. Pelo método dependente de cultivo, a população de bactérias mesófilas e BAL (bactérias do ácido lático) aumentou linearmente ao longo do tempo de análise. Para pH e análise a_w, houve aumento nas unidades de medida no final do tempo de estocagem. Apenas para três amostras, a_w permaneceu em 0,97, durante todo o período estudado. No método independente de cultivo, as bactérias deteriorantes predominantes foram *Lactobacillus sakei* e *Brochothrix thermosphacta*.

Palavras-chave: bactérias deteriorantes, linguiças, PCR-DGGE.

ABSTRACT

Fresh sausages are highly perishable and serve as substrates for several spoilage and pathogenic microorganisms. Information about microbial metabolites and SSOs (specific spoilage organisms) can ultimately be used to predict the shelf life of a product. Denaturing gradient gel electrophoresis (DGGE) is one of the most commonly used culture-independent methods for evaluating microorganisms. In this study, PCR–DGGE analysis was employed to identify spoilage bacterial communities in fresh pork sausages stored at 4°C for 0, 14, 28 and 42 days. Simultaneously, culture-dependent methods and pH and a_w measurements were performed. Culture-dependent methods showed that the populations of mesophilic bacteria and Lactic Acid Bacteria (LAB) increased linearly over storage time. An increase was observed in the pH and a_w values at the end of the storage time. Only 3 samples retained an a_w of 0.97 during the entire study. According to culture-independent methods, the predominant spoilage bacteria present were *Lactobacillus sake*i and *Brochothrix thermosphacta*.

Keywords: Spoilage bacteria, sausages, PCR-DGGE.

INTRODUCTION

Fresh sausages are highly perishable and serve as substrates for several spoilage and pathogenic microorganisms due to their high water content and abundance of essential nutrients (Cocolin *et al*. 2004). Spoilage can be defined as any change in a food product that makes it unacceptable to the consumer from a sensory point of view. Microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth (slime, colonies), as textural changes (degradation of polymers) or as off-flavors (Gram *et al*. 2002). In the case of meat and meat products, microbial spoilage leads to the development of off-flavors, oxidative rancidity, discoloration, gas production and, often, slime formation (Lloyd-Puryear *et al*. 1991; Cocolin *et al*. 2004).

Knowledge of the metabolites and Specific Spoilage Organisms (SSOs) can ultimately be used to predict the shelf life of a product, to aid the microbiological inspections and to design new preservation or production methods (Hansen and Huss 1998). Due to the limitations of conventional microbiological methods, the characterization of microorganisms that require selective enrichment and subculturing is difficult and sometimes not possible. Moreover, it was shown in the last decade that classical microbial techniques do not accurately detect microbial diversity (Hugenholtz *et al*. 1998; Diez *et al*. 2008). Alternative molecular methods, independent of cultivation, have become a very important tool in the study of microbial communities because they are believed to overcome the problems associated with selective cultivation and with the isolation of bacteria from natural samples (Jiang *et al*. 2010).

Denaturing gradient gel electrophoresis (DGGE) is perhaps the most commonly used culture-independent fingerprinting technique (Ben Omar and Ampe 2000; Ercolini 2004). Many scientists have been using this technique to monitor the dynamics of microbial populations and to characterize the dominant spoilage bacteria in pork meat and pork meat products (Cocolin *et al*. 2004;

Fontana *et al*. 2005; Rantsiou *et al*. 2005; Li *et al*. 2006; Vasilopoulos *et al*. 2008; Hu *et al*. 2009; Jiang *et al*. 2010). However, there are few studies that have characterized the spoilage bacteria in pork sausages in Brazil by molecular methods. Further investigation is necessary to obtain a more complete understanding of the microbial species in products responsible for spoilage. Therefore, the objective of this study was to characterize spoilage bacteria in fresh pork sausages by culture-dependent methods and PCR-DGGE analysis, as well as by monitoring of pH and water activity (a_w) values of the sausages during the time of storage.

MATERIALS AND METHODS

Samples and storage

Sealed packages of fresh industrial pork sausages of twelve different trademarks were collected from commercial establishments in the state of Minas Gerais, Brazil and transported in isothermal boxes under refrigeration. In the laboratory, sausages were portioned aseptically, packaged in sterile plastic bags (Cryovac, Brazil; O_2 transmission rate, 30 cm^3 m^{-2} atm^{-1} 24 h^{-1} at 20 °C) and stored at 4°C for a total of 42 days. At 0, 14, 28 and 42 days, samples of sausage were subjected to molecular analysis, and triplicate samples were used for microbiological, pH and a_w analyses.

Microbiological analysis

Ten grams of each sausage sample was homogenized in 90 ml of 0.1% peptone, pH 7.00 (Difco Laboratories, Detroit, Mich.) in a Stomacher (Mayo Homogenius HG 400, São Paulo, Brazil). Decimal dilutions were prepared, and the following analyses were performed on agar plates: total mesophilic aerobic counts on Plate Count Agar (PCA, Merck) for 48 h at 37°C and count of Lactic

Acid Bacteria (LAB) on Man-Rogosa-Sharpe (MRS) agar (Merck) at pH 6.5 for 48 h at 30°C.

pH and a_w measurements

The pH values were determined by homogenizing 10 g of sausage in 100 ml of distilled water using a pH meter PHS-3B (Labmeter Model PH equipped with an electrode T818-A, Shanghai, China). The a_w values were measured from 5 g of sausage using an AquaLab model 3 TE (Braseq, São Paulo, Brazil).

DNA extraction and PCR analysis

Total DNA was extracted from samples at different times of fermentation using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20 °C. For the DGGE analyses, genomic DNA was used as the template for the PCR amplification of bacterial ribosomal target regions. The bacterial community DNA was amplified with the primers 338fgc (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3′) (the GC clamp is underlined) and 518r (5′-ATT ACC GCG GCT GCT GG-3′) spanning the V3 region of the 16S rRNA gene (Ovreas *et al.* 1997). The PCR mix (25 µl) contained the following: 12.5 µl of TopTaq Master Mix (Qiagen, Hilden, Germany), 0,5 µl of each primer, 1 µl of DNA and 10,5 µl of RNase-free water. The amplification was performed as as follows: template DNA was denatured for 5 min at 95 °C, followed for 30 cycles of denaturing at 92 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s. The PCR tubes were then incubated for 10 min at 72 °C for the final extension. Aliquots (2 µl) of the amplified products were analysed by

electrophoresis on 1% agarose gels before they were subjected to DGGE analysis.

DGGE analysis and band sequencing

The PCR products were analysed by denaturing DGGE using a BioRad DCode Universal Mutation Detection System (BioRad, Richmond, CA, USA). Samples were loaded on 8% (w/v) polyacrylamide gels in 0.5× TAE. Optimal separation was achieved with a 15–55% urea–formamide denaturing gradient for the bacterial community (100% denaturant corresponds to 7 mol l^{-1} urea and 40% [v/v] formamide). The gels were run for 3 h at 200 V at 60 °C and were then stained with SYBRGreen I (Molecular Probes, Eugene, UK) (1:10,000 v/v) for 30 min. The gels were photographed with a laser FluorImager densitometer and analysed using Fragment Analysis (Amersham Biosciences, Sweden). Single pieces of DGGE bands were excised with a sterile scalpel, and the DNA from each band was eluted in 30 µl of sterile water overnight at 4 ºC. From the eluted DNA, 10 µl from each DGGE band was amplified. The PCR products were sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer, and the sequences were compared to those available in the GenBank database using the BLAST algorithm (National Centre for Biotechnology Information, Maryland, USA).

Statistical analysis

A randomized block design with three replicates was used for the microbiological analyses and pH and a_w measurements. The treatments were arranged in a factorial 12 X 4 design: 12 sausages of different trademarks and 4 time points (0, 14, 28 and 42 days). The parameters bacterial count, pH and a_w were subjected to analysis of variance (ANOVA), and the means were compared by a Scott-Knott test. The quantitative data were analyzed using regression in

relation to storage time. Data were considered significantly different when the *P* values were less than 0.05. The statistical analysis was performed using the SISVAR® (Lavras, Brazil) software version 4.5.

RESULTS

Microbiological analysis and pH and a_w measurements

Bacterial counts throughout storage are shown in Table 1. There was a significant interaction ($P<0.05$) between the sausages and time of evaluation of the mesophilic bacteria and LAB populations. The population of mesophilic bacteria increased linearly over the storage time. This increase ranged from 0.017 to 0.091 log units per day (CFU g^{-1}.) depending on the sample (trademark of sausage), as observed by the regression equation for the sausages in the study. At the end of the storage time, the highest population was detected in PCA agar for sausage of brand 12 (6.72 log CFU g^{-1}). For the other brands of sausage, the population was, in general, detected at the level of 10^5 CFU g^{-1}. For the LAB counts, no colonies (<10 CFU g^{-1}) were observed in sausages of brands 4, 5, 6, 9 and 11 at day zero. In sausage 9, LAB colonies were not detected at day 14 either. However, the LAB population increased linearly over time in all of the sausages analyzed, as can be explained by the first-degree equations (Table 1). The increase in the LAB population ranged from 0.027 to 0.098 log units per day according to the sausage brand analyzed.

Table 1 Population $\log_{10}$ (CFU g^{-1}) values of Mesophilic bacteria and LAB over different storage times at 4°C fresh industrial pork sausage samples of twelve different trademarks

Sausage	Mesophilic bacteria[1] ($\log_{10}$ CFU g^{-1})					LAB[2] ($\log_{10}$ CFU g^{-1})				
	Time (Days)				Equation	Time (Days)				Equation
	0	14	28	42		0	14	28	42	
1	2.30	3.66	3.94	4.52	0.05 x + 2.566 R^2 = 90.60%	7.63	7.81	8.33	8.82	0.029 x + 7.533 R^2 = 96.42%
2	2.75	3.36	4.103	5.35	0.061 x + 2.608 R^2 = 97.18%	5.52	6.33	7.62	8.07	0.063 x + 5.545 R^2 = 97.09%
3	2.53	3.15	4.66	5.63	0.077 x + 2.375 R^2 = 97.79%	5.88	6.51	6.66	7.09	0.027 x + 5.966 R^2 = 94.85%
4	2.20	3.34	4.37	5.53	0.078 x + 2.209 R^2 = 99.94%	< 2	2.64	3.33	4.32	0.098 x + 0.526 R^2 = 90.84%
5	2.51	3.36	4.63	5.76	0.078 x + 2.412 R^2 = 99.39%	< 2	2.51	3.09	3.39	0.077 x + 0.635 R^2 = 80.97%
6	2.08	3.37	4.79	5.86	0.091 x +2.111 R^2 =99.71%	< 2	2.63	2.79	3.64	0.079 x + 0.602 R^2 =82.64%
7	2.73	3.53	4.35	5.10	0.056 x + 2.736 R^2 = 99.97%	5.26	6.45	6.65	7.33	0.046 x + 5.463 R^2 = 92.19%

Table 1, continuation

8	2.39	3.09	4.52	5.09	0.068 x + 2.340 R^2 = 97.25%	2.98	3.42	4.59	5.49	0.062 x + 2.814 R^2 = 97.32%
9	2.21	2.80	3.00	3.09	0.020 x+ 2.350 R^2 = 85.78%	< 2	< 2	2.39	2.58	0.072 x – 0.278 R^2 = 82.94%
10	2.26	2.63	2.93	4.14	0.042 x + 2.101 R^2 = 88.75%	4.34	4.63	5.27	6.61	0.053 x + 4.095 R^2 = 90.57%
11	2.16	2.54	2.64	2.91	0.017 x + 2.209 R^2 = 95.74%	< 2	2.10	2.62	3.42	0.077 x + 0.417 R^2 = 90.73%
12	3.09	4.40	5.56	6.72	0.086 x + 3.135 R^2 = 99.92%	6.12	7.31	8.09	8.78	0.062 x + 6.260 R^2 = 98.22%

For each row, mean values with different letters are significant (P <0.005) according to the Scott–Knott test
[1]SE=0.0698
[2]SE= 0.0065

The results of the pH and a_w measurements are shown on Table 2. There was a significant interaction ($P<0.05$) between the sausages and the time of pH and a_w evaluation. The initial pH values ranged from 5.60 to 6.97 for all the samples. In sausages 1, 2, 3, 4, 5, 6, and 10, the pH increased linearly over storage time with the increases ranging from 0.039 to 0.064 pH units per day, according to regression equation for each sausage. According to the quadratic equations (Table 2), the pH values of samples from sausages 7, 8 and 12 showed a reduction up to days 26, 26 and 15, respectively, with minimum values of 6.05, 5.23 and 5.22, followed by an increase from these time points until the final evaluation time (42 days). The water activity (a_w) values showed a significant increase ($P<0.05$) of 0.01 and 0.02 units per day until the end of storage to a total of eight and one sausages brand analyzed, respectively. Sausages 3, 4 and 5 did not show changes in the aw values, which remained at 0.97 over the entire period of study.

Table 2 pH and a_w measurements over different storage times at 4°C for fresh industrial pork sausage samples of twelve different trademarks

Sausage	pH[1]					A_w[2]				
	Time (Days)				Equation	Time (Days)				Equation
	0	14	28	42		0	14	28	42	
1	5.60	5.91	7.20	8.16	0.064 x + 5.374 R^2 = 95.41%	0.96[a]	0.97[b]	0.97[b]	0.97[b]	*
2	6.16	6.96	7.20	8.26	0.047 x + 6.162 R^2 = 95.03%	0.97[a]	0.97[a]	0.97[a]	0.98[b]	*
3	6.97	7.32	8.00	8.56	0.039 x + 6.895 R^2 = 98.5%	0.97[a]	0.97[a]	0.97[a]	0.97[a]	*
4	6.52	6.11	7.84	8.20	0.049 x + 6.147 R^2 = 75.1%	0.97[a]	0.97[a]	0.97[a]	0.97[a]	*
5	6.61	6.29	7.62	8.41	0.048 x + 6.222 R^2 = 80.43%	0.97[a]	0.97[a]	0.97[a]	0.97[a]	*
6	6.64	6.27	7.33	8.20	0.041 x + 6.249 R^2 =75.87%	0.97[a]	0.97[a]	0.97[a]	0.98[b]	*
7	6.70	6.36	6.35	7.17	$0.001x^2$- 0.052x+ 6.728 R^2 = 97.22%	0.92[a]	0.92[a]	0.92[a]	0.93[b]	*

Table 2, continuation

8	6.66	5.64	5.98	7.22	$0.002x^2$- 0.106x+6.637 $R^2 = 99.3\%$	0.96	0.96	0.97	0.97	0.0003 x + 0.959 $R^2 = 80.00\%$
9	6.86[b]	6.38[a]	7.60[c]	7.76[d]	*	0.94	0.94	0.95	0.96	0.0005 x – 0.937 $R^2 = 89.09\%$
10	6.73	6.96	7.95	8.43	0.043 x + 6.604 $R^2 = 95.14\%$	0.96[a]	0.96[a]	0.96[a]	0.97[b]	*
11	6.61[b]	6.27[a]	6.71[c]	6.79[d]	*	0.96[a]	0.96[a]	0.96[a]	0.97[b]	*
12	5.92	5.35	5.94	8.03	$0.003x^2$ -0.093 x + 5.94 $R^2 = 99.86\%$	0.96[a]	0.96[a]	0.96[a]	0.97[b]	*

For each row, mean values with different letters are significant (P <0.005) according to the Scott–Knott test
[1]SE= 0.0064
[2]SE= 5.807
* There was no adjustment of the equation to observed data

Direct analysis of microbial diversity in sausages by DGGE

The results from the DGGE analysis were obtained by amplifying the V3 region of the 16S rRNA gene using the 338f (GC) and 518r primers. By applying a denaturing gradient of 15–55% to a 290 bp PCR product, a high microbial diversity at the beginning of the storage was observed, which was indicated by the presence of multiple bands (Figures 1 and 2). Individual bands observed in the DGGE profiles, named A to V, were excised from acrylamide gels, re-amplified for sequencing and identified (Table 3).

The spoilage microbiota of sausages consisted of the following microorganisms: *Lactobacillus* species: *L. sakei*, *L. fuchuensis* (or *L. sakei*), *L. plantarum*, *L. brevis*, *L. algidus* and *L. curvatus*; other LAB, such as *Leuconostoc mesenteroides* and *Weissella paramesenteroides*; *Pseudomonas fluorescens*, *Brochothrix thermosphacta*, *Carnobacterium divergens, Janthinobacterium lividum* and *Psychrobacter immobilis*; *Bacillus* species: *B. licheniformis* and *B. subtilis*; and species of the genera *Microbacterium, Enterococcus, Paenibacillus, Vibrio* and *Alcaligenes.*

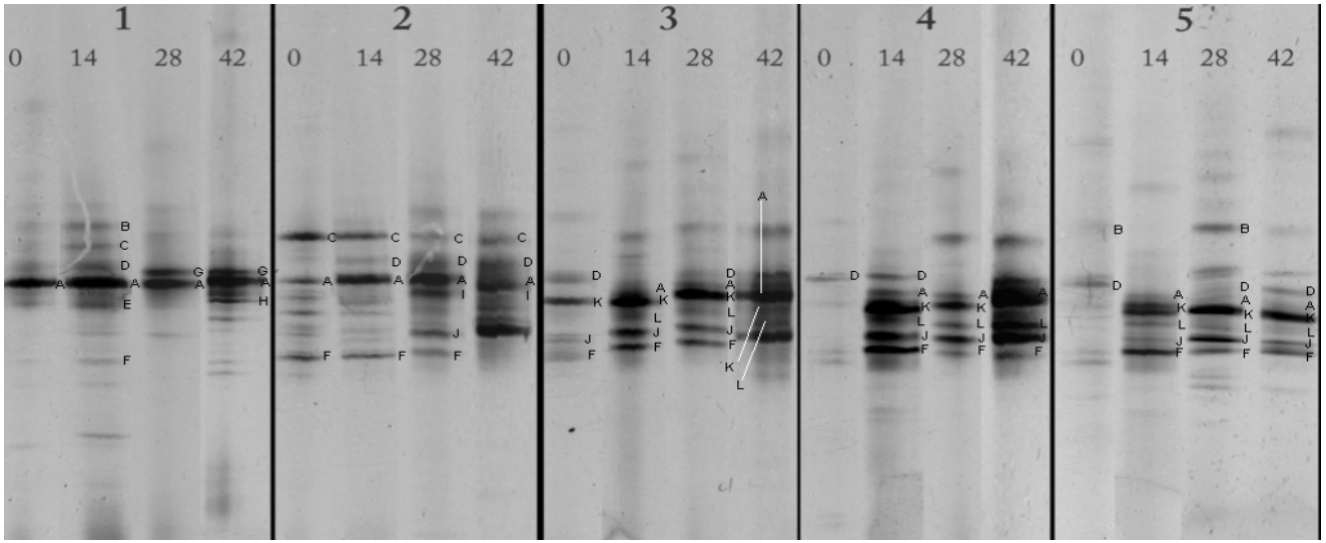

Figure 1 DGGE profiles of the bacterial community from the DNA directly extracted from sausage samples (1 to 5) at the indicated storage times (0, 14, 28 and 42 days). The letters represent the bands that were excised and subjected to sequencing. The letters from A to L correspond to the species listed in Table 3

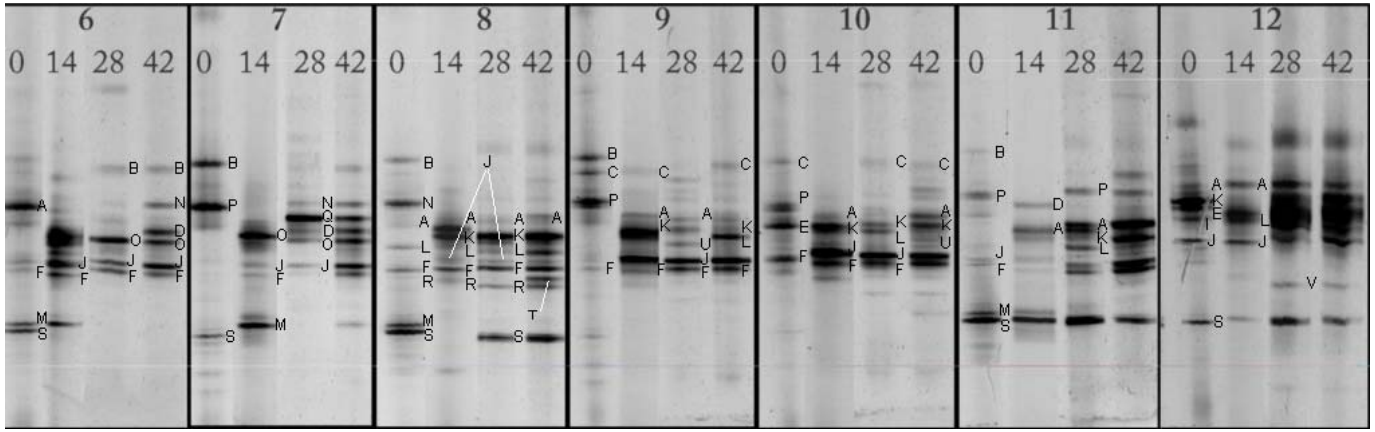

Figure 2 DGGE profiles of the bacterial community from the DNA directly extracted from the sausage samples (6 to 12) at the indicated storage times (0, 14, 28 and 42 days). The letters represent the bands that were excised and subjected to sequencing. Letters from A to V correspond to the species listed in Table 3

Table 3 Species identification of the DGGE band sequences of the V3 region of the 16S rRNA gene of the total bacterial community DNA directly extracted from the sausage samples.

Bands	Closest relatives	ID[a] (%)	Accession No.
A	*Lactobacillus sakei*	99	JF756323.1
B	*Lactobacillus plantarum*	99	GU430799.1
C	*Lactobacillus algidus*	98	AB289024.1
D	*Lactobacillus curvatus*	98	AY383042.1
E	*Carnobacterium divergens*	98	JF756331.1
F	*Brochothrix thermosphacta*	98	JF756334.1
G	*Lactobacillus fuchuensis*	98	AB289024.1
H	*Bacillus licheniformis*	97	HM640420.1
I	*Bacillus subtilis*	97	EU130453.1
J	*Janthinobacterium lividum*	98	HQ003440.1
K	*Psychrobacter immobilis*	97	HQ698589.1
L	*Pseudomonas fluorescens*	99	HM597248.1
M	*Paenibacillus* sp.	100	HM161756.1
N	*Leuconostoc mesenteroides*	98	FR852570.1
O	*Psychrobacter* sp.	99	GQ169116.1
P	*Lactobacillus brevis*	99	JF720006.1
Q	*Weissella paramesenteroides*	98	HQ721270.1
R	*Enterococcus* sp.	98	JF799879.1
S	*Microbacterium* sp.	100	AF390085.1
T	*Bacillus* sp.	97	HQ620634.1
U	*Vibrio* sp.	98	AB038029.1
V	*Alcaligenes* sp.	98	AY346136.1

[a] ID represents the identity with the sequences in the GenBank databases

DISCUSSION

Fresh sausages are highly perishable because of their characteristic pH and a_w values. The microbiology of fresh sausages has only been characterized by the presence of mesophilic, psychrotrophic microorganisms and pathogens thus far. Thus, more detailed studies focusing on the ecology of fresh sausages and the investigation of the population dynamics of these products should be performed (Cocolin *et al*. 2004). However, ecological studies using traditional microbiological methods have been repeatedly criticized because only easily cultivatable microorganisms can be detected, while members that need selective

enrichments for detection or that are in a particular physiological condition (in a sub-lethal or injured state) are unable to be detected (Rantsiou *et al*. 2005). The current study confirmed, by culture-dependent methods, that the LAB population gradually increased and later became the dominant bacterial population. However, as reported by Hu *et al.* (2009), the population of LAB could not be detected (<10 CFU g^{-1}) using culture-dependent methods at day zero for some samples of meat products. Nevertheless, using PCR–DGGE analysis, LAB populations were found in the initial stage of storage, similar to what was observed in our study.

In relation to total mesophilic aerobic counts, it was possible to establish that the sausages analyzed were of high quality. This is because the mesophilic population was $\leq 10^6$ UFC g^{-1}, which is indicative of good manufacturing practices because the products used were raw and not heat treated. According to Gram and Dalgaard (2002), the level of microorganisms detected, ‘‘total count’’, can be used to predict the shelf life of the product.

Even in the presence of high LAB populations, the pH values increased linearly during storage time in seven different sausages sampled. This fact can be explained because it is well established that glucose, lactic acid, and certain amino acids followed by nucleotides, urea and water-soluble proteins are catabolized by almost all the bacteria of the meat microbiota and consequently there was a generation of radicals alkaline (ammonia and amines), contributing to increase in pH values (Nychas *et al.* 2007, 2008). LAB species are able to produce decarboxylases, enzymes with proteolytic activity that generate amines and increase the matrix pH values (Bover-Cid *et al.* 2005). The a_w values did not decrease in any sample, which according to Borch *et al.* (1996) contributes to the stability of LAB.

L. sakei (Band A, Figure 1 and 2) was identified as the predominant spoilage bacterium by PCR–DGGE. *L. sakei* produces ropy slime that confers a

strong competitive ability to this species (Bjorkroth and Korkeala 1997). A specific spoilage phenomenon of commercial significance, characterized by long, stretchy, polysaccharide ropes between sausages or sausage slices, was also detected. *L. sakei* strains play a major role in this spoilage phenomenon. *Lactobacillus curvatus* has also been shown to be a common species in sausages (Korkeala and Björkroth 1997). *L. curvatus* (Band D, Figures 1 and 2) was detected in samples from sausages 1, 2, 3, 4, and 5 (Figure 1), as well as sausage 11 (Figure 2). *Lactobacillus plantarum* (Band B, Figure 1 and 2) was detected in sausages 1, 5, 6, 7, 8, 9 and 11 (Figures 1 and 2). Among LAB, *L. sakei*, *L. curvatus* and *L. plantarum* are the most widely described species in sausages, as also reported by Parente *et al.* (2001).

Lactobacillus algidus (Band C, Figures 1 and 2) was detected in four samples. A previous study reported *L. algidus* as a psychrophilic, predominant strain isolated from vacuum-packaged meat stored at 2 ºC for 3 weeks (Kato *et al.* 2000). In relation to band G (Figure 1), identified as *L. fuchuensis* (or *L. sakei*), this species is phylogenetically close to but distinct from *L. sakei* and also appears to be associated with (vacuum) packaged meat (Sakala *et al.* 2002). Solely based on the DGGE analysis of 16S rDNA amplicons, band G should thus be assigned to *L. sakei* and/or *L. fuchuensis*. It is possible that the use of housekeeping genes in DGGE-based population fingerprinting could result in a higher taxonomic resolution for the separation of closely related species such as *L. sakei* and *L. fuchuensis* (Audenaert *et al.* 2010). Other LAB were detected in this research: *Leuconostoc mesenteroides* (Band N, Figure 2) and *Weissella paramesenteroide* (Band Q, Figure 2) were detected in three and one sample, respectively. In general, the control of growth of spoilage LAB on processed meats is difficult because these bacteria are psychrotrophic, microaerophilic and resistant to nitrite, salt and smoke (Franz *et al.* 1996).

Brochothrix thermosphacta (Band F, Figures 1 and 2) was detected in 11 sausages and was present at almost all the sampling times. It was also a dominant spoilage bacteria in sausages. Both *L. sakei* and *B. thermosphacta* are of the main , if not the most important, cause of spoilage in meat and meat products, which can be recognized as sour off-flavours and off odours, slimy and pack swelling and/or greening (Nychas *et al.* 2008). A possible alternative for the conservation of sausages is to select biopreservative cultures that are able to produce bacteriocins on chilled meat. The inhibition of *B. thermosphacta* in the presence of selected bacteriocin-producing LAB strains was reported by Castellano and Vignolo (2006).

Janthinobacterium lividum (Band J, Figures 1 and 2) was detected in 11 samples and was present for more than one evaluation time, similar to *B. thermosphacta*. *J. lividum* was reported by Nichas *et al.* (2008) and Cavil *et al.* (2011) as the genera of spoilage bacteria commonly found in meat and processed meat. *Pseudomonas fluorescens* was another species detected in our study (Band L, Figures 1 and 2) and was present in eight samples. *Pseudomonas* has been demonstrated as one of the dominant spoilage microbiota in chilled pork (Li *et al.* 2006). In our work, *L. sakei* and *B. thermosphacta* were the most frequently detected spoilage bacteria.

The detection of unknown species of *Psychrobacter* (band O, Figure 2) in two samples and *P. immobilis* (Band K , Figure 1 and 2) in eight samples, in four times evaluated this study in pork sausages, corroborate with the data reported by Gennari *et al*. (1992) who reported the presence of this species in fresh sausage products. Although it has been reported as a spoilage bacterium of low importance in meat, *P. immobilis* is a lipolytic species and might be a cause of incidental infections (Lloyd-Puryear *et al.*1991).

The genus *Bacillus* (Band T, Figure 2) and the species *B. licheniformis* (Band I) and *B. subtilis* (Band H) were found in our study. Only *B. licheniformis*

was detected from the 14th day of storage; *Bacillus* sp. and *B. subtilis* were detected on the 42nd day. *Alcaligenes* and *Vibrio* were detected at the final stage of shelf life, when the pH became alkaline in sausages brand. The genera *Enterococcus*, *Microbacterium* and *Paenibacillus* were detected in few samples from different sampling times. These genera are commonly associated with the deterioration of processed meats (Nychas *et al.* 2008). The genus *Enterococcus* is indicative of fecal contamination; thus, the quality of sanitary hygiene of sausage 8 could be questioned.

PCR-DGGE allowed for the discrimination of fifteen species and seven genera of bacteria that frequently consistute the microbiota in sausage products. The most frequent spoilage bacteria identified from the sausages were *L. sakei* and *B. thermosphacta.* The samples of the sausages showed good sanitary hygienic quality, as the microbiota was composed of only spoilage microorganisms. *Enterococcus*, an indicator of fecal contamination, was detected in only one sample.

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ARTIGO 4

Screening of *Lactobacillus* isolated from pork sausages for potential probiotic use and evaluation of the microbiological safety in fermented product

Normas do periódico *Meat Science*

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RESUMO

O objetivo deste estudo foi selecionar estirpes probióticas de *Lactobacillus* para aplicação em linguiça suína. Estirpes de *Lactobacillus* isoladas de linguiça suína foram avaliadas em testes baseados em característics probióticas e segurança microbiológica. As estirpes UFLA SAU 14, 52 e 91 foram diferenciadas por coagregação com *L. monocytogenes*, produção de ácido lático e autoagregação. Estirpes UFLA SAU 172 e 187 mostraram alta coagregação com *S.* Typhi e *E. coli*, tolerância ao fluido pancreático e adesão ao clorofórmio. UFLA SAU 20 e 34 foram caracterizados pela produção de EPS, sobrevivência a pH 2 e suco intestinal e inibição de *E. coli* e *S.* Typhi. UFLA SAU 185, 238 e 258 foram eficientes na inibição de *L. monocytogenes*, na sobrevivência à bile e na adesão ao xileno. Um coquetel destas 10 estirpes de *Lactobacillus* com potencial probiótico foi inoculo em linguiça suína e melhorou a segurança microbiológica do produto.

Palavras-chave: probiótico, linguiça suína fermentada, *Lactobacillus plantarum,* segurança microbiologica

ABSTRACT

The aim of this study was to select strains of *Lactobacillus* isolated from pork sausage for probiotic use. *Lactobacillus* strains were evaluated in tests based on probiotic characteristics and microbiological safety. The strains UFLA SAU 14, 52 and 91 were differentiated by coaggregation with *Listeria monocytogenes*, production of lactic acid and autoaggregation. Strains UFLA SAU 172 and 187 showed high coaggregation with *S.* Typhi and *E. coli*, tolerance to pancreatic fluid and adhesion to chloroform. UFLA SAU 20 and 34 were characterized by EPS production, survival to pH 2 and intestinal juice and inhibiting the growth of *E. coli* and *S.* Typhi. UFLA SAU 185, 238 and 258 were efficient in inhibiting the growth of *L. monocytogenes* and exhibited resistance to bile and adhesion to xylene. A cocktail of these ten *Lactobacillus* strains with potential probiotic was inoculated in pork sausage and improved the microbiological safety of the product.

Keywords: probiotic, fermented pork sausage, *Lactobacillus plantarum,* microbiological safety.

1. Introduction

Meat has been shown to be an excellent vehicle for probiotics (Rivera-Espinoza & Gallardo-Navarro, 2010). Currently, the main use of microorganisms as probiotics is in the dairy industry, but their application to the meat industry is also promising (De Vuyst, Falony, & Leroy, 2008). Inoculated microorganisms may improve the product's nutritional properties (Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010), the technological and sensory characteristics of sausages and increase the value and reliability of the product (Lücke, 2000). Germany and Japan were the first two countries to incorporate probiotic Lactic Acid Bacteria (LAB) into meat products (Arihara, 2006).

Many of the bacteria used in probiotic preparations (Bifidobacteria and LAB) have been isolated from human fecal samples to maximize the likelihood of compatibility with the human gut microbiota and improve their chances of survival (Andersson et al., 2001). However, LAB isolated from non-dairy fermented foods have shown these abilities in *in vitro* studies (Rivera-Espinoza & Gallardo-Navarro, 2010). Selection of bacteria from meat is advantageous because the microorganisms are adapted to the substrate (or matrix) and possess the appropriate physiological requirements for meat colonization (De Vuyst et al., 2008). Therefore, selected LAB from sausages should be more competitive than LAB isolated from other sources (De Vuyst et al., 2008; Pennacchia et al., 2004; Pennacchia, Vaughan, & Villani, 2006).

Some features important for the selection of new probiotic strains are viability in an artificial simulation of gastrointestinal tract fluid (Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000), low aminogenic potential, organic acid production, antibiotic resistance pattern, hemolytic activity (Ruiz-Moyano et al., 2011), hydrophobicity (Pelletier, Bouley, Bouttier, Bourlioux, & Bellon-Fontaine, 1997), auto and coaggregation abilities (Kos et

al., 2003), production of exopolysaccharides (EPS) (Van Geel-Schutte, Flesch, Brink, Smith, & Dijkhuizen,, 1998) and pathogen inactivation (Lücke, 2000).

The inactivation of pathogens is very important for the microbiological quality of products. In Brazil, pathogens have been detected in pork sausage, including *Salmonella* (Borowsky, Schmidt, & Cardoso, 2007; Mürmann, Santos, & Cardoso, 2009), *E. coli* (Cortez, Carvalho, Amaral, Salotti, & Vidal-Martins, 2004; Marques, Boari, Brcko, Nascimento, & Piccoli, 2006) and *Listeria monocytogenes* (Lima, Von Laer, Trindade, & Silva, 2005; Silva et al., 2004). The use of probiotic cultures can be a new alternative to prevent sausages from serving as the vehicle for pathogenic strains. Therefore, the aim of this study was to investigate the potential of *Lactobacillus* bacteria isolated from pork sausage as a probiotic and to evaluate their safety in meat products.

2. Materials and methods

2.1. Pre-selected strains

Two pre-selection criteria were applied to 567 strains of *Lactobacillus* isolated from pork sausage in Minas Gerais, Brazil. The first selection was by catalase activity: 101 strains possessed catalase activity. The second selection was based on the ability of the strains to tolerate low pH. A total of 32 strains survived low pH and were thus used.

2.2. Simulation of tolerance to the Gastrointestinal Tract (GIT)

To simulate the survival to the GIT, the 32 pre-selected *Lactobacillus* strains were tested in an *in vitro* model that chemically simulates physiological conditions. In the tolerance to low pH test, the pH of MRS broth (Himedia) was adjusted to 2.0 with 1 N hydrochloric acid. In the bile tolerance test, the medium was prepared with MRS broth supplemented with bovine bile (Sigma-Aldrich)

at a concentration of 1.0%. For the pancreatic fluid tolerance test, 150 mM $NaHCO_3$, 1.9 mg/ml pancreatin (Sigma-Aldrich) and pH 8 were used, as suggested by Rönkä et al. (2003). To test the tolerance to intestinal juice, in accordance with Bao et al. (2010), 0.1 g of trypsin (Sigma, Aldrich) and 1.8 g of bile salts were added to a sterile solution of 1.1 g of sodium bicarbonate and 0.2 g of sodium chloride in 100 ml distilled water. The pH of the solution was adjusted to 8.0 with 0.5 M sodium hydroxide and sterilized by filtering through a 0.45 µm membrane.

The strains for each test were initially cultured for 24 h in MRS broth at 37 ºC. After this period, the strains were centrifuged for 5 minutes and washed 3 times in Phosphate Buffered Saline (PBS) pH 7.0. Individual tubes containing each strain and test medium were incubated for 3 h at 37 °C in a water bath. Viability was evaluated in duplicate at time 0 and 3 h on MRS agar (Himedia). Survival rates were calculated according to the following equation:

$$\text{Survival rate (\%)} = \frac{\log \text{CFU } N_1}{\log \text{CFU } N_0} \times 100$$

Where N1 represents the total viable count of strains at time 3 h, and N_0 represents the total viable count of strains at time 0 h.

2.3. Identification of *Lactobacillus* strains

The API 50CH kit (BioMérieux) was used to identify biochemically the pre-selected 32 strains and the final identification was performed using the API LAB Plus software (BioMérieux). The species names were confirmed using molecular identification. Bacterial DNA from each strain was extracted using a QIAamp DNA Mini Kit (Qiagen). The PCR reactions were carried out in a final volume of 50 µl containing 25 µl of TopTaq Master Mix (Qiagen), 1 µl of each primer (27f /1512r), 2 µl of DNA and 21 µl of RNase free water. The unpurified

PCR products were sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. Sequences were then compared to those in the GenBank database using the BLAST algorithm (National Centre for Biotechnology Information, Maryland, USA).

2.4. Hemolysis

The lactobacilli strains were cultured in MRS broth at 37 ºC for 15 h and then transferred onto blood agar (Himedia) plates supplemented with 5% defibrinated whole horse blood (Oxoid). After 48/72 h, the hemolytic reaction was evaluated by observing either the partial hydrolysis of red blood cells and the production of a green zone (α-hemolysis), the total hydrolysis of red blood cells producing a clear zone around bacterial colony (β-hemolysis) or no reaction (γ-hemolysis).

2.5. Decarboxylase activity of the UFLA SAU strains

The decarboxylase activity of the isolated microorganisms was evaluated according to Komprda et al. (2004). The *Lactobacillus* strains were inoculated into a physiological solution. After 24 h, 0.3 ml of the mixture was transferred to a base medium consisting of 0.5 g peptone, 0.3 g yeast extract, 0.3 g glucose, 1 ml bromocresol purple (BCP; 2% in 50% ethanol), 1 g L-amino acids (tryptophan, histidine, ornithine, lysine, phenylalanine and arginine) and 100 ml distilled water. Then, the mixture was overlaid by sterile paraffin oil. After incubation of 1, 4, 24 and 48 h at 37 ºC, the production of a violet or yellow color was considered positive or negative, respectively. A tube with base medium lacking amino acids was used as a negative control.

2.6. Antimicrobial susceptibility

The antimicrobials penicillin G (10 UI/disc), nitrofurantoin (300 µg/disc), teicoplanin (30 µg/disc), vancomycin (30 µg/disc), nalidixic acid (30 µg/disc), chloramphenicol (30 µg/disc), pipemidic acid (20 µg/disc), erythromycin (15 µg/disc), norfloxacin (10 µg/disc), gentamycin (10 µg/disc), ampicillin (10 µg/disc), ciprofloxacin (5 µg/disc), ofloxacin (5 µg/disc), clindamycin (2 µg/disc) and oxacillin (1 µg/disc) were used following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2011). *Lactobacillus* strains were grown on MRS agar for 24 hours at 37 °C. The strains were inoculated in 4 ml of sterile distilled water to achieve the *nº 0.5* McFarland turbidity standard (Probac, Brazil). A swab was used to spread the inoculum across the surface of Muller Hinton agar (Merck), and then antibiotic disks (DME Polisensidisc ® 4x6-Specialized Diagnostic Microbiology, São Paulo, Brazil) were applied to the plate. Strain resistance was assessed by measuring the zone of inhibition of bacterial growth after incubation for 24 h at 37 °C. *Escherichia coli* ATCC 25922 was used for quality control testing.

2.7. Lactic acid production

Strains were grown in MRS broth for 48 hours at 37 ºC, and the quantification of lactic acid was measured by High-Performance Liquid Chromatography (HPLC) equipped with a UV detector operated at 210 nm and a Shim-pack SCR-101H column (7.9 mm x 30 cm). Analysis was performed at 30 °C using 100 mM perchloric acid as the eluent at a flow rate of 0.6 ml/min with a sample volume of 20 µl. Lactic acid was identified by comparing the retention time to an authentic standard. The concentration of lactic acid was determined using a calibration curve obtained by the injection of different concentrations of a lactic acid standard injected under the same conditions used for sample analysis.

2.8. EPS production by *Lactobacillus*

Exopolysaccharide (EPS) production from *Lactobacillus* isolates was tested according to the method described by Van Geel-Schutte et al. (1998), with modifications. Briefly, *Lactobacillus* cultures were grown in conical flasks containing 20 ml MRS broth supplemented with 2% (w/v) glucose at 37 °C for 3 days. Bacterial cells were removed by centrifugation at 6000×g for 20 min and two volumes of 95% (v/v) of cold ethanol (Merck) were added to one volume of culture supernatant for EPS precipitation. Precipitates were recovered by filtration under vacuum, dried at 60 °C and their weight was measured to determine the amount of EPS produced.

2.9. Microbial Adhesion To Solvents (MATS) measurement

MATS was measured according to the method proposed by Pelletier et al. (1997) with modifications. In this study, three solvents (Merck) were tested for adherence to *Lactobacillus* and pathogenic strains; xylene (apolar solvent), chloroform (monopolar and Lewis-acid solvent) and ethyl acetate (monopolar and Lewis-base solvent). The microbial adhesion to xylene, chloroform and ethyl acetate reflect cell surface hydrophobicity as well as the electron donor/basic and electron acceptor/acidic characteristics of bacteria.

The pathogens utilized were: *E. coli* (ATCC 8739), *S.* Typhi (ATCC 6539) and *L. monocytogenes* (ATCC 7644). Stationary phase cells were washed twice in PBS and resuspended in 3 ml of 0.1 M KNO_3 to a final concentration of approximately 10^8 CFU/ml bacteria (cell suspension). One milliliter of each solvent was then added to the cell suspension to form a two-phase system. After a 10 min pre-incubation at room temperature, the two-phase system was mixed by vortexing for 2 min and incubated for 30 min at room temperature to allow phase separation. The aqueous phase (A_t) was carefully removed (200 µl) and added to a microplate (96 wells - Denmark®). The cell suspension (A_0) (200 µl)

was also added to a microplate. The absorbance at 620 nm of each sample was measured (Multiskan FC-ThermoScientific Uniscience), and the percentage of cell surface hydrophobicity (H%) was calculated using the formula: $H\% = (1-A_t/A_0)\times 100$.

2.10. Aggregation activity

2.10.1. Autoaggregation assays

Autoaggregation assays were performed as previously described by Kos et al. (2003), with minor modifications. Briefly, the cells were washed twice with PBS (pH 7.2). The cells were then resuspended in 4 ml to 10^8 CFU/ml by vortexing for 10 s and incubated for 4 h at room temperature. At times 0 and 4 h, 5 µl of the upper suspension was carefully removed, transferred to microplate containing 195 µl of PBS, and the absorbance (*A*) at 620 nm was measured. The autoaggregation percentage was expressed as a function of time until it was constant, using the formula: $1- (A_t/A_0) \times 100$, where A_t represents the absorbance at time t= 4 h and A_0 the absorbance at t=0.

2.10.2. Coaggregation assays of pathogens with *Lactobacillus* strains

The method for preparing the cell suspensions used for testing coaggregation was the same as the autoaggregation assay as suggested by Kos et al. (2003). Equal volumes (2 ml) of each *Lactobacillus* and pathogenic strain (section 2.9) were mixed by vortexing for 10 s. Control tubes were set up at the same time, containing 4 ml of each separate bacterial suspension. The *A* at 620 nm of the suspensions was measured after mixing and after 4 h of incubation at room temperature. Samples were taken in the same way as in the autoaggregation assay. The percentage of coaggregation was calculated using the equation:

$$\text{Coaggregation (\%)} = \frac{((A_{Lactob} + A_{pathog})/2) - A_{mix}}{A_{Lactob} + A_{pathog}} \times 100,$$

where A_{pathog} and A_{Lactob} represent the A620 nm of the separate bacterial suspensions, and A_{mix} represents the absorbance of the mixed bacterial suspension.

2.11. Agar disc diffusion - Antibacterial activity

The inhibitory effect of different strains of *Lactobacillus* over pathogens was tested using the agar disc diffusion method. *S.* Typhi and *E. coli* were grown in Brain Heart Infusion agar (BHI, Merck) and *L. monocytogenes* in Tryptic Soy Agar (Merck) with 0.6% Yeast Extract (Himedia, TSAYE) for 24 h at 37 °C. Each pathogen was suspended in 4 ml of sterile water and standardized to approximately 10^8 CFU/ml, compared to the standard turbidity nº 0.5 of McFarland. A sterile swab was soaked in the suspension and spread on the surface of a plate with BHI agar (*S.* Typhi and *E. coli*) or TSAYE agar (*L. monocytogenes*). After the inoculum was added and allowed to absorb, 6 mm sterile paper filter discs (Whatmann nº1) moistened with 20 µl of cell free supernatant from each strain of *Lactobacillus* in exponential growth phase were added. The supernatants were obtained by centrifugation (2500×g/10min). The susceptibility of pathogens to the discs was assessed by measuring the zone of inhibition of bacterial growth around the discs (radius - mm) after incubation for 24 h at 37 °C.

2.12. Inhibition of pathogens in fermented pork sausage

Pork sausages were manufactured to evaluate the inhibitory action of *Lactobacillus* against pathogens. The sausages were prepared in the laboratory under aseptic conditions using the following formula: 75% lean pork ham, 20% fat pork, 1.5% NaCl, 0.5% Antioxidant Ibracor L600® (IBRAC Additives &

Spices, São Paulo, Brazil), 0.5% Cure/IBRAC® (IBRAC Additives & Spices, São Paulo, Brazil), 1.0% lactose, 0.5% chili pepper and 1% cold water. The mass was divided into three batches of 400 g each. In the first batch (sausage 1st), one inoculum containing a mix of pathogenic bacteria suspended in 15 ml of BHI broth (10^5 CFU/ml) was added as a positive control. In the second batch (sausage 2nd), 15 ml of MRS broth containing 10^5 CFU/ml pathogenic bacteria and *L. plantarum* was added. In the third batch, there was no microbial inoculation: the sausage was the negative control. Each batch was filled into a natural casing with a 26 mm diameter. The sausages were stored at 10 °C for 30 days. Bacterial enumeration, pH and water activity a_w were performed on the days 0, 5, 10, 20 and 30 after preparation of the sausages.

2.12.1. Bacterial enumeration

The enumeration of *Lactobacillus, E. coli, S.* Typhi and *L. monocytogenes* in pork sausages was done using the following culture media: MRS, EMB (Merck), Rambach (Merck), Palcam (Himedia) with *L. monocytogenes* selective supplement (FD061 -5VL- Himedia), respectively. Twenty-five grams was aseptically removed from the central part of each sausage and then homogenized in the stomacher® (Mayo Homogenius HG 400) with 225 ml of 1% peptone water (Himedia). To enumerate *Listeria*, Listeria Enrichment broth (LEB, Himedia) containing selective supplement (FD061 -5VL- Himedia) was used. Serial dilutions were prepared and plates were incubated at 37°C for 24 hours. Typical colonies on each medium were enumerated.

2.12.2. pH value and a_w analysis

The pH value was determinate by homogenizing 10 g of sausage in 100 ml of distilled water using a pH meter (PHS-3B, Labmeter Model PH equipped

with an electrode T818-A, Shanghai, China). The a_w value was measured from 5 g of sausage using an AquaLab model 3 TE (Braseq, São Paulo, Brazil).

2.13. Statistical analysis

All tests were performed in triplicate, except the simulation of tolerance to the gastrointestinal tract, lactic acid production and antimicrobial susceptibility. For EPS and autoaggregation, the data were analyzed using ANOVA, and the means were compared by a Scott-Knott test.

A randomized complete design was used for the coaggregation, antibacterial activity, MATS methods and inhibition of pathogens in fermented pork sausage. For coaggregation and antibacterial activity, treatments were arranged in the factorial 32 X 3: 32 *Lactobacillus* strains, and three pathogenic microorganisms were tested. For the MATS test, the factorial was 35 X 3: 35 strains at three solvents. For measuring the inhibition of pathogens in fermented pork sausage, treatments were arranged in the factorial 2 X 5: 2 sausages (sausage with an inoculum of pathogens and sausage with an inoculum of pathogens plus *L. plantarum*) and 5 time points (0, 5, 10, 20 e 30 days). The data were analyzed using ANOVA and the means were compared by Scott-Knott test. Quantitative data were analyzed using regression. The statistical analysis was performed using SISVAR® (Lavras, Brazil) software, version 4.5.

All *Lactobacillus* properties were analyzed by Principal Component Analysis (PCA) using the software XLSTAT 7.5.2 (Addinsoft, New York, NY, USA).

3. Results and Discussion

3.1. Simulation tolerance of GIT

The results obtained by exposure of strains of *Lactobacillus* to pH 2.0, pancreatic fluid, 1% bile and intestinal juice are reported in Table 1. As a standard of tolerance to GIT, Bao et al. (2010) reported an 80% survival rate by several strains. In our study at pH 2.0, 19 strains showed a survival rate ≥ 90%, eight strains were in the range of 80-89%, four strains were in the range of 70-79% and one strain was in the range of 60-69% in the tolerance test to GIT. The lower rates of survival of the strains when challenged to pH 2.0 was probably due to its high selectivity, which often impedes the growth of many microorganisms (Pennacchia et al., 2004).

In response to pancreatic fluid (Table 1), the survival rate was > 90% for all strains tested. When incubated in 1% bile, 28 strains had a survival rate > 94%, and 4 strains had a survival rate between 87 and 90%. In intestinal juice, the survival rate was > 90% for 24 strains, and 8 strains showed a survival rate in the range of 80-89%. According to Cebeci and Gürakan (2003), the selection of strains of *L. plantarum* in low pH and bile can increase the properties of acid and bile tolerance of the population.

Strains that presented a high survival rate in the *in vitro* simulation of GIT are potential candidates for successfully crossing the human gastrointestinal tract. According to Klingberg and Budde (2006), strains of *L. plantarum* isolated from fermented meats and selected for acid and bile tolerance *in vitro* were able to persist in the human GIT in *in vivo* tests.

Table 1. Number of *Lactobacillus* isolates (total of 32) tolerant to pH 2.0, pancreatic fluid, bile (1%) and intestinal juice.

Survival rate (%)	Nº of isolates surviving at			
	pH 2	Pancreatic fluid	Bile (1%)	Intestinal juice
100 ≥ % ≥ 90	19	32	28	24
89 ≥ % ≥ 80	8	-	4	8
79 ≥% ≥ 70	4	-	-	-
69 ≥ % ≥ 60	1	-	-	-

3.2. Identification of *Lactobacillus* strains

The isolates identified by the API 50CHL test as *L. plantarum*-group were identified with 99% similarity by 16S rRNA gene sequencing as *L. plantarum* (AB603688.1, AB510750.1, EU419598.1, HM130542.1 and HM562999.1) (data no shown). Thirty-one strains were identified as *L. plantarum*. The strain UFLA SAU 130 was identified by the API 50CHL test as a member of the *Lactobacillus casei* group and was confirmed by molecular identification as *L. paracasei* (HM462419.1). *L. plantarum* and *L. paracasei* have potential applications for probiotic use in innovative starter cultures in meat products (Pennacchia et al., 2006).

3.3. Safety aspects and probiotic features of UFLA SAU strains

In the hemolysis tests, all strains exhibited γ-hemolytic activity when grown in horse blood agar. The determination of hemolytic activity is required in recognition of the importance of assuring safety, even among a group of bacteria that is Generally Recognized as Safe (GRAS) (Joint FAO/WHO, 2002).

The isolates showed no decarboxylation activity on the decarboxylase medium containing amino acids. This was a pleasant result because the decarboxylation of amino acids is not a desirable feature of candidates for probiotic use. The microbial decarboxylation of amino acids produces an

accumulation of biogenic amines in food and has toxicological implications to consumers (Latorre-Moratalla et al. 2007).

Table 2 shows the antibiotic susceptibility of the 32 *Lactobacillus* strains in this study; 16 strains were susceptible to only one antibiotic, three strains were susceptible to two antibiotics, seven strains were susceptible to three antibiotics and six strains were susceptible to four of the antibiotics tested. Thirty strains were susceptible to erythromycin, which is the antibiotic usually active against the *Lactobacillus* species studied (Cebecci, & Gürakan, 2003; Ruiz-Moyano et al. 2009). Six strains showed the same profile of susceptibility to four antibiotics: erythromycin, ampicillin, chloramphenicol and gentamycin (Table 2). The investigation of the resistance pattern of *Lactobacillus* strains is important because the commercial introduction of probiotics containing antibiotic resistance strains, the genes encoding antibiotic resistance can be transferred to intestinal pathogens (Mathur, & Singh, 2005).

The average lactic acid production by all of the *Lactobacillus* strains was 13.28 g/l. Twenty strains produced lactic acid above the average (19.81 g/l), and 12 strains produced lactic acid below the average (<2.65 g/l) (Table 2). Ruiz-Moyano et al. (2009) identified cultures with lactic acid production ranging from 16-21% as a potential probiotic for manufacture of Iberian sausages. Additionally, Saarela et al. (2000) demonstrated that the ability of lactobacilli strains to produce lactic acid or other low molecular weight metabolites might show a wide inhibitory spectrum against many harmful organisms.

The difference in EPS production among the *Lactobacillus* strains was not significant ($P>0.05$), and the average production was 21.87 mg/l (Table 2). The *Lactobacillus* strains did not show good production of EPS. Van Geel-Schutte et al. (1998) reported that strains of *Lactobacillus* produced EPS molecules in relatively large amounts (>100 mg/l), predominantly in media containing glucose. Additionally, Badel, Bernardi and Michaud (2011) showed

that the optimization to production of EPS depends on the carbon and nitrogen sources as well as the physico-chemical conditions for bacterial growth. The optimization of the EPS production by LAB is important because, according to De Vuyst and Degeest (1999), exopolysaccharide contributes to the formation of bacterial cell aggregates and adhesion to the surface, thereby facilitating colonization in various ecosystems.

Table 2 Antimicrobial susceptibility, lactic acid (g/l) and exopolysaccharides (EPS) (mg/l) production of *Lactobacillus* strains

Strains UFLA SAU	Antimicrobial susceptibility[1]	Lactic acid (g/l)	EPS[2] (mg/l)
1	eri	19.81	26.33
11	eri-amp-gen-clo	11.21	26.00
14	eri	16.89	26.33
18	eri	17.33	21.33
20	eri-amp-gen-clo	16.79	29.00
34	eri-amp-gen-clo	13.64	35.00
52	eri	15.66	20.00
73	eri	15.66	32.00
86	eri-amp-gen-clo	15.45	23.33
87	eri	17.24	25.67
91	eri- gen	18.23	19.00
101	eri	14.22	22.00
125	eri	14.98	10.33
127	eri	5.47	13.67
130	eri	10.93	34.00
131	eri-amp-gen-clo	6.72	19.67
132	eri-gen-clo	14.54	26.67
135	amp-gen-clo	12.17	14.67
145	eri-amp	13.62	17.00
172	eri	13.48	10.67
185	eri	12.10	22.00
186	eri	11.99	21.33
187	eri	17.40	19.33
204	eri-gen-clo	2.65	25.00
213	eri-amp-gen	8.70	25.67
217	eri	7.41	30.33
220	eri-amp-gen	13.87	25.67
226	eri-amp-clo	14.92	20.00
238	eri-amp-clo	8.05	18.33
245	clo	11.65	21.00
258	eri-amp-gen-clo	18.22	15.67
265	eri-clo	13.87	2.80
Average	-	13.28	21.87

[1] eri= erythromycin, amp= ampicillin, gen= gentamycin, clo= chloramphenicol
[2]There was not difference statistical by Scott–Knott test

The *Lactobacillus* strains showed a range of percent adherence to the apolar solvent (Table 3). The percentage values ranged from 10.4 to 54.75. Among the strains, UFLA SAU 132 showed the highest percentage (54.75) of adherence to xylene, whereas other strains showed lower percentages of adherence to this apolar solvent, which has a hydrophilic surface (UFLA SAU 14, 18 and 91). *Lactobacillus* showing an affinity to an apolar solvent above 40% generally presented more elevated hydrophobic characteristics (Giarous, Chapot-Chartier, & Briandet, 2009). In this study, five *L. plantarum* strains presented a hydrophobic surface (UFLA 11, 125, 132, 220 and 258). According to Del Re, Sgorbati, Miglioli and Palenzona (2000) and Giarous et al. (2009), strains should present a hydrophobic surface for a high capacity of adhesion to intestinal cells and solid materials.

In this study, the percentage of adhesion of pathogens to solvents was tested for comparison with *Lactobacillus* (Table 3). Compared to lactobacilli, *L. monocytogenes* showed a higher ability to adhere to xylene, an apolar solvent (64.61%); this high percentage of adhesion to xylene can be justified because the bacteria possess the ability to form biofilms. Adhesion, facilitated by bacterial cell surface hydrophobicity, is defined as the first phase of biofilm formation (Tresse, Lebret, Benezech, & Faille, 2006). *E. coli* and *S.* Typhi showed percentages of adherence to xylene that were slightly higher than the average of the UFLA SAU strains (Table 3).

There was an interaction between the strains and solvents tested ($P < 0.05$). Twenty-nine strains of *Lactobacillus* (Table 3), as well as the pathogenic strains tested, showed a strong overall affinity to chloroform, an acidic solvent and electron acceptor, and a low affinity for ethyl acetate, a basic solvent. Lactobacilli and the pathogens have a strong basic and a weak acidic characteristics; thus, the strains in this study are strong electron donors and weak electron acceptors. In the MATS test, almost all of the strains were electron

donors because their affinity to the Lewis-acid chloroform was higher than that to the apolar solvent. These results were similar to those reported by Giaouris et al. (2009) who analyzed *Lactobacillus lactis* strains isolated from animal and vegetables.

The autoaggregation ability of the strains ranged from 26.99 to 77.2% (P<0.05). The average autoaggregation of *Lactobacillus* strains was 44.90% (Table 4). The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains used as probiotics. Aggregation is a phenotype related to cell adherence properties (Kos et al., 2003; Peletier et al., 1997). Our strains showed significant autoagreggation with values above 10%. Strains with values lower than 10% are designed as non-autoaggregating (Del Re et al., 2000). In general, probiotic strains should show higher autoaggregation capabilities than pathogenic strains (Collado, Meriluoto, & Salminen, 2007). Compared to the capacity of autoaggregation of pathogens, 31 and 18 *Lactobacillus* strains were more efficient than *E. coli* and *S.* Typhi, respectively. Strain UFLA SAU 52 was the only strain to show a greater capacity to autoaggregate than *L. monocytogenes*.

Table 3 Percent (%) adhesion of *Lactobacillus* and pathogenic strains to the three solvents: xylene, ethyl acetate and chloroform[1]

Strains UFLA SAU	Xylene (%)	Ethyl acetate(%)	Chloroform (%)
1	38.91^{eB}	19.27^{bA}	80.95^{hC}
11	50.54^{gA}	75.27^{iB}	80.91^{hC}
14	10.41^{aA}	74.54^{iC}	63.38^{eB}
18	11.35^{aA}	75.25^{iC}	69.29^{fB}
20	28.28^{cA}	28.35^{dA}	41.00^{bB}
34	16.38^{bA}	28.24^{dB}	43.65^{bC}
52	28.12^{cB}	20.94^{bA}	60.96^{eC}
73	29.70^{cA}	43.53^{gB}	59.98^{eC}
86	25.69^{cA}	29.11^{dA}	67.97^{fB}
87	26.68^{cB}	12.34^{aA}	35.34^{aC}
91	11.30^{aA}	21.05^{bB}	49.42^{cC}

Table 3, continuation

101	32.76^{dB}	24.24^{cA}	56.29^{dC}
125	42.45^{fB}	34.31^{eA}	75.22^{gC}
127	36.29^{eA}	32.58^{eA}	55.77^{dB}
130	31.57^{dA}	30.63^{dA}	59.72^{eB}
131	27.80^{cA}	33.41^{eB}	63.74^{eC}
132	54.75^{hB}	30.16^{dA}	74.62^{gC}
135	38.98^{eA}	38.22^{fA}	64.37^{eB}
145	31.01^{dA}	35.51^{eB}	78.86^{hC}
172	32.44^{dA}	77.34^{iC}	62.53^{eB}
185	38.78^{eB}	34.18^{eA}	65.46^{fC}
186	33.20^{dA}	37.46^{fB}	42.97^{bC}
187	35.15^{dA}	35.28^{eA}	85.98^{iB}
204	39.83^{eA}	39.14^{fA}	65.76^{fB}
213	37.58^{eB}	27.11^{cA}	63.80^{eC}
217	38.54^{eB}	24.47^{cA}	41.32^{bB}
220	46.03^{fB}	27.90^{dA}	67.96^{fC}
226	31.89^{dB}	26.90^{cA}	43.60^{bC}
238	33.36^{dB}	26.37^{cA}	51.43^{cC}
245	33.05^{dA}	35.12^{eA}	64.88^{fB}
258	43.02^{fB}	29.48^{dA}	88.44^{iC}
265	30.89^{dA}	44.14^{gB}	62.28^{eC}
Average *Lactobacillus*	32.71^{A}	35.98^{A}	62.12^{B}
E. coli	34.52^{dA}	34.27^{eA}	66.12^{fB}
S. Typhi	39.49^{eA}	37.63^{fA}	74.54^{gB}
L. monocytogenes	64.61^{iB}	60.17^{hA}	94.98^{jC}

Mean values bearing the same superscript in upper (rows) or lower (columns) case letters are not significantly different ($P<0.05$) according to the Scott- Knott test
[1] SE= 1.37

There was an interaction ($P<0.05$) between the lactobacilli strains and the three pathogenic strains in the coaggregation tests (Table 4). All of the strains coaggregated with the pathogens except strain UFLA SAU 132, which did not show any coaggregation with the pathogens tested. The coaggregation abilities of the *Lactobacillus* species with potential pathogens might prevent the colonization of the gut by pathogenic bacteria (Bao et al., 2010). Thus, probiotic

strains should show the ability to coaggregate with the pathogenic strains tested, but the percentage of coaggregation is strain-specific (Collado et al., 2007). In our study, strains of *Lactobacillus* UFLA SAU 185, 91 and 52 showed greater coaggregation with *E. coli*, *S.* Typhi and *L. monocytogenes*. In relation to the pathogenic strains tested, the UFLA SAU strains showed the highest average coaggregation with *Listeria monocytogenes*. This property may be related to the formation of a mixed species biofilm. Mixed species biofilms of *L. monocytogenes* and *L. plantarum* have been reported by Veen and Abee (2011).

The *Lactobacillus* strains were examined for their antimicrobial activity against potentially pathogenic bacteria (Table 5). The statistical analysis showed the interaction among strains of *Lactobacillus* against the pathogens tested. *L. monocytogenes* (P=0.05) was more sensitive to the *Lactobacillus* strains, and the highest inhibitory activity against this pathogen was presented by strains UFLA SAU 135, 226, 238 and 258. These results confirmed that antagonistic substances produced by lactobacilli are active against Gram-positive bacteria (Aymerich, Garriga, Monfort, Nes, & Hugas, 2000). The inhibitory action of the UFLA SAU strains to *E. coli* and *S.* Typhi was very low (average halo: 2.02 mm), and no significant differences were found for either pathogen (Table 4). Ruiz-Moyano et al. (2009) reported that *Lactobacillus* strains do not inhibit Gram-negative bacteria; however, they showed moderate or high antimicrobial activity against strains of *L. monocytogenes*.

Table 4 Percent (%) autoaggregation[1] and coaggregation[2] of 32 strains of *Lactobacillus*

Strains UFLA SAU	Autoaggregation (%)	Coaggregation *E. coli* (%)	Coaggregation *S.*Typhi (%)	Coaggregation *L.monocytogenes* (%)
1	50. 09^{w}	29.29^{bA}	36.67^{bA}	18.73^{bA}
11	43.32^{s}	36.38^{bB}	0.00^{aA}	39.76^{cB}
14	41.76^{o}	39.83^{bB}	25.82^{bA}	50.16^{cB}
18	41.21^{m}	27.67^{bA}	40.28^{bA}	58.91^{dB}
20	42.57^{q}	0.00^{aA}	10.24^{aA}	31.00^{bB}
34	39.04^{i}	35.73^{bA}	23.20^{bA}	27.9^{bA}
52	77.20^{af}	23.45^{bA}	19.89^{bA}	70.07^{dB}
73	34.98^{f}	0.00^{aA}	0.00^{aA}	36.32^{cB}
86	57.35^{aa}	28.76^{bA}	31.52^{bA}	40.35^{cA}
87	58.37^{ac}	34.29^{bA}	31.25^{bA}	52.83^{dB}
91	42.85^{d}	25.77^{bA}	42.01^{bB}	19.35^{bA}
101	57.81^{ab}	30.89^{bA}	26.98^{bA}	43.43^{cA}
125	26.99^{a}	20.09^{bA}	32.70^{bA}	26.16^{bA}
127	38.62^{r}	23.17^{bA}	15.79^{aA}	44.14^{cB}
130	33.03^{e}	29.12^{bA}	22.36^{bA}	36.86^{cA}
131	39.18^{i}	37.42^{bA}	27.31^{bA}	43.50^{cA}
132	58.81^{ad}	0.00^{aA}	0.00^{aA}	0.00^{aA}
135	42.26^{p}	38.18^{bA}	31.02^{bA}	29.21^{bA}
145	53.55^{y}	34.35^{bA}	38.31^{bA}	40.25^{cA}
172	40.92^{l}	34.03^{bA}	34.86^{bA}	43.82^{cA}
185	33.55^{d}	43.08^{bA}	25.64^{bA}	24.88^{bA}
186	36.32^{g}	42.34^{bA}	28.25^{bA}	42.31^{cA}
187	40.67^{k}	33.33^{bA}	40.59^{bA}	46.38^{cA}
204	55.47^{z}	27.92^{bA}	31.25^{bA}	40.26^{cA}
213	49.02^{v}	35.23^{bA}	29.26^{bA}	14.26^{bA}
217	45.63^{t}	34.36^{bA}	34.82^{bA}	32.00^{bA}
220	46.88^{u}	37.49^{bA}	29.25^{bA}	63.61^{dB}
226	53.27^{x}	10.47^{aA}	0.00^{aA}	0.00^{aA}
238	45.58^{t}	18.23^{bA}	16.69^{aA}	19.04^{bA}
245	34.24^{e}	34.16^{bA}	24.23^{bA}	46.05^{cA}
258	39.99^{j}	35.42^{bA}	30.01^{bA}	23.49^{bA}
265	38.53^{h}	20.27^{bA}	22.08^{bA}	20.17^{bA}
Average *Lactobacillus*	44.90	28.21^{A}	25.07^{A}	35.16^{B}
E. coli	28.70^{b}	-	-	-
S. Typhi	41.49^{n}	-	-	-
L. monocytogenes	62.24^{ae}	-	-	-

Mean values bearing the same superscript in upper (rows) or lower (columns) case letters are not significantly different (P <0.05) according to the Scott- Knott test

[1] SE= 0.07

[2] SE= 6.77