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CARACTERIZAÇÃO, BIOACUMULAÇÃO E ESPECIAÇÃO DE SELÊNIO NA BIOMASSA DE *Enterococcus durans*

SIMONE PIENIZ

Porto Alegre, Rio Grande do Sul, Brasil
Março de 2013

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"A persistência é o menor caminho para o êxito".

(Charles Chaplin)

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Caracterização, bioacumulação e especiação de selênio na biomassa de *Enterococcus durans*[1]

Autora: Simone Pieniz

Orientador: Adriano Brandelli

RESUMO

O objetivo deste estudo foi caracterizar o potencial probiótico, antimicrobiano e antioxidante, bem como analisar os genes envolvidos em relação aos fatores de virulência e de resistência. Do mesmo modo, objetivou-se avaliar a capacidade de bioacumulação e a especiaçao de selênio (Se(IV)) na biomassa microbiana. O micro-organismo utilizado neste estudo foi isolado de queijo "Minas Frescal" e caracterizado por métodos fenotípicos (Vitek® 2 system) e moleculares (sequenciamento do gene 16s rRNA e análise pelo gene *ddl* (D-Ala–D-Ala ligase)). O isolado LAB18s foi identificado fenotipicamente e genotipicamente como *Enterococcus durans*. Além disso, apresentou características probióticas; atividade antimicrobiana e capacidade antioxidante. Quando analisados os genes relacionados aos fatores de virulência (*ace*, *agg*, *asa*, *bopA*, *bopB*, *bopC* e *bopD*), e de resistência à vancomicina (*vanA*, *vanB*, v*anC*1 e *vanC2/3*) não foi verificada a presença destes genes pela *E. durans* LAB18s. Da mesma forma, o isolado mostrou-se sensível aos antibióticos testados (eritromicina, tetraciclina, vancomicina, gentamicina e penicilina) e apresentou ainda capacidade de adesão e hidrofobicidade, assim como forte capacidade para a formação de biofilme. Para a bioacumulação de Se(IV) pelo isolado *E. durans* LAB18s foram encontrados como parâmetros ótimos de crescimento a temperatura de 30°C e pH 7. Através da especiação foi demonstrado que em todas as análises o isolado *E. durans* LAB18s pode bioacumular concentrações consideráveis de Se(IV), sendo que a maior percentagem de selênio orgânico foi encontrada na fração de proteínas totais e, quando analisada as extrações por diferentes solventes, observou-se maior conteúdo de selênio em meio alcalino. As análises de Microscopia Eletrônica de Varredura (MEV), Microscopia Eletrônica de Transmissão (MET) e Espectrometria de Energia Dispersiva (MEV-EDS) corroboraram com os resultados obtidos em termos de bioacumulação de selênio pelo *E. durans* LAB18s. De acordo com os resultados obtidos pode-se inferir que o isolado *E. durans* LAB18s pode ser considerado um micro-organismo com potencial probiótico, biologicamente seguro e com ampla capacidade em bioacumular selênio na biomassa microbiana.

Palavras-chave: *Enterococcus durans*; selênio; caracterização probiótica; bioacumulação; especiação.

[1]Tese de Doutorado em Microbiologia Agrícola e do Ambiente (Área de concentração: Microbiologia Industrial), Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil (154p.), Março de 2013.

Characterization, bioaccumulation and speciation of selenium in biomass of *Enterococcus durans*[1]

Author: Simone Pieniz

Advisor: Adriano Brandelli

ABSTRACT

The aim of this study was to characterize the potential probiotic, antimicrobial and antioxidant activity as well as analyze the genes involved in virulence factors and resistance. Similarly, it was aimed to evaluate the ability of selenium (Se (IV)) bioaccumulation and speciation through the microbial biomass. The micro-organism used in this study was isolated from "Minas Frescal" cheese and characterized by phenotypic (Vitek® 2 system) and molecular methods (16S rRNA gene sequencing and analysis by gene *ddl* (D-Ala-D-Ala ligase)). The isolate LAB18s was identified phenotypically and genotypically as *Enterococcus durans*. Moreover, it exhibited probiotic characteristics, antimicrobial and antioxidant activity. The genes related to virulence factors (*ace*, *agg*, *asa*, *bopA*, *bopB*, *bopC* e *bopD*), and vancomycin resistance (*vanA*, *vanB*, v*anC*1 e *vanC2/3*) were not verified the presence of these genes by LAB18s. Likewise, the isolate showed susceptibility to the antibiotics tested (erythromycin, tetracycline, vancomycin, gentamicin and penicillin) and also it showed adhesion and hydrophobicity ability, as well as a strong ability to form biofilms. For the Se(IV) bioaccumulation by isolated *Enterococcus durans* LAB18s have been found as optimal parameters growth by isolate at temperature of 30°C and pH 7. Analysis for Se(IV) speciation had shown in all analyzes that the isolate *E. durans* LAB18s can bioaccumulate considerable concentrations of Se(IV), and the highest percentage of organic selenium was found in the fraction of total protein; and when analyzed different solvent extractions, it was observed a higher content of selenium in alkaline medium. Analyses from Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Energy Dispersive Spectroscopy (SEM-EDS) corroborate with the results obtained with Se(IV) bioaccumulation by *E. durans* LAB18s. In according to the obtained results it can be infer that the isolated *E. durans* LAB18s can be considered a micro-organism with potential probiotic, biologically safe and with wide capacity to bioaccumulate selenium in microbial biomass.

Keywords: *Enterococcus durans*; selenium; probiotic characterization; bioaccumulation; speciation.

[1]*Doctoral Theses in Agricultural and Environmental Microbiology (Major: Industrial Microbiology), Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil (154p.), March 2013.*

SUMÁRIO

LISTA DE ABREVIATURAS

BAL – bactérias ácido lácticas
GRAS – generally recognized as safe
TGI – trato gastrointestinal
DNA – ácido desoxirribonucléico
RNA – ácido ribonucléico
ORFs – *open reading frames*
GSH-Px – glutationa peroxidase
DMB – doença do músculo branco
SeMet – selenometionina
SeCis – selenocisteína
Na_2SeO_3 ou Se(IV) – selenito de sódio
T3 – tri-iodotironina
T4 – tiroxina
AVC – acidente vascular cerebral
LDL – β-lipoproteína de baixa densidade
dNTPs – desoxirribonucleotídeos fosfatados
Se – selênio
TrxR – tioredoxina redutase
SeP – selenoproteína P
FAD – flavina adenina dinucleotídeo
NADPH – nicotinamida adenina dinucleotídeo fosfato reduzido
NF-κB – fator de transcrição
H_2O_2 – peróxidos de hidrogênio
Hg – mercúrio
ANVISA – agência nacional de vigilancia sanitária
IDR – ingestão diária recomendada
NRC – national research rouncil
Na_2SeO_4 – selenato de sódio
AIDS – síndrome de imunodeficiência adquirida
Se^0 – selênio elementar
ddl – D-Ala–D-Ala ligase
BHI – infusao de cérebro e coração
BHA – agar infusao de cérebro e coração
CFU – unidades formadoras de colônias
PBS – tampão fosfato salina
NaCl – cloreto de sódio
OD ou DO – densidade óptica
HCl – ácido cloridríco
NaOH – hidróxido de sódio
TBARS – substâncias reativas ao ácido tiobarbitúrico
ABTS•+ – ácido 2,2'-azinobis-(3-etilbenzotiazolina-6-sulfônico)
DPPH – 2,2-difenil-1-picrilhidrazil
EC_{50} – concentração requerida para reduzir em 50% o DPPH inicial
*bop*A – putative glycosyltransferase
*bop*B – beta-phosphoglucomutase
*bop*C – aldose 1-epimerase
*bop*D – sugar-binding transcriptional regulator

ace – proteína de adesão do colágeno
agg e *asa* – substâncias de agregação
$MgCl_2$ – cloreto de magnésio
CLSI – instituto de padrões clínicos e laboratoriais
ERI – eritromicina
TET – tetraciclina
VAN – vancomicina
GEN – gentamicina
PEN – penicilina
MIC – concentração inibitória mínima
VRE – *Enterococcus* resistentes a vancomicina
ICP-OES – espectrometria de emissão óptica com plasma indutivamente acoplado
SDS-PAGE – eletroforese desnaturante em gel de poliacrilamida
SEM ou MEV – microscopia eletrônica de varredura
TEM ou MET – microscopia eletrônica de transmissão
SEM/EDS ou MEV/EDS – microscopia eletrônica de varredura e espectrometria de energia dispersiva
kDa – quilodalton
C – carbono
N – nitrogênio
O – oxigênio
Na – sódio
K – potássio
Zn - zinco
Si – silício
H – hidrogênio
He – hélio
Li - lítio

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1. INTRODUÇÃO

O desenvolvimento de novos produtos alimentícios torna-se cada vez mais desafiador, à medida que procura atender à demanda dos consumidores por produtos que, concomitantemente, sejam saudáveis e atrativos. Produtos probióticos têm sido amplamente comercializados, visto a busca constante por uma alimentação saudável e equilibrada, que forneça nutrientes básicos à dieta e possua componentes ou substâncias que desempenhem papel benéfico à saúde do hospedeiro, por incremento das propriedades da microbiota nativa. O desenvolvimento de produtos probióticos, cientificamente válidos, requer um conhecimento precedente, amplo e detalhado dos micro-organismos a serem utilizados, visto que a eficácia do produto é estritamente dependente da quantidade e características das linhagens utilizadas na elaboração do produto probiótico.

Vários micro-organismos têm sido utilizados como probióticos, entre eles bactérias ácido lácticas (BAL), bactérias não ácido lácticas e leveduras. BAL têm sido investigadas cientificamente visando à produção de derivados lácteos, produtos fermentados e aditivos alimentares, devido à capacidade de produzirem compostos antimicrobianos, apresentarem atividade antitumoral, promoverem redução do colesterol, redução da intolerância à lactose, estimulação do sistema imunológico, estabilização da microflora intestinal, dentre outros efeitos benéficos. Estes micro-organismos possuem longa história de uso seguro em produtos alimentícios (GRAS - Generally Recognized as Safe) sendo reconhecidos e largamente empregados como probióticos.

Contudo, para que micro-organismos probióticos, sejam empregados na alimentação humana ou animal, é necessária a investigação de alguns parâmetros básicos. Devem ser inócuos; manterem-se viáveis durante a

estocagem e transporte; sobreviverem ao pH do lúmen gástrico; à ação da bile e às secreções pancreática e intestinal. Além disso, não serem transportadores de genes transmissores de resistência e de virulência; competir por sítios de adesão, impedindo aderência de outros micro-organismos patógenos e, exercer benefícios ao sistema imunológico e a saúde em geral do hospedeiro.

Devido aos avanços tecnológicos nas áreas de genética e nutrição, a avicultura vem buscando excelência na produção animal, aliada ao baixo custo e qualidade dos produtos finais, visando atender desta forma, a demanda e as exigências dos consumidores. Deste modo, a busca por nutrientes que permitam o aumento da produtividade animal, aprimore a qualidade dos produtos finais e reduzam os custos de produção, sem prejudicar o desempenho animal e, ainda, minimizando o impacto ambiental, é constante.

A pouca diversidade da microbiota intestinal indígena de aves recém nascidas, além de ser um fator limitante para a digestão, possibilita a colonização intestinal por bactérias enteropatogênicas. Os efeitos negativos desse processo têm sido contornados com uso indiscriminado de antimicrobianos nas rações de cria, recria, engorda e terminação dos frangos de corte. Entretanto, o uso de antimicrobianos no controle da microbiota intestinal gram-positiva e gram-negativa é questionável, uma vez que o resíduo deixado no leite, ovos e demais produtos de origem animal tem sido associado com a indução de multiresistência aos antimicrobianos, além de reação de hipersensibilidade e surgimento de neoplasias em humanos. Tais resistências podem ocorrer não apenas em bactérias patogênicas, que podem ser transferidas a partir de produtos de aves para a população humana, mas também em bactérias comensais, o que constitui um reservatório de genes de resistência para bactérias patogênicas. Desta forma, pesquisas têm sido focadas principalmente em incorporar na alimentação animal, substâncias derivadas de plantas, animais, bactérias e fungos, assim como ácidos orgânicos e óleos essenciais.

Com a finalidade de atender a este mercado consumidor sensibilizado com a situação e em franca ascensão, estuda-se alternativas que visam à substituição dos antibióticos das rações por culturas probióticas, sem causar redução na produtividade avícola e, da mesma forma, sem causar aumento nos

custos de produção. Dentre estas alternativas destaca-se o uso de probióticos, como o emprego de BAL na alimentação animal, considerando uma alternativa eficaz para a melhoria das condições animais, aprimorando o crescimento e as características de produção, sem deixar resíduos prejudiciais na carne. Concomitantemente com a utilização dos probióticos, tem-se administrado micronutrientes que beneficiam a saúde e o desempenho animal. O selênio (Se), considerado um micronutriente essencial, tem sido utilizado na elaboração de aditivos probióticos com potencial aplicação na alimentação animal. Muniz-Naveiro et al. (2005) sugerem que o selênio orgânico é um aditivo ideal, pois os animais e os seres humanos, absorvem e retém maiores concentrações deste, do que o selênio na forma inorgânica.

Diante do exposto, este trabalho teve por objetivo investigar uma linhagem de *Enterococcus*, isolado de queijo “Minas Frescal”, quanto a sua característica probiótica, segurança em termos de virulência e resistência, bem como quanto à capacidade de bioacumulaçao de selênio na biomassa bacteriana. Futuramente, após a caracterização e elaboração deste composto probiótico enriquecido com selênio (considerada uma forma orgânica de selênio), pretende-se aplicar este produto na alimentação animal, como por exemplo, em frangos de corte. Supõe-se, que a bioacumulação de selênio na biomassa bacteriana, pode ser de grande contribuição para a alimentação animal, bem como ser uma alternativa eficaz e economicamente viável na criação de frangos de corte, almejando a obtenção de benefícios significativos para os animais como melhores condições físico-químicas, melhor desempenho, melhor perfil lipídico e bioquímico, maior ganho de peso, melhor índice de conversão alimentar, maior rendimento de carcaça, dentre outros.

2. REVISÃO BIBLIOGRÁFICA

2.1 *Enterococcus*

Enterococcus, um gênero pertencente ao grande grupo das bactérias ácido lácticas (BAL), são cocos Gram-positivos que geralmente se dispõem em pares e em cadeias curtas. São catalase-negativos, anaeróbios facultativos, e possuem crescimento entre as temperaturas de 10°C a 45°C, sendo que a temperature ótima de crescimento é de 35°C (Cai, 1999; Franz et al., 2011).

Os *Enterococcus* compartilham muitas características com os gêneros *Streptococcus* e *Lactococcus*, e apesar de sua morfologia tipicamente formada por cocos, os *Enterococcus* distinguem-se dos *Streptococcus* e *Lactococcus* principalmente pela sua capacidade de crescimento em meio contendo cloreto de sódio (NaCl) 6,5%, toleram a presença de 40% de sais biliares e, por hidrolisar a esculina (Facklan et al., 1999).

Os *Enterococcus* possuem importância tanto na fermentação de alimentos quanto na deterioração dos alimentos, ou seja, podem ser responsáveis pela deterioração de produtos cárneos coccionados, porém, ao contrário, podem contribuir para a maturação e o desenvolvimento do aroma de certos queijos e produtos cárneos fermentados, devido às atividades glicolíticas, proteolíticas e lipolítica (Settanni; Corsetti, 2008; Gomes et al., 2010). Da mesma forma, certas espécies de *Enterococcus* também têm sido utilizadas como culturas probióticas como adjuvante no tratamento de doenças em humanos (Aggarwal et al., 2012) e animais (Huff et al., 2006). Entretanto, outras espécies de *Enterococcus*, estão associadas a infecções nosocomiais e são causas de doenças humanas como endocardite, bacteremia ou infecções

do trato urinário, além disso, algumas linhagens patogênicas muitas vezes são carreadoras de resistência múltipla aos antibióticos e fatores de virulência (Gomes et al., 2010). Desse modo, uma seleção criteriosa da linhagem de *Enterococcus* a ser utilizada na alimentação de humanos ou de animais, com relação ao potencial probiótico, ausência de genes transmissores de resistência a antibióticos e ausência de fatores de virulência, através de testes *in vitro*, torna-se de extrema importância antes de qualquer aplicação *in vivo* (Gaggìa et al., 2010).

2.2 Probióticos

As BAL abrangem a classe mais representativa dos micro-organismos probióticos, e têm sido amplamente utilizadas e comercializadas como culturas probióticas (Todorov et al., 2011). Os primeiros benefícios à saúde associados com o uso de probióticos tiveram início no século 20, quando o cientista russo Eli Metchnikoff atribuiu à longa vida de camponeses búlgaros ao consumo de leite fermentado contendo BAL (Gaggìa et al., 2010). A partir deste relato, muitos estudos foram conduzidos com os diversos gêneros que compõe o grupo das BAL, dentre os quais destacam-se *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Leuconostoc, Pediococcus*, relacionando os efeitos da atividade probiótica com a saúde humana e animal (Holzapfel et al., 2001; Huff et al., 2006 ; Culligan et al., 2009 ; Gaggìa et al., 2010; Todorov et al., 2011 ; Aggarwal et al., 2012).

Segundo a FAO-WHO (2001), os probióticos são definidos como "micro-organismos vivos que, quando administrados em quantidades adequadas conferem benefícios à saúde do hospedeiro". As BAL são comumente utilizadas como culturas probióticas em diversos produtos alimentícios como iogurtes, leites fermentados e não fermentados, fórmulas infantis e produtos farmacêuticos. O alegado dos efeitos benéficos inclui: (i) tratamento da diarréia associada com a terapia antibiótica, infecções virais e quimioterapia; (ii) inibição de micro-organismos patogênicos; (iii) fortalecimento da mucosa intestinal; (iv) atividades antimutagênicas e anticarcinogênicas; (v) estimulação do sistema imunológico; (vi) prevenção de úlceras relacionadas com infecção

por *Helicobacter pylori* e (vii) redução do colesterol (Schrezenmeir; Vrese, 2001; Arihara, 2006; Khan et al., 2011).

FAO-WHO (2002) também afirmam que a seleção apropriada de culturas probióticas exige conformidade com os seguintes critérios: (i) capacidade de sobrevivência no trato gastrointestinal (TGI) de seres humanos e de animais; (ii) atividade de sais biliares hidrolase; (iii) capacidade de adesão a mucosa ou as células epiteliais; (iv) exclusão ou redução de patógenos de aderência e (v) atividade antimicrobiana contra agentes patogênicos. Além dessas características, somam-se outras condições complementares necessárias às culturas probióticas como rápido crescimento, ausência de propriedades tóxicas, alérgicas e mutagênicas, e ainda, não conter genes transmissores de resistência a antibióticos (Holzapfel et al., 2001; Culligan et al., 2009).

2.3 Bactérias ácido lácticas e a colonização do trato gastrointestinal

Para entender melhor os funcionamento das BAL no trato gastointestinal (TGI) é necessário conhecer os aspectos fisiológicos relacionados a estes micro-organismos na microbiota intestinal. A colonização do TGI pela microbiota é um processo complexo e multifatorial, que envolve fatores ambientais e alimentares, associados aos micro-organismos e relacionado ao hospedeiro. Para que ocorra a colonização do colón, as bactérias necessitam encontrar um habitat apropriado para adesão ao intestino. Quatro habitats bacterianos característicos foram descritos no intestino: o lúmen intestinal, a camada de muco, as criptas intestinais e as superfícies das células epiteliais (Mountzouris et al., 2002; Kim; Ho, 2010; Huidrom et al., 2012). O estômago e o intestino delgado são ambientes desfavoráveis à colonização e à proliferação bacteriana pela ação do suco gástrico, da bile, da secreção pancreática e pelo intenso peristaltismo do intestino delgado. O íleo é um sítio de transição bacteriológica, entre a escassa população bacteriana do jejuno e a densa microbiota do cólon. No cólon as bactérias encontram condições favoráveis para sua proliferação devido ao peristaltismo lento e abundante suprimento nutricional (Brandt et al., 2006; Kim; Ho, 2010).

O TGI abriga uma complexa e dinâmica população de micro-organismos, em sua maioria, bactérias que constituem a microbiota intestinal. Algumas dessas bactérias são adquiridas desde o nascimento e colonizam o intestino permanentemente e, por este motivo, são denominadas nativas ou indígenas, enquanto outras transitam apenas temporariamente pelo trato intestinal (Andoh; Fujiyama, 2006). Como essa microbiota desempenha importante papel no desenvolvimento e na manutenção da resposta imune intestinal (Tlaskalová-Hogenová et al., 2011), as investigações nessa área têm atraído interesse nos últimos anos. Um aspecto interessante diz respeito à possibilidade de se promover a manipulação terapêutica da microbiota intestinal para melhorar a saúde do hospedeiro, pela administração de diversas espécies de micro-organismos, coletivamente denominados probióticos (Isolauri et al., 2004).

2.4 Influência dos probióticos com a microbiota intestinal e o sistema imune na mucosa intestinal

O termo microbiota intestinal refere-se ao ecossistema essencialmente bacteriano que reside normalmente no intestino do homem e dos animais. O conhecimento a respeito da sua própria composição ainda é escasso, pois, calcula-se que pelo menos 40% das suas espécies ainda não foram cultivadas (Rastall et al., 2005). A microbiota intestinal é também conhecida como flora indígena dos intestinos (grosso e delgado), microflora intestinal ou simplesmente flora normal do intestino. Como funções da microbiota intestinal podem-se citar a resistência à colonização por outros micro-organismos, a imunomodulação e a contribuição nutricional resultante das interações locais e dos metabólitos produzidos (Rastall et al., 2005).

O TGI humano é um micro-ecossistema cinético que possibilita o desempenho normal das funções fisiológicas do hospedeiro. Manter um equilíbrio adequado da microbiota pode ser assegurado por uma suplementação sistemática da dieta com probióticos. Em virtude desse fato, nos últimos anos, o conceito de alimentos funcionais concentrou-se de maneira intensiva nos aditivos alimentares que podem exercer efeitos benéficos sobre a composição da microbiota intestinal. Os probióticos são atualmente os aditivos

alimentares que compõem os alimentos funcionais (Ziemer; Gibson, 1998; Bielecka et al., 2002; Siró et al., 2008).

A modulação da microbiota intestinal pelos micro-organismos probióticos ocorre através de um mecanismo denominado “exclusão competitiva”. Esse mecanismo impede a colonização da mucosa por micro-organismos potencialmente patogênicos, através da competição por sítios de adesão, competição por nutrientes e/ou produção de compostos antimicrobianos (Fuller, 1989; Puupponen-Pimiä et al., 2002; Culligan et al., 2009).

De acordo com Kim; Ho (2010); Sekirov et al. (2010) o epitélio intestinal é coberto por uma camada de mucosa semelhante a um gel composto por glicoproteínas (mucina). A secreção de mucina a partir das células caliciformes é estimulada por fatores neurogênicos e por mudanças nos fatores ambientais, incluindo a flora indígena e infecções microbianas. A defesa do epitélio em contato direto com o conteúdo luminal, age como uma barreira para a adesão de micro-organismos patogênicos e toxinas. Os vários mecanismos pelos quais a camada de muco protege a superfície intestinal contra enteropatógenos incluem: barreira física sobre as células epiteliais, ligação competitiva e a liberação de muco dentro do TGI. Além disso, a formação do complexo antígeno-anticorpo no revestimento da mucosa, juntamente com o peristaltismo, resulta em uma rápida eliminação de antígenos/micro-organismos no intestino delgado.

Estudos recentes têm demonstrado que probióticos ingeridos oralmente podem exercer efeito anti-infeccioso, influenciando na regulação da expressão do gene mucina e na liberação de muco (Sekirov et al., 2010; Howarth; Wang, 2013). Dados experimentais indicam que diversos probióticos são capazes de modular algumas características da fisiologia digestiva como a imunidade da mucosa e a permeabilidade intestinal (Fioramonti et al., 2003; Kim; Ho, 2010). A ligação de bactérias probióticas aos receptores da superfície celular dos enterócitos também desencadeia reações em cascata que resultam na síntese de citocinas (extenso grupo de moléculas envolvidas na emissão de sinais entre as células durante o desencadeamento das respostas imunes) (Isolauri et al., 2004; Ohashi; Ushida, 2009; Ng et al., 2009; Kim; Ho, 2010).

O mecanismo de ação dos probióticos relacionados com o sistema imunológico ainda não está completamente elucidado, mas sabe-se que alguns probióticos são fagocitados pelas células especializadas no reconhecimento de antígeno (células M). Estas células por sua vez apresentam esses antígenos às células T que são estimuladas a produzirem citocinas. Além disso, elas estimulam os linfócitos B a produzirem anticorpos, especialmente IgA, ativando dessa forma o sistema imunológico (Ohashi; Ushida, 2009). Outro mecanismo de ação proposto seria de que as bactérias patogênicas ativam o fator de transcrição NF-κB induzindo, desta forma, resposta inflamatória nas células epiteliais intestinais. Em contrapartida, os probióticos atenuariam essa reposta inflamatória por bloquear a via de transcrição do NF-κB (Gregorieff et al., 2009; Ng et al., 2009).

Relatasse na literatura que os probióticos podem estimular tanto a resposta imune não-específica quanto específica, tanto *in vivo* quanto *in* vitro. Este estímulo pode ser mediado por uma ativação dos macrófagos por meio da elevação nos níveis de citocinas, da atividade das células destruidoras naturais (NK - “natural killer”) e/ou dos níveis de imunoglobulinas. A resposta imune pode ser aumentada, quando um ou mais probióticos são consumidos concomitantemente e atuam sinergicamente como, por exemplo, no caso de linhagens de *Lactobacillus* administrados em conjunto com linhagens de *Bifidobacterium* (Kopp-Hoolihan, 2001; Van de Water, 2003).

Relatos bibliográficos de estudos com BAL têm demonstrado que os probióticos têm efeito imuno-estimulante nos homens e nos animais. Esse efeito pode estar relacionado à capacidade de os micro-organismos probióticos interagirem com as placas de Peyer e as células epiteliais intestinais, estimulando as células B produtoras de IgA e a migração de células T do intestino. Também tem sido demonstrado que os probióticos favorecem a atividade fagocítica inespecífica dos macrófagos alveolares, sugerindo uma ação sistêmica por secreção de mediadores que estimulariam o sistema imune (Cross, 2002; Delcenserie et al., 2010).

2.5 Probióticos e os benefícios atribuídos ao seu consumo

Vários são os benefícios atribuídos ao consumo de culturas probióticas à saúde humana como controle e estabilização da microbiota intestinal; promoção da resistência gastrointestinal à colonização por patógenos; estimulação do sistema imune; aumento da absorção de minerais e vitaminas; diminuição do risco de câncer de cólon e de doença cardiovascular; redução das concentrações plasmáticas de colesterol, dentre outros (Kaur et al., 2002; Puupponen-Pimiä et al., 2002; Servin; Coconier et al., 2003; Tuohy et al., 2003).

Da mesma forma, os probióticos têm sido administrados em animais com a finalidade de colonizar o ambiente intestinal e promover um melhor equilíbrio da flora (Fuller, 1989). Pesquisas com probióticos na alimentação animal têm sido instigadas principalmente pela preocupação da comunidade científica com o surgimento de linhagens de micro-organismos resistentes e com o uso indiscriminado de agentes antimicrobianos, que pode ter resultado no desenvolvimento da resistência bacteriana (Tenover, 2001). Em função disso, uma área que tem se mostrado promissora é a da utilização de probióticos como promotores de crescimento e de sanidade de animais de produção, em substituição às doses sub-terapêuticas de antibióticos, comumente utilizadas nas rações de animais criados intensivamente, como aves e suínos (Choudhari et al., 2008). De acordo com Jin et al. (1998); Kabir (2009); Dibner e Richards (2005), o uso de probiótico como promotor de crescimento pode resultar em maior ganho de peso, melhor índice de conversão alimentar, maior rendimento de carcaça e melhor palatabilidade da carne.

A eficácia do probiótico é estritamente dependente da quantidade e das características das linhagens do micro-organismo utilizado na elaboração de aditivos alimentares (Jin et al., 1998). O conhecimento genético básico das bactérias probióticas é crucial para o entendimento de suas características essenciais e dos sistemas de sinalização e respostas que são necessários para a sua funcionalidade *in vivo* (O'Flaherty; Klaenhammer, 2010). Para que uma linhagem probiótica seja aplicada *in vivo* de forma segura, a correta

identificação ou caracterização molecular, diferenciando os diversos gêneros probióticos existentes, é de extrema importância uma vez que a identificação inequívoca desses micro-organismos poderá acarretar consequências no desenvolvimento de produtos finais destinados ao consumo humano e animal (Leroy e De Vuyst, 2004; Corcionivoschi et al., 2010). O desenvolvimento da tecnologia do DNA (ácido desoxirribonucléico) não apenas acelerou o desenvolvimento da pesquisa fundamental, como também ampliou o conhecimento para a produção de células e organismos para as indústrias de alimentação e farmacêutica. Além disso, as facilidades do sequenciamento automatizado do DNA e o avanço de softwares que convertem sequências de nucleotídeos a ORFs (*Open Reading Frames*) e ligam sequências de nucleotídeos formando contigs têm permitido que genomas bacterianos inteiros sejam sequenciados (Bolotin et al., 2001).

Ressalta-se ainda que, para a seleção de linhagens com potencial probiótico, análises dos fatores de virulência e da resistência de genes são de grande relevância, pois a resistência aos antimicrobianos é um fenômeno genético, relacionado à existência de genes contidos nos micro-organismos que codificam diferentes mecanismos bioquímicos que impedem a ação das drogas (Davies; Davies, 2010). A resistência pode ser originada em mutações que ocorrem no micro-organismo durante seu processo reprodutivo e resultam em erros de cópia na sequência de bases que formam o DNA cromossômico, responsáveis pelo código genético (Levin et al., 2000; Toomey et al., 2009; Davies; Davies, 2010). A outra origem da resistência é a importação dos genes causadores do fenômeno, consistindo na resistência transferível. Esta resistência faz-se através dos mecanismos de transdução, transformação e conjugação, e frequentemente envolve genes situados em plasmídios e transposons (Toomey et al., 2009; Davies; Davies, 2010). Neste contexto, enfatiza-se a importância de análises de atividade probiótica, dos fatores de virulência, da resistência de genes, da susceptibilidade antimicrobiana e da identificação molecular em nível de DNA dos micro-organismos, quando se pretende aplicá-los como aditivo alimentar tanto na alimentação animal quanto na alimentação humana.

Os probióticos vêm sendo aplicados na alimentação animal por exercer efeitos benéficos na saúde destes, como já relatado anteriormente. Simultaneamente, têm sido utilizados micronutrientes, que auxiliam similarmente na promoção da saúde destes animais. O selênio é um dos micronutrientes minerais utilizado concomitantemente em rações animais, como aditivo alimentar, ao qual tem sido atribuído efeitos benéficos com relação ao crescimento, ganho de peso, ação antioxidante e melhoria do sistema imunológico.

2.6 Selênio

O selênio (Se) é um nutriente essencial na dieta de humanos e de animais e está relacionado ao sistema antioxidante do organismo por fazer parte da enzima glutationa peroxidase (GSH-Px) (Stephensen et al., 2007). Estudado inicialmente devido à incidência de intoxicação (selenose), este elemento descoberto em 1817, passou a ser visto como micro-elemento essencial para diversos processos da vida de todos os animais, incluindo as aves (Wang; Xu, 2008). A forma biologicamente ativa foi encontrada em 1973, quando a GSH-Px foi identificada (Rotruck et al., 1973).

A importância do selênio na fisiologia animal foi primeiramente reportada em 1957, quando a deficiência deste mineral foi associada com a vitamina E, a qual resultou na doença do músculo branco (DMB) (Muth et al., 1958). No entanto, o seu significado biológico como parte estrutural de selenoenzimas foi entendida em 1973 com a descoberta da GSH-Px e o seu papel na regulação de processos oxidativos e de proteção da membrana da célula (Rotruck et al., 1973) e, deste modo, pode-se inferir que a deficiência de selênio danifica principalmente as membranas celulares e mitocôndrias (Behne; Kyriakopoulos, 2001; Hefnawy; Tórtora-Pérez, 2010).

Há duas fontes principais de suplementação de selênio para as aves: uma fonte na forma de selenoaminoácidos, também chamada de selênio orgânico, que inclui a selenometionina (SeMet) e selenocisteína (SeCis), e o selênio inorgânico como selenitos ou selenato de sódio sendo que a principal fonte de selênio usada nas rações para as aves está na forma inorgânica,

como selenito de sódio, normalmente incluídos como suplementação mineral (Surai et al., 2006). Na Figura 1, é apresentada a estrutura das moléculas de SeMet e selenito de sódio (Na_2SeO_3).

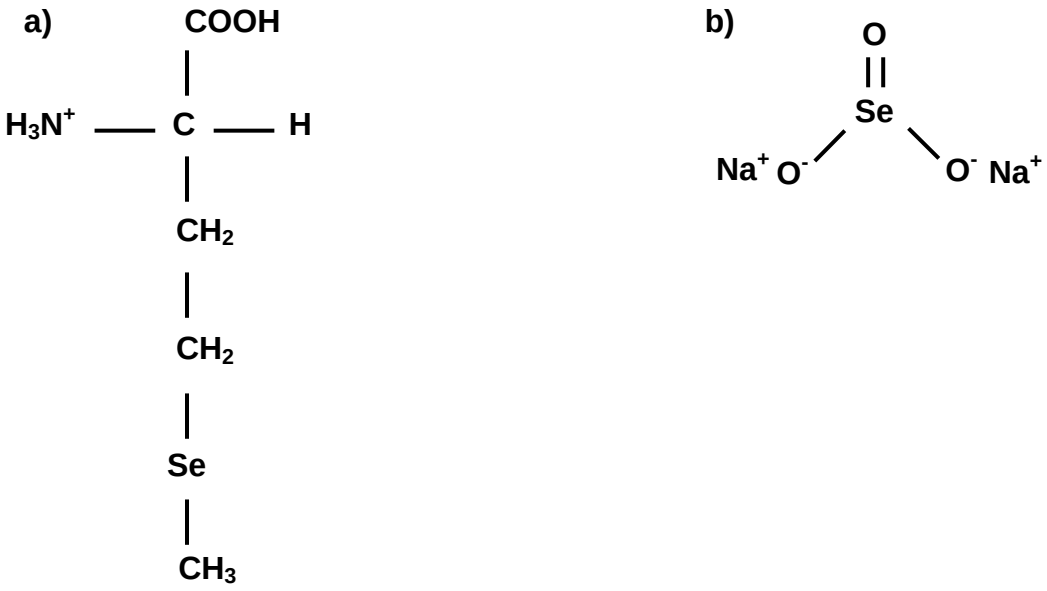

Figura 1: Estrutura molecular da SeMet (a) e Selenito de sódio (b)

O selênio na forma de SeMet, por ser absorvido no intestino por transporte ativo, através do mecanismo de absorção das proteínas, e não de forma passiva como classicamente é o transporte dos minerais, desta maneira torna-se facilmente disponível à utilização dos tecidos. Assim sendo, a forma conjugada da proteína pode ser metabolizada como aminoácido e ter uma reduzida perda por excreção, proporcionando maior disponibilidade (Cornelis et al., 2005).

O selênio é parte do sítio ativo das enzimas, por se tornar uma SeCis (Negro, 2008; Terpiłowska; Siwicki, 2011). A capacidade de catalisar processos de oxido-redução está associada com o aumento da capacidade de ionização do selenol ao pH fisiológico, em grupos tiol (enxofre) de cisteína. Substituíndo a SeCis por cisteína reduz-se drasticamente a capacidade de oxido-redução das selenoenzimas (Driscoll and Copeland, 2003).

2.7 Selenoproteínas e o efeito funcional do selênio no organismo

O equilíbrio fisiológico do organismo é influenciado de forma expressiva pela função das substâncias antioxidantes no controle da produção de radicais livres. A produção de radicais livres é uma consequência natural dos processos celulares, porém pode estar elevada em algumas situações patológicas, como no câncer, na aterosclerose, no acidente vascular cerebral (AVC) e, em doenças neurodegenerativas, como doença de Alzheimer, doença de Parkinson e esclerose múltipla, sendo relatado ainda na literatura, o envelhecimento precoce (Rahman, 2007). Dentre as diversas substâncias antioxidantes que participam do metabolismo celular, destaca-se o selênio.

O selênio é um micronutriente essencial para o crescimento e manutenção tanto do organismo humano quanto do organismo animal. No corpo humano está presente em quase todas as células, sendo mais abundante nos rins, fígado, baço, pâncreas, glândula tireóide e testículos (Nyman et al., 2004). Este mineral possui diversas funções, dentre as quais destacam-se a regulação da atividade da GSH-PX, dos hormônios da tireóide e prostaglandinas, aumento da eficiência da vitamina E, melhora na atuação do sistema imune e suporte nas funções reprodutivas. Em frangos de corte, a deficiência de selênio pode levar à necrose hepática, redução da quantidade de proteínas, diátese exsudativa, redução na secreção de enzimas digestivas, além de reduzir o crescimento (Moreira et al., 2001).

A absorção do selênio ocorre principalmente no duodeno, ceco e cólon, onde a SeMet (forma orgânica) é absorvida por um mecanismo de transporte ativo; o selenito de sódio (forma inorgânica) é absorvido por difusão simples; o selenato de sódio (forma inorgânica) é absorvido em conjunto com o sulfato por meio de carreadores mediados por sódio e, a SeCis pode compartilhar o sistema de transporte ativo comum aos aminoácidos básicos (histidina, lisina e arginina) (Letavayová et al., 2006; Fairweathertait et al., 2011). A absorção do selênio na forma inorgânica pode sofrer ação antagônica à absorção de outros ânions como sulfatos. Já a absorção da SeMet ocorre por meio de transporte ativo através do sistema de transporte de aminoácidos neutros, por canais sódio dependente, o que torna o processo de absorção do selênio orgânico

mais eficaz que o da fonte inorgânica (Thiry et al., 2012). Na molécula de SeMet, o selênio substitui o enxofre, dentro da molécula de metionina, ligado a dois carbonos por uma ligação covalente e por isso não sofre dissociação no processo de absorção intestinal. Essa característica diferencia o selênio orgânico dos minerais quelatados, nos quais ocorre apenas uma complexação com grupos funcionais das proteínas (Suzuki, 2005; Gierus, 2007; Pessione, 2012). A selenometilselenocisteína não é incorporada como SeMet e, desta forma, é convertida rapidamente em metilselenol (Letavayová et al., 2006; Fairweathertait et al., 2011). Nos enterócitos, o selênio é reduzido a selenito e, desta forma, é transportado no sangue ligado a proteínas, principalmente frações de β-lipoproteína de baixa densidade (LDL) e, em menor quantidade, a outros tipos de proteínas como a albumina, especialmente quando a SeMet é a principal forma presente nos alimentos. Os compostos de selênio, tanto aqueles que entram no pool de selenito como os convertidos a metilselenol, são metilados por metiltransferases-tióis e geram diferentes formas metabólicas metiladas de selênio que serão excretadas e contribuem para a homeostase do mineral (Cominetti et al, 2011).

Quanto aos efeitos benéficos do selênio relacionados ao câncer, ressalta-se a capacidade de elevar a resposta imune ou a capacidade de produzir metabólitos antitumorais (metilselenol e precursores), os quais possuem a capacidade de diminuir o metabolismo da célula tumoral, inibir a angiogênese e induzir a apoptose na célula tumoral. Este papel antitumoral seria realizado por componentes do selênio, como a SeMet (Yu et al., 1997; Letavayová et al., 2006).

As funções do selênio no organismo humano e animal são exercidas por meio das selenoproteínas, muitas das quais apresentam ação antioxidante. Foram identificadas até o momento 25 selenoproteínas, porém somente algumas foram caracterizadas funcionalmente. A maioria possui função enzimática redutora via SeCis, o que promove atividades catalíticas ou antioxidantes. Os processos celulares que necessitam da presença de selenoproteínas incluem a biossíntese de desoxirribonucleotídeos fosfatados (dNTPs) para o DNA; a remoção de peróxidos que promovem danos às células; a redução de proteínas ou lipídios oxidados; a regulação da sinalização

redox; o metabolismo dos hormônios tireoidianos e o transporte e armazenamento do selênio (Papp et al., 2007; Cominetti et al., 2011). As funções especificas das selenoproteínas tioredoxina redutase (TrxR), GSH-Px e selenoproteína P (SeP) estão descritas a seguir.

O sistema tioredoxina é composto pela TrxR, tioredoxina e nicotinamida adenina dinucleotídeo fosfato reduzido (NADPH), sendo o maior sistema redox celular presente nos organismos vivos. As TrxRs agem controlando a função da tioredoxina, que é a molécula central do sistema redox a qual age na redução de diversos substratos. As TrxRs contêm um domínio flavina adenina dinucleotídeo (FAD), um domínio ligante de NADPH, um domínio de interfase e um resíduo de SeCis, responsável por sua função enzimática. A presença desta SeCis no sítio ativo da enzima demonstra a importância do selênio para sua atividade, e explica porque este elemento é necessário para a proliferação celular, uma vez que o controle do estado redox necessário à produção de desoxirribonucleotídeos ou à ativação de fatores de transcrição depende de tioredoxina (Papp et al., 2007; Cominetti et al., 2011). A selenoproteína TrxR possui uma particularidade especifica e esta relaciona-se com a sua sensibilidade à oxidação, ocorrendo uma alteração em sua conformação, o que pode comprometer a interação com outras moléculas e apresentar algum tipo de efeito no início da sinalização celular em resposta ao estresse oxidativo (Sordillo, 2013).

O sistema tioredoxina tem função central na regulação da expressão gênica por meio do controle redox de fatores de transcrição como NF-κB, Ref-1, AP-1, P53, receptores de glicocorticóides e quinases reguladoras da apoptose, modulando indiretamente as atividades celulares como proliferação, morte programada e ativação da resposta imune (Papp et al., 2007). A indução de enzimas cito-protetoras em resposta a situações de estresse ocorre, sobretudo à nível transcricional e, é mediada pelo elemento de resposta antioxidante encontrado na região promotora de diversos genes que codificam enzimas de destoxificação (Dinkova-Kostova et al., 2010).

A GSH-Px é uma enzima que protege o tecido celular contra o estresse oxidativo, catalisando as reações que convertem o peróxido de hidrogênio em água, e os hidroperóxidos de lipídeos a ácidos graxos alcoólicos (Amanullah et

al., 2012). O nível de atividade desta enzima é indicativo do aprovisionamento de selênio para o organismo, pois sua atividade nos eritrócitos, no plasma e em outros tecidos apresenta proporção direta com a ingestão de selênio (Halver; Hardy, 2002). A GSH-Px possui 4 selenoproteínas com função conhecida. A GSH-Px clássica (GSH-Px1), a selenoproteína mais abundante em mamíferos, está presente no citosol das células, no qual funciona como antioxidante reduzindo peróxidos de hidrogênio (H_2O_2) e hidroperóxidos orgânicos livres e transformando-os respectivamente em água e álcool. A GSH-Px gastrintestinal (GSH-Px2) é a selenoproteína antioxidante mais importante no cólon e protege o organismo de mamíferos da toxicidade causada por hidroperóxidos lipídicos. A GSH-Px extracelular (GSH-Px3) tem expressão elevada nos rins e pode ter função antioxidante nos túbulos renais ou espaços extracelulares. A GSH-Px fosfolipídio hidroperóxido (GSH-Px4) é diretamente responsável pela redução de hidroperóxidos lipídicos (Tapiero et al., 2003; Huang et al., 2012).

A SeP é uma proteína extracelular rica em selênio que contêm aproximadamente 10 SeCis que são codificadas pelo códon de terminação UGA no ORFs do RNAm (RNA mensageiro). A questão-chave que permanece sem conhecimento específico é a função catalítica da SeP. Esta selenoproteína é considerada uma proteína plasmática altamente glicosilada, produzida e, sobretudo, secretada pelo fígado, porém sendo encontrada em outros órgãos, incluindo coração, rins e cérebro (Saito et al., 1999; Saito; Takahashi, 2000; Dewing et al., 2012). Devido à sua localização no plasma e origem predominante no fígado, a SeP tem sido considerada como sendo uma proteína transportadora de selênio (Saito; Takahashi, 2000). Inicialmente, após a identificação desta selenoproteína, acreditou-se que como o selênio estaria covalentemente ligado à proteína, seria necessária a quebra desta para a liberação do mineral. No entanto, após estudos conduzidos em ratos para o gene da SeP, pode-se inferir que esta selenoproteína é responsável pelo transporte de selênio do fígado para outros tecidos (Burk et al., 2003; Richardson, 2005).

2.8 Importância do selênio na nutrição humana e animal

O selênio é um micronutriente essencial com propriedades antioxidantes que tem recebido atenção especial na dieta humana, principalmente pelo considerável papel potencial na prevenção de câncer e, na dieta animal, por aumentar a biodisponibilidade e resultar em um produto final de maior qualidade para o consumidor (Bleys et al., 2008; Arpášová et al., 2010). O provável mecanismo responsável pela prevenção e inibição de câncer, ainda não está completamente elucidado, mas acredita-se que a ação do selênio em inibir o crescimento tumoral possa ocorrer pela atividade antioxidante sobre as selenoproteínas, efeito anti-inflamatório derivado de interações com o sistema imune e a via de cicloxigenase-lipoxigenase, e também mudanças na expressão global de genes que podem bloquear a progressão do ciclo celular ou induzir a apoptose em ambos precursores tumorais ou células estromais (Felix et al., 2004; Sonn et al., 2005). Níveis reduzidos de selênio nas células e tecidos têm como consequência, concentrações menores da enzima antioxidante GSH-Px, resultando em maior suscetibilidade das células e do organismo aos danos oxidativos induzidos pelos radicais livres (Zhou et al., 2009).

Além disso, o selênio exerce outras funções no organismo humano, entre as quais, a regulação da atividade da GSH-Px, dos hormônios da tireóide e prostaglandinas; aumento da eficiência da vitamina E; melhoria na atuação do sistema imune e suporte nas funções reprodutivas. Similarmente, o selênio pode influenciar positivamente na qualidade da carne de frangos, no empenamento, na conversão de tiroxina (T4) em tri-iodotironina (T3), nas funções reprodutivas, na imunocompetência e na resistência ao calor (Bartholomew et al., 1998; Ševčíková et al., 2008). Payne e Southern (2005) e Ryu et al. (2005) relataram em estudo que o selênio na dieta de frangos aplicado sob as diferentes formas (orgânico e inorgânico) promoveu o aumento da biodisponibilidade, aumento do peso corporal e diminuição dos níveis de oxidação lipídica. Soetan et al., 2010 e Mahima et al. (2012) ressaltam ainda que a deficiência de selênio em aves pode levar à necrose hepática, redução

da quantidade de proteínas, diátese exsudativa, redução na secreção de enzimas digestivas, além de reduzir o crescimento.

O selênio tem importância especial na nutrição de animais como bovinos, ovinos, suínos, e em particular, em aves de corte. A presença do selênio suplementar em frangos de corte e em galinhas poedeiras pode levar ao aumento do conteúdo de selênio na carne de frangos e nos ovos, e o consumo destes alimentos por humanos pode promover benefícios à saúde através da ingestão de alimentos ricos em selênio (Şara; Odagiu, 2009).

As funções biológicas do selênio são exercidas principalmente pelas selenoproteínas que participam do controle redox em vários processos biológicos (Rayman, 2000; Gan et al., 2002; Hill et al., 2007; Papp et al., 2007). O melhor exemplo da função redox é a redução do peróxido de hidrogênio a produtos inócuos como água e alcoóis, pela família das enzimas peroxidases, selênio-dependentes tais como GSH-Px (Rayman, 2000; Gan et al., 2002; Papp et al., 2007). Estas enzimas ajudam a manter a integridade da membrana, reduzem a probabilidade de propagação de danos oxidativos de biomoléculas, como lipídeos, lipoproteínas e DNA, que estão associados com o aumento de câncer e aterosclerose (Angstwurm; Gaertner, 2006). A TrxR reduz a GSH-Px, assim como outros peróxidos lipídicos, sendo sua forma oxidada regenerada por diferentes subtipos de TrxR selênio-dependentes. Estudos em ratos demonstram que a atividade desta enzima aumenta com a atividade da GSH-Px após a suplementação com selênio, indicando uma hierarquia na síntese de seleno-enzimas (Gan et al., 2002; Hill et al., 2007). O reconhecimento do papel das selenoproteínas no metabolismo ajuda a explicar os efeitos adversos da deficiência de selênio na saúde humana e animal. A manutenção da concentração ótima de selênio no organismo, bem como da atividade das selenoproteínas, torna-se imprescindível para a prevenção de doenças (Rayman, 2000).

Assim sendo, uma ingestão diária de selênio é recomendável, pois contribui com as funções fisiológicas do organismo e na prevenção de doenças. De acordo com a Agência Nacional de Vigilancia Sanitária – ANVISA (1998) a Ingestão Diária Recomendada (IDR) é de 70 µg/dia para indivíduos adultos e para crianças com idade entre 0-10 anos a recomendação varia entre

10 e 30 µg/dia. Este valor aumenta para 65 e 75 µg/dia no caso de gestantes ou lactentes, respectivamente. A ingestão elevada de selênio pode causar a toxicidade, isto é, uma selenose. Ao exceder a ingestão do limite máximo tolerável de 400 µg/dia pode ocorrer a toxicidade, mas existem evidências científicas que o risco de selenose aumenta para uma ingestão igual ou superior a 800 µg de selênio por dia (German Nutrition Society, 2002). Nos Estados Unidos da América, um grupo de pessoas que faziam a ingestão de suplementos impróprios devido à quantidade excessiva de 27 mg de selênio por cápsula, tiveram sintomas de toxicidade, tais como náuseas, dores abdominais, diarréias, enfraquecimento das unhas, perda de cabelo, neuropatia periférica, fatigabilidade e irritabilidade. Em casos extremos de ingestão pode ser fatal, quando os sintomas progridem resultando em hipotensão, edema pulmonar e colapso cardiovascular (Stipanuk, 2006).

Em relação à recomendação de selênio para animais como bovinos é de 0,3 mg kg^{-1} de massa seca, de acordo com o National Research Council (NRC) (2001) e para frangos é de 0,15 mg Kg^{-1} de acordo com a NRC (1994).

Através do exposto na literatura em relação aos efeitos benéficos dos probióticos e os efeitos benéficos da ingestão de quantidades adequadas de selênio tanto para os humano quanto para os animais, pesquisas vêm sendo desenvolvidas visando a bioacumulação de selênio na biomassa microbiana com potencial probiótico.

2.9 Bioacumulação de selênio por bactérias ácido lácticas

Muitos estudos têm sido realizados em relação à bioacumulação de selênio pela biomassa de BAL visando à produção e a utilização em produtos para a nutrição animal. A biomassa bacteriana possui maior teor de proteína, e desta forma, em comparação com as plantas, a biomassa bacteriana pode incorporar maior concentração de selênio, devido à substituição do enxofre participante na formação da proteína (Ponce de Leon et al., 2002; Yin et al., 2010). Os micro-organismos, como as leveduras, podem utilizar açúcares solúveis e ácidos orgânicos para produção de biomassa com alto teor de proteína (Yin et al., 2010).

Culturas probióticas enriquecidas com selênio em concentrações e em condições ambientais adequadas podem permitir a conversão eficiente de selênio inorgânico em forma orgânica, tais como a SeMet e SeCis. Desse modo, o enriquecimento de probióticos com selênio pode desempenhar ambos os efeitos benéficos à saúde animal, através do potencial probiótico e, da mesma forma, pela função benéfica atribuída ao consumo de selênio no desenvolvimento do organismo e na melhoria das condições de saúde dos animais (Arpášová et al., 2010; Yin et al., 2010).

Na literatura têm sido descritos estudos com leveduras enriquecidas com selênio (Ponce de Leon et al., 2002; Stabnikova et al., 2008), da mesma forma, estudos similares têm sido conduzidos com BAL enriquecidas com selênio (Calomme et al., 1995; Zhang et al., 2009; Svoboda et al., 2010). Outros estudos têm sido conduzidos em animais com a suplementação de selenito de sódio (forma inorgânica do selênio); SeMet (forma orgânica sintetizada quimicamente), ou ainda, na forma combinada (orgânica e inorgânica) (Givens et al. 2004; Reis 2009; Miezeliene et al. 2011).

O enriquecimento de leveduras com selênio ou de BAL enriquecidas com selênio, representam uma forma orgânica de suplementação de selênio, produzida a partir de uma cultura probiótica enriquecida com selênio inorgânico (selenito de sódio). O selênio produzido por culturas probióticas sofre um tipo de biossíntese por meio da qual sais de selênio inorgânico são convertidos para uma forma orgânica via incorporação intracelular nos micro-organismos. Estes derivados orgânicos de selênio biossintetizados pelos probióticos são melhores fontes nutritivas de selênio por serem menos tóxicos e mais facilmente metabolizados que seus correlativos inorgânicos (Pessione, 2012).

Calomme et al. (1995) relataram em seu estudo que espécies de *Lactobacillus* poderiam concentrar selênio intracelular como SeCis na biomassa e, desse modo, fornecer uma dieta com potencial fonte de selênio orgânico. Similarmente, tem sido relatado na literatura (Arpášová et al., 2010; Ren et al., 2011) que leveduras enriquecidas com elevado teor de sais minerais (selenito de sódio) têm sido utilizadas para a obtenção de selênio orgânico. Arpášová et al. (2010) reportam que quando o meio de cultura é pobre em enxofre, as leveduras podem incorporar o selênio como metionina e SeCis em

quantidades maiores, quando comparadas com a suas necessidades nutricionais, sendo capazes de formar 97% de SeMet, e desta maneira, o organismo de frangos de corte absorve e metaboliza a SeMet da mesma maneira como o aminoácido metionina.

Reis (2009) relata em seu estudo que o uso de selenito de sódio na nutrição de reprodutoras permite a suplementação de um nutriente essencial e de baixo custo. Por ser uma fonte de selênio altamente concentrada, o selenito de sódio é incluído em baixíssimas quantidades na dieta animal (NRC – 0,15 mg Kg^{-1}) e, desta forma, pode-se inferir que o enriquecimento de BAL enriquecidas com selênio inorgânico, pode ser uma alternativa de fonte orgânica viável, promissora, com maior biodisponibilidade de selênio e de baixo custo.

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3. CAPÍTULO I

Bioacumulação de selênio em células de bactérias ácido lácticas: uma alternativa para a produção animal

Bioacumulação de selênio em células de bactérias ácido lácticas: uma alternativa para a produção animal[1]

Selenium bioaccumulation by lactic acid bacterial: An alternative to animal production

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Resumo – O selênio é um micronutriente essencial a todos os seres vivos. Esse elemento, quando em baixas concentrações nos animais pode comprometer a capacidade imunológica, produtiva e até mesmo a capacidade reprodutiva dos animais. As bactérias ácido lácticas (BAL) provenientes do trato gastrintestinal, e da mesma forma, quando presente nos alimentos, apresentam diversos aspectos benéficos ao seu hospedeiro como: atividade antimicrobiana, controle de patógenos e contaminações microbiológicas nos alimentos, atividade antioxidante, proteção da mucosa do trato gastrintestinal, entre outros benefícios. Devido à grande necessidade mundial da produção de alimentos, estudos com o enriquecimento de BAL com selênio podem ser promissores na produção de carne no setor agropecuário, e ainda, reduzindo custos com doenças e patógenos naturais existentes no cenário agroindustrial. Desta forma, a bioacumulação de selênio por BAL pode ser uma alternativa eficiente na melhoria e no incremento da produção animal.

Palavras-chave: selênio, bactérias ácido lácticas, bioacumulação, enriquecimento.

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Abstract – The selenium is an essential micronutrient for all living organisms. Low concentrations of this element in the animals can reduce the immunological, production and reproduction capacity of the animals. Lactic acid bacteria (LAB) from gastrointestinal and even in the food have shown many beneficial aspects to the host such as: antimicrobial activity, antioxidant activity, control pathogens and microbial contamination, mucous protection of the gastrointestinal system, and other benefits. Due to the high world production necessity, studies with the LAB enrichment with selenium can promote the meat production, and still can reduce the costs with diseases and natural pathogens existents in the agricultural scenario. Thus, the selenium bioaccumulation by LAB can be an efficient alternative in increasing animal production.

Keywords: Bioaccumulation, selenium, enrichment, acid lactic bacteria.

Introdução

As bactérias ácido lácticas (BAL) têm sido foco de intensivas pesquisas nacionais e internacionais pelo seu papel essencial na produção de derivados lácteos e fermentados, devido a sua capacidade de produzir compostos antimicrobianos, bem como apresentar atividade antitumoral, redução do colesterol, redução da intolerância à lactose, estimulação do sistema imunológico, estabilização da microflora intestinal, bem como propriedades probióticas (isolauri et al., 2001; Jackson et al., 2002; Hilde et al., 2003; Khedid et al., 2009; Nueno-Palop e Narbad, 2011; Ripamonti et al., 2011). Basicamente, o termo probiótico refere-se a micro-organismos vivos, que melhoram o equilíbrio microbiano do intestino e possuem efeitos benéficos para a saúde do hospedeiro (López-Brea e Domingo, 2007).

Os efeitos benéficos dos probióticos na saúde humana e na nutrição estão sendo cada vez mais reconhecidos. Pesquisadores que estudam as propriedades e a funcionalidade dos micro-organismos vivos na dieta sugerem que os probióticos desempenham um papel importante nas funções digestivas, imunológicas e respiratórias, assim, poderiam ter um efeito significativo no

tratamento das enfermidades infecciosas, especialmente em crianças e em populações de alto risco (López-Brea e Domingo, 2007). Desta forma, existe a legislação da União Européia que regula a comercialização e uso dos probióticos, suplementos dietéticos e alimentos funcionais (López-Brea e Domingo, 2007). No Brasil, o uso de probióticos foi regulamentado pela Resolução RDC nº 2, de 07 de janeiro de 2002, que aprova o regulamento técnico de substâncias bioativas e probióticos isolados com alegação de propriedades funcional e/ou de saúde (Brasil, 2002).

A utilização de probióticos também tem sido aplicada em ração animal. Os probióticos são produtos constituídos por organismos vivos ou em suas *L-formas* (sem parede celular). Uma vez introduzidos no organismo animal, podem colonizar o novo ambiente, promover um melhor equilíbrio microbiano intestinal, produzir enzimas digestivas e, ainda, estimular a imunidade da mucosa intestinal, protegendo-a contra toxinas pré-formadas por micro-organismos patogênicos (Toma e Pokrotnieks, 2006).

Juntamente com os probióticos têm sido utilizados micronutrientes que beneficiam a saúde dos homens e dos animais. O selênio, considerado um micronutriente essencial, tem sido utilizado na elaboração de suplementos probióticos. Este elemento tanto na sua forma inorgânica (selenito de sódio (Na_2SeO_3) e selenato de sódio (Na_2SeO_4)) ou na sua forma orgânica (selenometionina (SeMet) e selenocisteína (SeCis)) através de leveduras enriquecidas, tem recebido atenção especial recentemente com relação ao seu papel na prevenção de várias doenças, entre elas o câncer (Nogueira et al., 2003).

As BAL têm sido utilizadas em pesquisas visando à produção de biomassa com consequente utilização em produtos alimentícios, bem como, em ração animal. A biomassa produzida por este tipo de bactérias tem sido utilizada como fonte de micronutrientes, em especial de selênio. Em comparação com as plantas, como fonte de selênio, a biomassa bacteriana apresenta maior teor de proteína, consequentemente, maiores concentrações desse elemento podem ser incorporadas na biomassa, devido à substituição do enxofre participante na formação da proteína (Ponce de Leon et al., 2002). Além disso, o estudo da suplementação de probióticos associado com

micronutrientes utilizados para a alimentação animal tem despertado o interesse de diversas pesquisas, visando à substituição de antibióticos por culturas probióticas (Guerra et al., 2007).

Assim, a bioacumulação de selênio na biomassa bacteriana pode ser de grande contribuição a alimentação, bem como, ser uma alternativa eficaz para a obtenção de maior ganho de peso, melhor índice de conversão alimentar, maior rendimento de carcaça, melhor palatabilidade da carne, dentre outros benefícios. Por este motivo, justifica-se a importância deste trabalho de revisão bibliográfica, abordando os principais benefícios do enriquecimento de BAL com selênio, e desta forma, contribuindo com estudos futuros, utilizando a biomassa bacteriana na elaboração de suplemento probiótico enriquecido com selênio para uso em ração animal.

Bactérias ácido lácticas (BAL)

As BAL compreendem um grupo amplo de micro-organismos, dentre os principais destacam-se *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Leuconostoc* e *Pediococcus*. São micro-organismos Gram positivos, não formadores de esporos, anaeróbios, aerotolerantes, fastidiosos, ácido tolerantes, com metabolismo estritamente fermentativo, apresentando o ácido láctico como principal produto da fermentação dos carboidratos (De Martinis et al., 2002). São bactérias acidófilas sendo que a principal função das BAL nos alimentos é a acidificação dos produtos alimentares em um pH próximo a 4,0, o que impede o desenvolvimento de bactérias indesejáveis pela produção de ácidos orgânicos. Isso permite que o período de conservação dos produtos fermentados seja muito maior que a dos produtos onde a matéria-prima não seja fermentada (Bromberg et al., 2006).

As BAL constituem um grupo de micro-organismos amplamente distribuídos nos alimentos, sendo produtoras de uma variedade de compostos antimicrobianos, incluindo: ácidos, diacetil, peróxido de hidrogênio, dióxido de carbono, álcool, aldeído e substâncias antimicrobianas de natureza protéica, denominadas bacteriocinas. Todos esses compostos podem antagonizar o crescimento de bactérias deterioradoras e patogênicas presentes nos

alimentos (Leroy e De Vuyst, 2004; Bromberg et al., 2006). Isso explica a ampla utilização das BAL em produtos alimentícios, sendo suas propriedades utilizadas na elaboração de queijos, iogurtes, leites fermentados, bebidas, salsichas e outros produtos cárneos. Mais recentemente, com o desenvolvimento dos produtos probióticos, as BAL têm sido fonte de várias pesquisas devido aos possíveis benefícios à saúde que advêm de seu consumo (Feord, 2002).

Kinouchi (2006) demonstrou recentemente em seu trabalho que ao suplementar fêmeas de camundongos com câncer de mama biologicamente induzido, com uma dieta contendo um produto a base de soja fermentado com *Enterococcus faecium* CRL 183 e *Lactobacillus helveticus* ssp *jugurti* 416, estes apresentaram atividade anticarcinogênica. Os animais que consumiram este produto apresentaram um volume menor do tumor, comparados aos demais grupos. Ainda com relação à atividade anticarcinogênica do *E. faecium,* Sivieri et al. (2007) observaram uma redução de 40% na incidência de tumores de cólon em ratos quimicamente induzidos e que ingeriram uma suspensão oral desse micro-organismo em uma concentração diária de 10^8 UFC/mL.

Selênio

O selênio pertence ao grupo 16 da tabela periódica localizado entre o enxofre e o telúrio, podendo ser encontrado em quatro estados de valência que são II, 0, IV e VI (Papp et al., 2007). Este micronutriente é considerado essencial em muitos processos bioquímicos e fisiológicos, podendo ser encontrado no solo, nas plantas, no corpo humano e nos animais, cuja principal fonte para humanos e animais é a dieta (Hiil et al., 2003; Menon et al., 2011; Ren et al., 2011; Visser et al., 2011). No corpo humano está presente em quase todas as células, sendo mais abundante nos rins, fígado, baço, pâncreas, glândula tireóide e testículos (Nyman et al., 2004).

Em algumas áreas do mundo há certa deficiência de selênio em humanos, isto porque, algumas populações possuem o hábito de se alimentar majoritariamente com alimentos de origem vegetal, como na China, sendo que o solo deste país possui deficiência de selênio. Portanto, torna-se

indispensável a ingestão de alimentos fonte de selênio como frutos do mar, peixes, cereais, carne, nozes, cogumelos e ovos (Muniz-Naveiro et al., 2005) ou mesmo, na forma de suplemento dietético.

Na alimentação humana, existem algumas recomendações, que foram sumarizadas pelo Comitê sobre Aspectos Médicos da Política Alimentar - COMA (1991): 0,060 a 0,075 mg Se/dia para homens e mulheres, respectivamente; 0,075 mg/dia para mulheres gestantes; 0,45 mg/dia consumo máximo. Valores acima de 0,91 mg/dia (0,015 mg/kg de peso corpóreo para um adulto de 60 kg), indicam efeito de toxicidade. Dosagem nutricional e segura recomendada tem sido de 0,35 a 0,40 mg/dia, baseada em um humano de 70 kg de peso, subsistindo em uma dieta normal e 0,2 mg adicional Se/dia na forma de suplemento nutricional.

O selênio possui três níveis de atividade biológica: 1) pequenas concentrações são requeridas para o crescimento e desenvolvimento normais; 2) concentrações moderadas que podem ser estocadas para manutenção das funções homeostáticas; e 3) concentrações elevadas que podem resultar em efeitos tóxicos (Hamilton, 2004). Do mesmo modo, Zuberbuehler et al. (2006) e Ren et al. (2011), relatam que o Se(IV) (selenito de sódio) pode ser tanto um elemento essencial, quanto tóxico, dependendo da concentração utilizada. Tanto os sinais de deficiência quanto os efeitos tóxicos do excesso de selênio incluem: redução do crescimento, letargia, diminuição das funções hepáticas, diminuição do peso do fígado, diminuição no desempenho reprodutivo, catarata, diminuição dos níveis de hemoglobina e aumento na incidência de câncer.

Níveis reduzidos de selênio nas células e tecidos têm como consequência, concentrações menores da enzima antioxidante glutationa peroxidase, resultando em maior suscetibilidade das células e do organismo aos danos oxidativos induzidos pelos radicais livres (Monteiro et al., 2007). Dados epidemiológicos também mostraram que o selênio pode interagir com as vitaminas A e E na prevenção do desenvolvimento de tumores e na terapia da Síndrome de Imunodeficiência Adquirida (AIDS). Entretanto, outros resultados mostraram que a suplementação com esse mineral antioxidante pode aumentar

os processos de carcinogênese, recomendando cautela na administração de selênio para os seres humanos (Rayman, et al., 2002; Borek, 2004).

Sabe-se que nos tecidos o selênio está presente em duas formas: SeMet e SeCis. A SeMet não pode ser sintetizada no organismo e deve ser fornecida pela dieta. Esta forma pode substituir a metionina em uma variedade de proteínas (Burtis e Ashwood, 2001). A SeMet é considerada como uma forma de depósito de selênio no organismo, ou seja, quando o suporte deste elemento da dieta é interrompido, este aminoácido é movimentado e repõe o selênio no organismo. O selênio constituinte de proteínas, denominadas selenoproteínas, algumas das quais tem funções enzimáticas importantes, está na forma de SeCis sendo a forma biológica ativa do selênio (Hiil et al., 2003).

O selênio entra para a cadeia alimentar animal através do consumo de vegetais, que o absorvem a partir do solo na forma inorgânica. Nas plantas, o selênio é convertido em formas orgânicas, como o aminoácido SeMet e SeCis. SeMet é considerado o principal composto de selênio em cereais, grãos, legumes e soja, e serve de precursor para a síntese de SeCis em animais. O aminoácido SeCis é encontrado no sítio de pelo menos 25 diferentes selenoproteínas, todas com importantes funções biológicas. SeCis é estruturalmente similar ao aminoácido cisteína, entretanto, o selênio é um melhor nucleófilo do que o enxofre (Chen e Berry, 2003; Papp et al., 2007; Navarro-Alarcon e Cabrera-Vique, 2008).

São exemplos de selenoproteínas que contém SeCis: glutationa peroxidase, tiorredoxina redutase, iodotironina deiodinase, SeP, selenoproteína W e metionina sulfóxido redutase, e várias destas selenoproteínas estão envolvidas nas defesas antioxidantes e regulações redox. O selênio nestas enzimas pode atuar como centro redox, por exemplo, quando a selenoenzima, tiorredoxina redutase, reduz nucleotídeos na síntese do DNA e ajuda a controlar o estado redox intracelular (Angstwurm e Gaertener, 2006).

O melhor exemplo da função redox é a redução do peróxido de hidrogênio a produtos inócuos como água e alcoóis, pela família das enzimas peroxidases, selênio-dependentes tais como glutationa peroxidase (Rayman, 2000). Estas enzimas ajudam a manter a integridade da membrana, reduzem a probabilidade de propagação de danos oxidativos de biomoléculas, como

lipídeos, lipoproteínas e DNA, que estão associados com o aumento de câncer e aterosclerose (Burtis e Ashwood, 2001). O maior papel na defesa antioxidante é atribuído a enzima glutationa peroxidase. Ela reduz o peróxido de hidrogênio, lipídeos e fosfolipídeos hidroperóxidos impedindo que ocorra propagação dos radicais livres (Angstwurm e Gaertener, 2006).

A tiorredoxina é uma proteína pequena (12 kDa) com dois resíduos de cisteína, que é regulada e contribui para a tolerância de endotoxinas. A tiorredoxina reduz a glutationa peroxidase, bem como outros peróxidos lipídicos, sendo sua forma oxidada regenerada por diferentes subtipos de tiorredoxinas redutases selênio-dependentes. Estudos em ratos demonstram que a atividade desta enzima aumenta com a atividade da glutationa peroxidase após a suplementação com selênio, indicando uma hierarquia na síntese de seleno-enzimas (Hill et al., 2003). A metionina sulfóxido redutase, que repara resíduos de metionina oxidada em proteínas, requer tiorredoxina para sua subsequente redução (Moskovitz et al., 2002).

Aproximadamente 60% do selênio no plasma é incorporado em SeP, a qual contém 10 átomos de selênio por molécula de selenocisteína. A glutationa peroxidase extracelular e a SeP representam mais de 90% do selênio no plasma e ambas podem servir como proteína transporte para o selênio. No entanto, a SeP também é expressa em muitos tecidos e, tem sido associada com membranas celulares, o que sugere que embora possa facilitar o transporte de selênio para todo o corpo, esta pode não ser sua única função, pois pode atuar também como uma enzima antioxidante (Brown e Arthur, 2001).

Em estudos com animais, os efeitos do consumo de selênio na concentração da selenoproteína W nos tecidos, têm sido relatados como sendo necessário para o metabolismo muscular. A calcificação nos músculos esqueléticos ovinos e bovinos, conhecida como distrofia muscular nutricional ou doença do músculo branco, uma afecção miodegenerativa hiperaguda, aguda ou subaguda dos músculos cardíaco e/ou esquelético, é prevenida pela suplementação de selênio e vitamina E. A doença é caracterizada por necrose segmentar com calcificação de segmentos necróticos das fibras musculares. A importância da selenoproteína W para o metabolismo muscular esquelético

humano, ainda não é totalmente compreendida, mas a recente clonagem do seu cDNA vem reforçando pesquisas sobre distrofias musculares em humanos, que tem demonstrado resposta à suplementação de selênio (Brown e Arthur, 2001).

Dentre os órgãos do corpo humano, a glândula tireóide é a que apresenta maior concentração de selênio. Neste órgão, o selênio está envolvido no metabolismo hormonal pela ação catalizadora da selenoenzima iodotironina deiodinase na conversão do hormônio da tireóide (tiroxina – T4) para a sua forma ativa (tri-iodotironina – T3) produz o hormônio da tireóide ativo a partir do precursor inativo (Combs et al., 2009).

Estudos epidemiológicos em humanos têm demonstrado relação inversa entre a ingestão de selênio e a incidência de câncer (Felix et al., 2004; Sonn et al., 2005). O aumento de evidências experimentais em estudos sugere que a suplementação com selênio na dieta poderia inibir tumores induzidos quimicamente em várias espécies de animais, como também, inibir cânceres provocados por vírus (Whanger, 2004). Animais que receberam dieta suplementada com selênio, mostram uma redução na incidência e no tamanho dos tumores. Contudo, o mecanismo responsável por este fenômeno ainda não é conhecido, mas acredita-se que a ação do selênio em inibir o crescimento tumoral possa incluir os seguintes processos: atividade antioxidante sobre as selenoproteínas, efeito antiinflamatório derivado de interações com o sistema imune e a via de cicloxigenase-lipoxigenase, e também mudanças na expressão global de genes, que podem bloquear a progressão do ciclo celular ou induzir a apoptose em ambos precursores tumorais ou células estromais (Felix et al., 2004; Sonn et al., 2005).

O reconhecimento do papel das selenoproteínas no metabolismo ajuda a explicar os efeitos adversos da deficiência de selênio na saúde humana e animal. A manutenção da concentração ótima de selênio no organismo, bem como, da atividade das selenoproteínas, torna-se imprescindível para a prevenção de doenças. Algumas destas doenças estão relacionadas à ingestão de baixa concentração de selênio como na incidência de infarto do miocárdio, aumento do risco de câncer (Rayman, 2000; Burtis e Ashwood, 2001), complicações em pacientes com doença renal, agravamento do quadro de

indivíduos infectados com o vírus HIV do tipo I. Outros estudos de relevância clínica do selênio têm sido relatados, como a sua importância na reprodução. Estudos realizados com homens inférteis suplementados com selênio e vitamina E mostraram melhora na morfologia do esperma. Indivíduos tratados com selenito de sódio mostraram aumento dos níveis de selênio no fluído seminal, e aqueles que receberam selênio na forma de levedura enriquecida, tiveram um aumento também na atividade da glutationa peroxidase (Rayman, 2000).

A importância do selênio na alimentação animal e nos processos fisiológicos tem sido relatada na literatura. Lacetera et al. (1996) e Givens et al. (2004) verificaram aumentos significativos na produção de leite, em vacas suplementadas com 5 mg de selenito de sódio para cada 100 Kg^{-1} de peso vivo. De acordo com estudo realizado por Wang et al. (2009), o selenito e selenato de sódio podem ser reduzidos a selênio elementar (Se^0) pelos micro-organismos ruminais como um meio de detoxificação. Os micro-organismos podem também incorporar selênio na biomassa microbiana e, desse modo, o selênio presente na ração na forma inorgânica, transformar-se-ia na forma orgânica, podendo ser absorvido no intestino delgado.

Propriedades probióticas das BAL

Considera-se como probiótico as cepas de micro-organismos que possuem a capacidade de resistir à condições ácidas, à ação da bile e lisozima, e de colonizar o trato intestinal humano, ao menos temporariamente, mediante a adesão às células intestinais. Além dessas características, somam-se outras condições complementares necessárias às culturas probióticas: capacidade de ativação, rápido crescimento, resistência aos antibióticos normalmente presentes nos alimentos, e ausência de propriedades patogênicas, tóxicas, alérgicas, mutagênicas ou carcinogênicas (Holzapfel et al., 2001).

Os maiores benefícios à saúde humana atribuídos à ingestão de culturas probióticas são: controle e estabilização da microbiota intestinal; promoção da resistência gastrintestinal à colonização por patógenos; diminuição da

população de patógenos através da produção de ácido acético e láctico e de outros compostos antimicrobianos; promoção da digestão da lactose em indivíduos intolerantes à lactose; estimulação do sistema imune; alívio da constipação; aumento da absorção de minerais e produção de vitaminas. Outros efeitos atribuídos a essas culturas são a diminuição do risco de câncer de cólon e de doença cardiovascular. É sugerida também, a diminuição das concentrações plasmáticas de colesterol, efeitos anti-hipertensivos e antitumorais, redução da atividade ulcerativa de *Helicobacter pylori,* controle da colite induzida por rotavírus e por *Clostridium difficile*, prevenção de infecções urogenitais, além de efeitos inibitórios sobre a mutagenicidade (Kaur et al., 2002; Puupponen-Pimiä et al., 2002; Servin et al., 2003; Tuohy et al., 2003; Saad, 2006).

Pesquisas com probióticos também têm sido realizadas em animais por dois aspectos complementares. A demanda crescente por métodos mais conservacionistas para o tratamento e a prevenção de infecções, fruto da maior conscientização ecológica da população leiga. Por outro lado, a preocupação da comunidade científica com o surgimento de cepas de micro-organismos resistentes e com o uso indiscriminado de agentes antimicrobianos. Em função disso, uma área que tem se mostrado bastante promissora é a da utilização de probióticos como promotores de crescimento e de sanidade de animais de produção, em substituição às doses subterapêuticas de antibióticos, comumente utilizadas nas rações de animais criados intensivamente, como aves e suínos (Guerra et al., 2007). O uso indiscriminado dos antibióticos na alimentação animal pode ter resultado no desenvolvimento de populações bacterianas resistentes (Gundogan et al., 2005).

Torna-se evidente, a necessidade de estudos de produtos alternativos que possam substituir os antibióticos na alimentação animal, sem causar perdas na produtividade e na qualidade dos produtos finais. Os prováveis substitutos promotores de crescimento devem manter as ações benéficas dos antibióticos e eliminar as indesejáveis, como a resistência bacteriana (Pelícia et al., 2004).

Bioacumulação de selênio e BAL

A bioacumulação de metais por bactérias é uma ferramenta que tem sido amplamente utilizada em várias técnicas, como a biorremediação de locais contaminados com metais pesados (Andreazza et al., 2010). Além disso, normalmente a bioacumulação de metais por bactérias pode ser realizada via sorção na parede celular, e também, estes metais podem ser reduzidos e, posteriormente bombeados para dentro da célula bacteriana pelas ATPases, envolvendo gasto de energia (Andreazza et al., 2011).

Em estudo realizado por Pieniz (2010) BAL, mais especificamente algumas espécies do gênero *Enterococcus*, demonstraram possuir propriedade antioxidante e atividade antimicrobiana e, além disso, capacidade em acumular Se(IV) na biomassa bacteriana. Deste modo, a aplicação de BAL enriquecidas com selênio pode proporcionar melhor qualidade à ração animal, pois estas podem ser utilizadas, além de fonte protéica e potencial probiótico, como fonte de micronutrientes para a dieta animal (Pieniz, 2010).

Resultados obtidos em estudo prévio demonstrou alto potencial de bioacumulação de selênio por espécies de *Enterococcus faecium* e *Enterococcus faecalis* (Pieniz et al., 2011), contudo necessita-se a continuidade de estudos nesta área. Assim, pode-se dizer que as BAL podem ser consideradas como uma fonte promissora de micronutrientes como o selênio, e podem ser uma alternativa eficiente na melhoria das características bioalimentares e, desta forma, promovendo efeitos benéficos à saúde animal.

Considerações Finais

A utilização de BAL enriquecidas com selênio pode proporcionar vários benefícios à alimentação animal, com incrementos na produção, tanto de carne quanto reprodutiva, aumentando a resistência às doenças, dentre outros benefícios como citados anteriormente. Além disso, altas concentrações de selênio são incorporadas em células de *Enterococcus*, sendo um probiótico de baixo custo e de grande potencial na utilização da alimentação de aves, suínos e bovinos. Sabe-se que a pesquisa nesta área é importante, porém os estudos

ainda são insuficientes para a implementação em grande escala destas técnicas. Deste modo, estudos com o isolamento, caracterização e aplicação de BAL enriquecidas com selênio são determinantes para melhorias no desenvolvimento da produção animal, não somente em termos de pesquisas laboratoriais, mas também para a aplicação em nível de propriedade rural.

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4. CAPÍTULO II

Probiotic potential, antimicrobial and antioxidant activities of *Enterococcus durans* strain LAB18s

Probiotic potential, antimicrobial and antioxidant activities of *Enterococcus durans* strain LAB18s

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Abstract

Enterococci represent Gram-positive lactic acid bacteria (LAB) which belong to enteric commensally microbiota with a widespread occurrence in the different ecosystems, and they still are considered probiotic cultures. Human nutrition has already been using probiotics especially in dairy products. Animal nutrition is another area for successful application of probiotics; the *Enterococococcus* spp. seems to be the most used microorganisms as additive. Hence, the aim of this study was to characterize the probiotic potential of LAB18s, isolated from "Minas Frescal" cheese and identified as *E. durans*, through acid and bile salts resistance, survival in simulated gastrointestinal tract and also, evaluated the antimicrobial and the antioxidant properties. The *E. durans* LAB18s showed resistance to acid conditions, except for pH 2; and it was able to survive in the presence of simulated gastric juice at pH 3, while at pH 2 it was observed viability of cells only at initial time (0 h) and it demonstrated also survival capability in the presence of simulated intestinal juice with or without bile salts. The antimicrobial activity of *E. durans* LAB18s was tested with two extract (culture supernatant and intracellular extract) against differents indicator pathogenic microorganisms: *Listeria monocytogenes*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella Typhimurium*, *Salmonella*

Enteritidis, *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Corynebacterium fimi*. The results showed that the *E. durans* LAB18s exhibited a high inhibition zone for all indicator microorganisms tested, except to *B. cereus*, *S. aureus* and *S. Enteritidis* when used the culture supernatant, and to *S. Typhimurium* when used the intracellular extract. It was analyzed the antioxidant activity for culture supernatant and intracellular extract of *E. durans* LAB18s by $ABTS^{\bullet+}$ and DPPH methods, and it was observed that only culture supernatant presented ability to scavenge the radical $ABTS^{\bullet+}$ and DPPH. However, when it was analyzed the sample by thiobarbituric acid reactive substances (TBARS) method, it was observed high antioxidant activity with both extracts. Thus, this isolate has strong potential use as probiotic as well as antimicrobial and antioxidant characteristics.

Keywords: *Enterococcus durans*, probiotic potencial, antimicrobial activity, antioxidant properties.

1. Introduction

Enterococci are lactic acid bacteria (LAB) that have an important role in the environment, food and clinical microbiology. Furthermore, they are regular habitants of the gastrointestinal tracts of both humans and animals (Bhardwaj et al., 2011). Enterococci also occur naturally into, or are deliberately added to fermented foods, contributing to the sensory properties (Giraffa, 2003). Moreover, several strains of the genus *Enterococcus* have also been used as probiotics, which may improve the microbial balance of the intestine or can be used in the treatment of gastroenteritis in humans and animals (Giraffa, 2003; Foulquié-Moreno et al., 2006; Bhardwaj et al., 2011).

The microflora that inhabits the human intestinal tract is part of an extremely complex ecological system. These microorganisms interact not only with other microorganisms, but also with their host. Among intestinal microflora, lactic acid bacteria (LAB) play significant roles in the gut ecosystem (Lin and Chang, 2000). The gram positive LAB has been well known for thousands of years for their important role in the food industry due to their fermentative

capacities. In current years, the role of these bacteria in health and functionality of human and livestock animal intestine have been well emphasized, mainly because of their ability to growth in low pH and to produce antimicrobial agents (Collins et al., 1998). Likewise, the antioxidant potential of LAB has been suggested by several researchers (Lin and Chang, 2000; Ou et al. 2009; Lobo et al. 2010).

Promising probiotic strains include members of the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* (Buntin et al., 2008). Many LAB, including the genus *Enterococcus*, are proved with probiotic functions, which are beneficial to the host's health when ingested in sufficient quantities. The colonization of the gut by probiotic bacteria prevents growth of harmful bacteria by competition exclusion and by the production of organic acids and antimicrobial compounds. The acid and bile tolerance are two fundamental properties that indicates the ability of a probiotic microorganism to survive through the upper gastrointestinal tract (Hyronimus et al., 2000; Erkkila and Petaja, 2000). The viability and activity of probiotic bacteria are important for survival in food during shelf life and transition through the acidic conditions of the stomach. To be potentially probiotics, bacteria must also be resistant to degradation by hydrolytic enzymes and bile salts in the small intestine (Belma and Gulcin, 2009).

Probiotics are nonpathogenic bacteria that contribute to the health and balance of the intestinal tract (Fuller, 1989). Addition of probiotics to feed is one of the alternatives to be used as a replacement for antibiotics, which have created great public concerns due to emergence of antimicrobial resistance (Patterson and Burkholder, 2003). Thus, the aim of this study was to characterize the probiotic potential of the *E. durans* LAB18s through acid and bile salts resistance, survival in simulated gastrointestinal tract conditions, and also to evaluate the antimicrobial and the antioxidant properties.

2. Materials and methods

2.1 Microorganism

The LAB18s isolated from “Minas Frescal” cheese (typical Brazilian’s soft cheese) from the collection of the Laboratory of Applied Microbiology and Biochemistry - ICTA, Federal University of Rio Grande do Sul (Porto Alegre, Brazil), was used in this study. This bacterium was identified as *E. durans* by phenotypic (Vitek® system) and molecular methods (16S rRNA gene sequence and species-specific *ddl* (D-Ala–D-Ala ligase) gene analysis).

The strain was maintained as frozen stock cultures in Brain Heart Infusion (BHI, Oxoid) containing 20% (v/v) glycerol. Bacteria was grown in BHI broth at 37°C and thereafter inoculated into plates of Brain Heart Agar (BHA, Oxoid) solidified with 1% agar and incubated at 37°C for 24 h.

2.2 Acid tolerance

The resistance of isolated under acid conditions was carried out according to Erkkila and Petaja (2000) with some modifications. *E. durans* LAB18s cells were grown in BHI without shaking at 37ºC for 24 h. Then, the culture was measured spectrophotometrically and standardized at optical density (OD_{600}) = 1.0 ± 0.05. One milliliter of standardized culture was added into tubes containing 10 mL of sterile BHI broth with the following pH values: 2.0, 3.0, 4.0 and 7.0 (adjusted using 5 M HCl), in which pH 7.0 was used as a control. Viable cell counts were determined after exposure to acidic condition for 0, 1, 2, 3 and 4 h at 37ºC. The experiment was performed in triplicate. Survival cell counts were expressed as log values of colony-forming units per mL (CFU mL^{-1}). The survival percentage was calculated as follows: % survival = final (CFU mL^{-1}) / control (CFU mL^{-1}) × 100.

2.3 Resistance to bile salts

After *E. durans* LAB18s was grown in BHI broth, bacteria were harvested by centrifugation (10.000 x g for 10 min at 4ºC) and the assessment of bacterial resistance to bile salts was performed in 10 mL of sterile BHI supplemented with a mixture of sodium cholate and sodium deoxychotale (Sigma) in a ratio of 1:1, achieving final concentrations of 0.1, 0.25, 0.5, 1.0 and 1.5% (w/v). Total

viable counts were determined after exposure to bile salt at 0, 1, 2, 3 and 4 h of incubation after serially diluting the sample and incubated at 37ºC for 24 h, and expressed as log values of colony-forming units per mL (CFU mL^{-1}) (Perelmuter et al., 2008).

2.4 Survival in simulated gastrointestinal tract

Survival in simulated gastrointestinal tract was performed according to Huang and Adams (2004). After 24 h of incubation in BHI medium, bacteria cells were harvested by centrifugation (10.000 x g for 10 min at 4 ºC), washed three times in 0.1 M PBS (phosphate buffered saline) (pH 7.2) and resuspended in 0.5% NaCl solution. Then, a 0.2 mL aliquot of bacterial suspension was inoculated into 1.0 mL of simulated gastric or intestinal juices and incubated at 37ºC for 4 h. Survival cells count were determined at initial time (0 h) and 1, 2, 3 and 4 h for the gastric transit tolerance and intestinal transit tolerance, and expressed as log values of colony-forming units per mL (CFU mL^{-1}).

Simulated gastric juice was prepared fresh daily containing 3 mg of pepsin (Sigma), 1 mL of NaCl solution (0.5%) and acidified with HCl to pH 2.0 and 3.0. Simulated intestinal juice was consisted of 1 mg of pancreatin (Merck), 1 mL of NaCl solution (0.5%) and adjusted to pH 8.0, with or without 1.5% of bile salts (1:1 mixture of sodium cholate and sodium deoxycholate). Both solutions were sterilized by filtering through 0.22 µm membranes (Millipore, Bedford, USA).

2.5 Growth profile

The growth profile of *E. durans* LAB18s was determined using a bacterium inoculum prepared in BHI and incubated at 37ºC for 24 h. Then, tubes containing 10.0 mL of BHI were inoculated with 100 µL of *E. durans* LAB18s (OD_{600}=0.127), in triplicate. Biomass development was determined after 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation at 37ºC. It also was verified the weight of biomass in all evalueted periods (0, 2, 4, 6, 8, 10, 12 and 24 h). Firstly, it was weighted in Sartorius® analytical balance (AY Precision Series -

220g capacity and precision of 0.0001 g) the petri dishes containing the filter paper. Then, 1 mL of homogenized inoculum was added on filter paper. The samples were dried into the oven at 37°C until constant weight. After drying, the weight biomass was measured in the same balance. Likewise, it was performed the viable cell count (CFU mL^{-1}) in all periods of incubation.

2.6 Preparation culture supernatant and intracellular extract

The *E. durans* LAB18s was inoculated to 10 mL of BHI and incubated at 37°C for 24 h. Aliquots of the culture were transferred to 2 mL polypropylene tubes, and centrifuged at 10.000 x g for 15 min at 4ºC. The resulting supernatant was neutralized (pH 7.0) with 1 M NaOH and heated at 95°C for 5 min (Bromberg et al. 2006). This culture supernatant was used to evaluate the antimicrobial and antioxidant activity.

For preparation of intracellular extracts, the cell pellet was washed twice with ultrapure water and resuspended in ultrapure water followed by ultrasonic disruption (40 kHz, Unique USC 700). The sonication was performed during 15 min, with five intervals of 1 min in an ice bath, with maximum potential. Cellular debris was removed by centrifugation at 10.000 x *g* for 15 min at 4ºC. The resulting supernatant was used as cell extract to evaluate the antimicrobial and antioxidant activity.

2.7 Antimicrobial activity

The indicators microorganisms used to evaluate antimicrobial activity were: *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 8739, *Bacillus cereus* ATCC 9634, *Staphylococcus aureus* ATCC 1901, *Salmonella Typhimurium* ATCC 14078, *Salmonella Enteritidis* ATCC 13076, *Pseudomonas aeruginosa* (isolated from food), *Aeromonas hydrophila* 00318 IOC/FDA 110-36 and *Corynebacterium fimi* NCTC 7547. They were suspended in 0.85% NaCl solution standardized to OD_{600} of 0.150 in spectrophotometer, which corresponded to a 0.5 McFarland turbidity standard solution. One aliquot of 20 μL of culture supernatant of *E. durans* LAB18s was applied on sterilized

cellulose discs (5 mm) onto BHA plates previously inoculated with a swab soaked in a culture of each indicator bacteria. The plates were incubated at 37ºC and inhibition zones were measured after 24 h. The same procedure was performed to evaluate the antimicrobial activity of intracellular extract. The diameter of inhibition zones was measured using a caliper and halos ≥ 7 mm were considered inhibitory (Bromberg et al., 2006). The experiment was performed in triplicate.

2.8 Thiobarbituric acid reactive substances (TBARS)

The reaction to thiobarbituric acid was performed according to the methodology of Ohkawa et al. (1979). Test tubes containing ultrapure water, extra virgin olive oil were subjected to oxidation with 100 µM ferrous sulfate and incubated in a water bath at 80ºC, for 10 min. Thereafter, to each tube was added the sample (culture supernatant or intracellular extract of the bacteria), 81 mg mL^{-1} of sodium dodecyl sulfate (SDS), buffered with acetic acid at pH 3.44 and 6 mg mL^{-1} thiobarbituric acid (TBA). The reaction mixture was further incubated in a water bath at 100ºC for 1 h. For each sample tested had a blank to either the culture supernatant or intracellular extract, and a standard control for all comparisons. The products of reaction were determined by measurement of absorbance at λ=532 nm with a spectrophotometer. The concentration of TBARS was calculated using a standard curve developed with known concentrations of 1,1,3,3-tetramethoxypropane, and results were expressed as nmol of malonaldehyde (MDA) mL^{-1} of sample. The experiment was performed in triplicate.

2.9 Antioxidant capacity using $ABTS^{\bullet+}$ method

The antioxidant activity was determined using $ABTS^{\bullet+}$ (2,2 azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation method (Re et al., 1999) with some modifications described by Rossini et al. (2009). $ABTS^{\bullet+}$ was dissolved in water (7 mM). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final

concentration) and allowing the mixture to stand in dark at room temperature for 16 h before use. Stock solution was used for a maximum of 3 days. Before the use, the $ABTS^{\bullet+}$ solution was diluted with ethanol, to an absorbance of 0.700 ± 0.020 at 734 nm. Samples were diluted with ethanol to obtain inhibition between 20 to 95% of the blank absorbance. Ascorbic acid was used as the standard in the range 0–9 μg mL^{-1}. After addition of 10 μL of sample (or standards) in 1.0 mL of $ABTS^{\bullet+}$ solution, the absorbance was read at 30 sec interval for 5 min. Likewise, a same proportion (10 μL) of culture medium or ultrapure water with 1.0 mL of $ABTS^{\bullet+}$ solution, were used as a control. All determinations were carried out at least three times. The percentage inhibition of absorbance at 734 nm was calculated using ascorbic acid standard curve.

2.10 Scavenging ability on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals

The DPPH method was used as described by Brand-Williams et al. (1995), based on the capture of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical for antioxidants, producing a decrease in absorbance at 515 nm. The DPPH was used at a concentration of 60 μM, dissolved in methyl alcohol. The solution was homogenized and transferred to a dark glass bottle. The prepared solution was used only in the day of analysis. In the dark, aliquots of 0.1 mL of sample (culture supernatant or intracellular extract) were transferred to test tubes with 3.9 mL of radical DPPH (60 μM DPPH solution) and homogenized by shaking. As a control, it was used 0.1 ml control solution (prepared from 50% methyl alcohol, 70% acetone and water) mixed with 3.9 ml of DPPH performed under the same conditions for the samples. Methyl alcohol was used as a blank. The standard curve was DPPH in the range between 0 to 60 μM. The results were expressed as EC_{50} (μg mL^{-1}), which is the minimum antioxidant concentration required to reduce 50% of the initial DPPH reaction from the time the extract reached stability.

3. Results

3.1 Tolerance to pH, bile salts and gastrointestinal juices

The isolate *E. durans* LAB18s exhibited high tolerance after exposition to acidic conditions (Table 1). The resistance of the isolate was observed in all acidified media, except for pH 2, which confirmed the presence of colonies only in the initial time (>7 log CFU mL^{-1}). In the other treatments, pH 3 and pH 4, no significant difference was observed when the samples were compared to the control (pH 7) during the incubation time, ranging between 0 and 4 h.

Table 1. Acid tolerance of *E. durans* LAB18s after exposure to acidic conditions in HCl (pH 2, 3 and 4) during 4 h of incubation at 37°C with shaking.

Time (h)	Control (pH 7)	pH 2	pH 3	pH 4
	log CFU mL^{-1}			
0	7.49±0.009*	7.18±0.023	7.35±0.013	7.40±0.011
1	7.97±0.020	0.00±0.000	7.33±0.008	7.33±0.022
2	8.33±0.007	0.00±0.000	7.06±0.037	7.33±0.022
3	8.69±0.013	0.00±0.000	6.97±0.036	7.31±0.020
4	8.81±0.020	0.00±0.000	6.91±0.031	7.32±0.015

* Values represent the mean ± standard error.

The tolerance of the *E. durans* LAB18s in the presence of different concentrations of bile salts was analyzed (Figure 1). The results showed that *E. durans* LAB18s was able to survive at all bile salt concentrations under study (0.1, 0.25, 0.5, 1.0 and 1.5%) to give an exponential growth from the inoculation (0 h) up to 4 h of incubation. There were no significant differences between control (BHI, pH 7, without the addition of bile salts) and different treatments with bile salts.

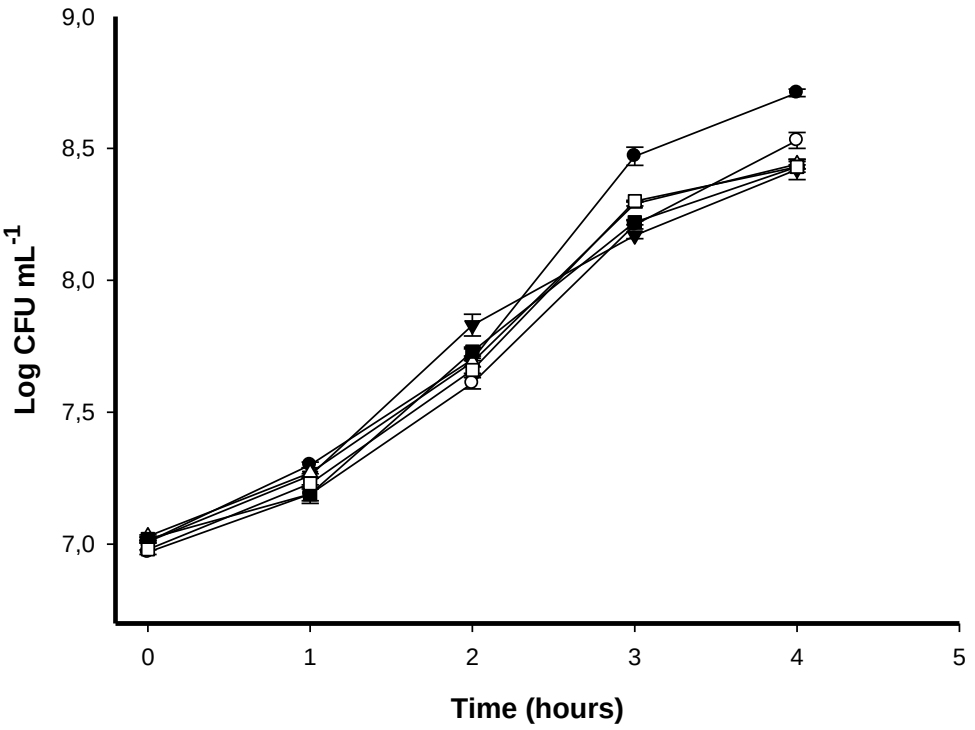

Figure 1. Tolerance of the *E. durans* LAB18s to bile salts concentration, containing 0.1% of bile salts (○); 0.25% (▼); 0.5% (∆); 1.0% (■); and 1.5% (□) after 4 h of incubation at 37°C without shaking. The pH 7.0 without bile salts was used as a control (●). Error bars represent standard errors.

To analyze the ability of *E. durans* LAB18s survive in the presence of simulated gastric juice, the strain was incubated at 37°C for 4 h (Figure 2). It was observed that pH 3 showed no significant difference in viability cell compared to the control in any of the evaluated times. When it was analyzed the resistance in pH 2, it was observed that viability of cells was apparent only at time 0 (6.53 log CFU mL^{-1}), and after 1 h, the cell counts was below the detection limit. It shows that the isolate *E. durans* LAB18s was not resistant to simulated gastric juice at pH 2.

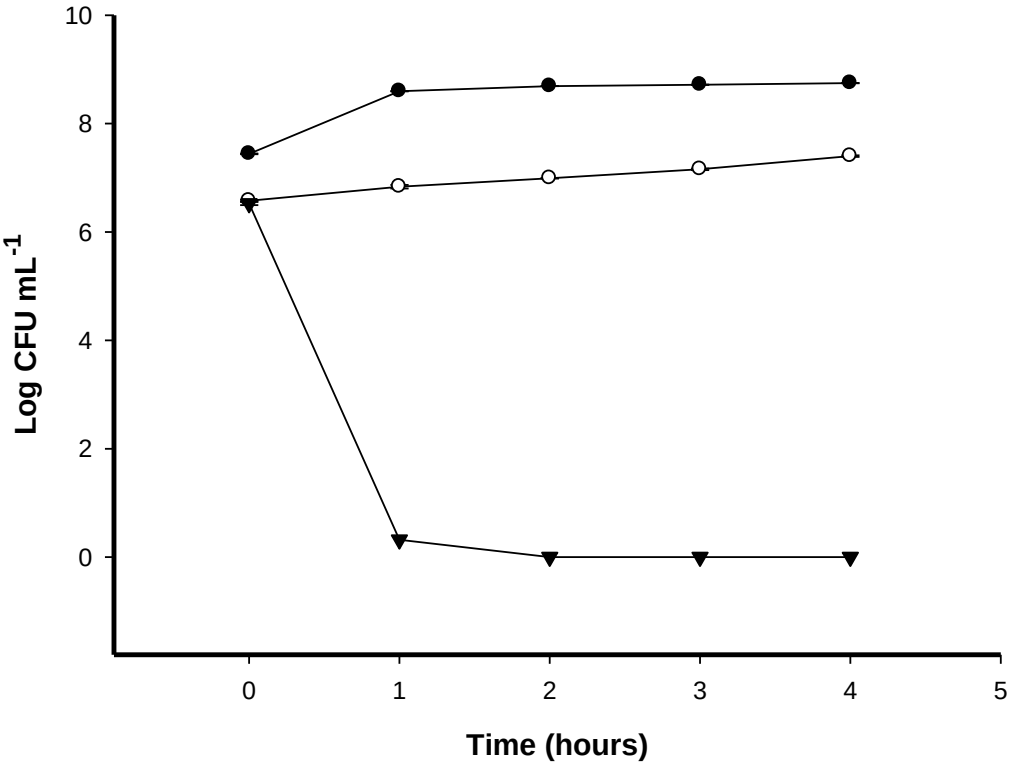

Figure 2. Resistance of the *E. durans* LAB18s to simulated gastric juice containing pepsin and acidified at pH 2.0 (▼) and 3.0 (○) after 4 h of incubation at 37°C without shaking. The pH 7.0 without pepsin was used as acontrol (●). Error bars represent standard errors.

Figure 3 shows the survival of *E. durans* LAB18s in the presence of simulated intestinal juice. The results indicate that no significant decrease in final viability (after 4 h incubation) of *E. durans* LAB18s to pH 8.0 with pancreatin (8.34 log CFU mL^{-1}), and pancreatin at pH 8.0 containing 1.5 % of bile salts (8.56 log CFU mL^{-1}) in relation to the control (8.75 log CFU mL^{-1}), thus demonstrating that the *E. durans* LAB18s presents resistance when exposed to the simulated intestinal juice.

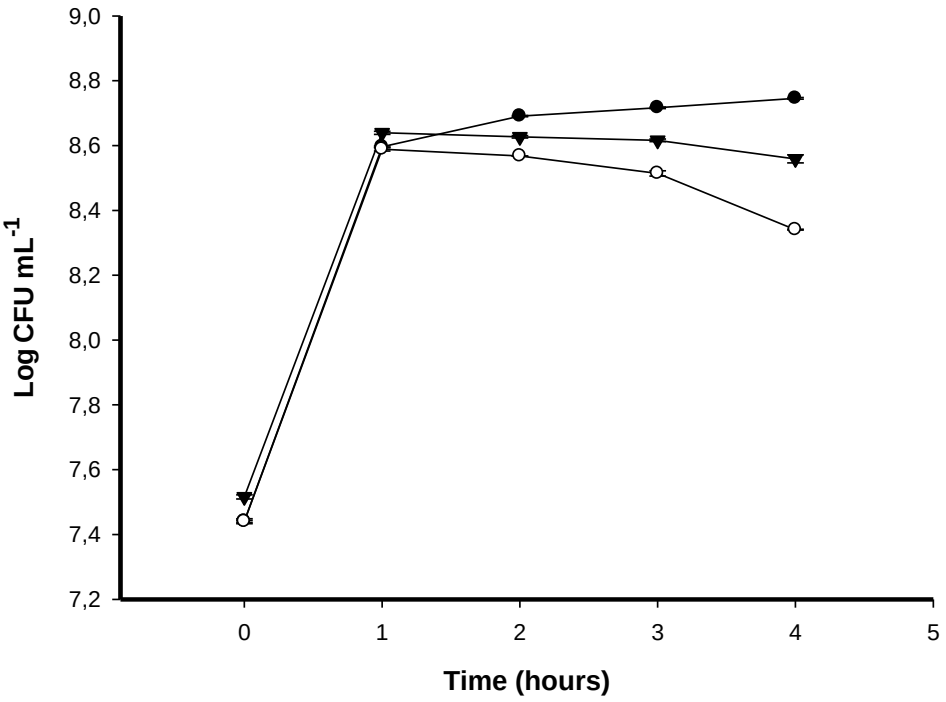

Figure 3. Resistance to simulated intestinal juice of *E. durans* LAB18s containing pancreatin (pH 8) (●); pancreatin containing 1.5% bile salts (sodium cholate and sodium deoxycholate, 1:1) (▼); and medium at pH 7.0 without treatment as the control (○) after 4 h of incubation at 37°C without shaking. Error bars represent standard errors.

3.2 Growth profile

Figure 5 shows the growth profile of the isolate *E. durans* LAB18s during the time course. The results showed that the isolate *E. durans* LAB18s had an exponential growth until 4 h of incubation, and after that it was maintained the same levels of OD and biomass production around 1.4 and 38 mg mL^{-1}, respectively. On the other hand, the isolate colony-forming unit was declined after 12 h of incubation from 9.2 to 9 Log CFU mL^{-1}.

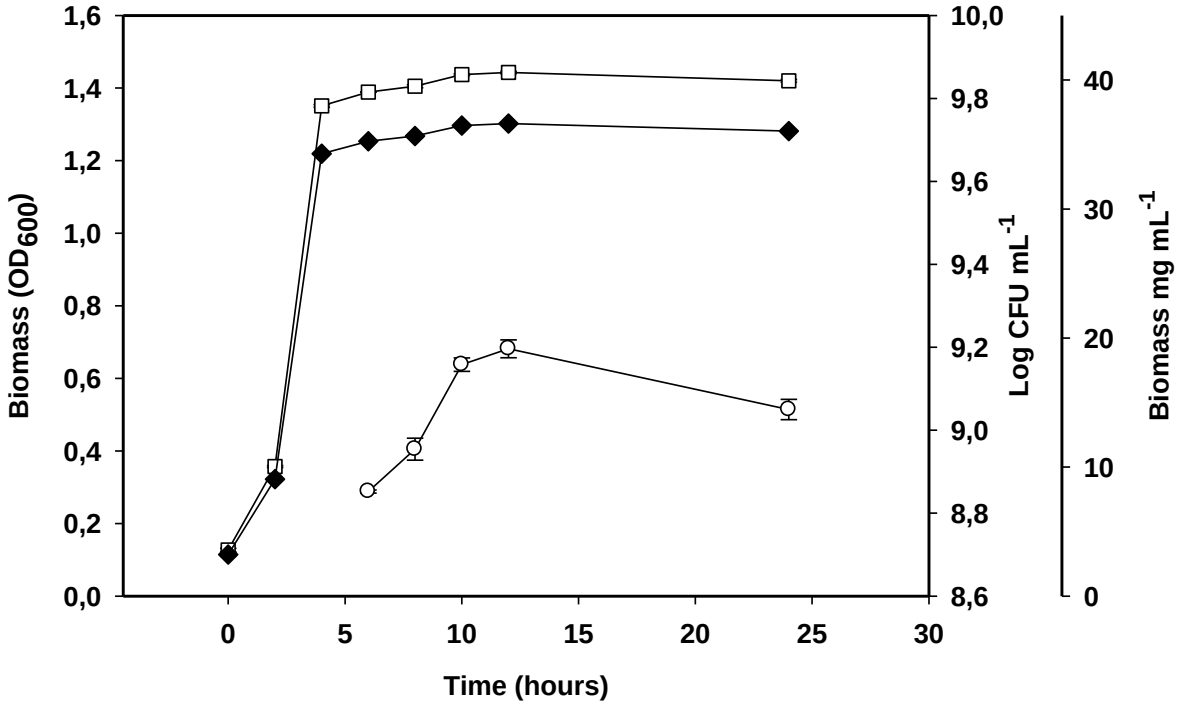

Figure 4. Biomass production for optical density (OD_{600}) (□); colony-forming units (Log CFU mL^{-1}) (○); and weight biomass (mg mL^{-1}) (♦) after growth of the isolate *E. durans* LAB18s in BHI at 37°C for 24 h. Error bars represent standard errors.

3.3 Antimicrobial activity

The isolate *E. durans* LAB18s exhibited antimicrobial activity against different indicator microorganisms, including *L. monocytogenes*, *E. coli*, *B. cereus*, *S. aureus*, *S. Typhimurium*, *S. Enteritidis*, *P. aeruginosa*, *A. hydrophila* and *C. fimi* (Table 2).

The highest inhibitory activity using the intracellular extract was observed against the microorganism *P. aeruginosa*, followed by *S. aureus*, *A. hydrophila* and *C. fimi*. When the culture supernatant was tested, it was observed that the highest antimicrobial activity was against the indicator microorganism *L. monocytogenes* (8.7 mm).

Table 2. Antimicrobial activity using the culture supernatant and intracellular extracts of *E. durans* LAB18s against indicators microorganisms. The results were expressed as means (inhibition zones (mm)) ± standard error of three independent experiments.

Indicators microorganisms	Culture supernatant	Intracellular extract
	Inhibition zone (mm)	
L. monocytogenes	8.70±0.29*	7.30±0.07
E. coli	7.20±0.14	7.15±0.07
B. cereus	*0.00±0.00	7.50±0.35
S. aureus	*0.00±0.00	9.10±0.07
S. Typhimurium	7.55±0.42	*0.00±0.00
S. Enteritidis	*0.00±0.00	7.30±0.14
P. aeruginosa	7.50±0.35	10.5±0.03
A. hydrophila	7.80±0.14	8.70±0.56
C. fimi	7.25±0.18	8.50±0.35

*Inhibition zones with values ≤ 7 mm, means no antimicrobial activity.

The smaller inhibition halos observed with both culture supernatant and intracellular extract were against *E. coli* (about 7.2 mm). Moreover, it was noted a positive antimicrobial activity in both culture supernatant and intracellular extract, against the indicator microorganisms *L. monocytogenes*, *E. coli*, *P. aeruginosa*, *A. hydrophila* and *C. fimi*.

3.4 Antioxidant activity

The antioxidant activity was evaluated by three different methods: $ABTS^{\bullet+}$, DPPH and TBARS (Table 3).

The culture supernatant of *E. durans* LAB18s exhibited high ability to scavenge the radical $ABTS^{\bullet+}$, whereas the sample of intracellular extract showed weak antioxidant activity, with an inhibitory percentage of 9.4%.

Table 3. Determination of antioxidant activity of the culture supernatant and intracellular extract of *E. durans* LAB18s by $ABTS^{\bullet+}$, DPPH and TBARS methods. The results were expressed as mean ± standard error of three independent experiments.

Methods	**Control**	*E. durans* LAB18s
$ABTS^{\bullet+}$	**--- % ---**	
Culture supernatant	100[a]	71.9±3.52 [b]
Intracellular extract	100[a]	9.4±0.21[a]
DPPH	**--- EC_{50} µg mL^{-1} ---**	
Culture supernatant	9.8±0.01[a]	3.6±0.01[b]
Intracellular extract	9.5±0.08[a]	8.8±0.12[a]
TBARS	**--- nmol MDA mL^{-1} ---**	
Culture supernatant	0.875±0.02[a]	0.468±0.01[b]
Intracellular extract	0.755±0.02[a]	0.485±0.09[b]

For DPPH method, the analysis conducted with culture supernatant showed high antioxidant activity (EC_{50} = 3.6) compared to the control (EC_{50} = 9.77). The intracellular extract of *E. durans* LAB18s showed low antioxidant activity (EC_{50} = 8.79) compared to the control (EC_{50} = 9.48).

When the samples were evaluated with culture supernatant or intracellular extract by the TBARS method, it was observed that both extracts showed high antioxidant activity as compared to the control (Table 3). These results indicate that *E. durans* LAB18s can be considered a bacterium with high antioxidant potential and may be useful reducing the oxidative damage in food and feed.

4. Discussion

The *E. durans* LAB18s isolated from “Minas Frescal” cheese showed in this study probiotic characteristics. The *Enterococcus* species most commonly isolated from cheese are *E. faecalis* and *E. faecium*, followed by *E. durans* (Giraffa, 2003; Martín-Platero et al., 2009). Enterococci can be used in the food industry as starter or probiotic cultures (Gomes et al., 2008). A higher prevalence of enterococci in processed foods may be attributed to their resistance to heat, extreme salinity and harsh conditions during ripening of fermented foods (Giraffa, 2003; Jurkovic et al., 2006). In contrast to human nutrition, where lactobacilli are frequent probiotics, *Enterococcus* sp. and *Saccharomyces* are frequently used as probiotics for animal nutrition (Strompfová et al., 2006; Marciňáková et al., 2006; Lauková et al., 2008). The major factors determining survival of these bacteria include particular characteristics of the strains (e.g., acid and bile tolerance, and resistance to gastric and intestinal juice), composition of food ingested, and competition of microflora in the intestine (Succi et al., 2005).

In order to select isolates with probiotic characteristics, the resistance to pH and bile salts is an importance factor in survival and growth of bacteria in animal gastrointestinal tract. Results from this study showed that *E. durans* LAB18s has acid and bile tolerance, surviving to exposure in pH 3.0 and 4.0, and likewise, in all concentrations of bile salts tested (from 0.1% to 1.5%). Bhardwaj et al. (2010) mention that the reports concerning the in vitro probiotic characteristics of lactobacilli are abundant, while those dealing with probiotic characteristics of enterococci are relatively scarce. The probiotic strain of *E. faecium* Fargo 688® could survive in the porcine gastric juice at pH 2.0 only for 8 min (Gardiner et al. 1999). Sun et al. (2010) reported that strain *E. faecium* SF68 retained viability and increased in number between 30 and 60 min of exposure to bovine bile, exhibiting an intrinsic tolerance towards bovine bile. When exposed to simulated gastric juice for 20 and 60 min, the *E. faecium* SF68 exhibited a survival rate (62 and 56%, respectively) that would allow it to pass through the stomach. Also, this strains retained 92% viability of the initial counts when exposed to simulated small intestinal juice for 120 min, indicating

that *E. faecium* SF68 can be classified as tolerant to the gastrointestinal secretions. In this study, the *E. durans* LAB18s survived in all times tested (1, 2, 3 e 4 h) at pH 3 and pH 4. In general, the acid tolerance of LAB depends on the pH profile of H^+-ATPase and the composition of the cytoplasmatic membrane. This is largely influenced by the type of bacterium, the composition of growth medium and the incubation conditions (Madureira et al., 2005).

High acidity in the stomach and the high concentration of bile components in the proximal intestine of the host, influence the selection of potential probiotic strains (Hyronimus et al. 2000). However, small intestine tolerance is potentially more important than gastric survival. With the development of new delivery systems and the use of specific foods, some studies indicate that acid-sensitive strains can be buffered through the stomach. However, to promote a positive effect to the host, probiotics need to survive and colonize the small intestine, and is the condition of this environment that may be an essential criterion for future probiotics (Huang and Adams, 2004).

In this study, the *E. durans* LAB18s demonstrated high ability to survive in the presence of simulated gastric juice containing pepsin (pH 3.0) and simulated intestinal juice containing pancreatin (pH 8, with or without addition of bile salts). It also demonstrated the viability of *E. durans* LAB18s was satisfactory when exposed to pH 3.0 and 4.0, although it has shown a reduced in cell counts in pH 2 even in the first hour.

The pH of the stomach is between 2.5 and 3.5; although, it may be lower during prolonged fasting (pH 1.5), or higher after a meal (pH 4.5) (Huang and Adams, 2004). This work is part of a larger research project, which aims to use the *E. durans* LAB18s enriched with selenium for use in feed for broilers. Thus, the isolate survived in a short time at pH 2.0, this data can not interfere with the probiotic ability, because it is intended to apply the strain concurrently with the feed, and thus the pH of the stomach is likely to be greater than 2.0. So, the *E. durans* LAB18s survived upper to gastrointestinal transit, and this tolerance to simulated gastric juice and simulated intestinal juice is very important to *E. durans* LAB18s be considered as an alternative source for development of future probiotic supplements.

The antimicrobial and antioxidant activity from LAB has been related in the literature (González et al., 2007; Ou et al., 2009; Belgacem et al., 2010; Lobo et al., 2010). The strain *E. durans* LAB18s showed high broad spectrum of antimicrobial activity, inhibiting *L. monocytogenes*, *E. coli*, *S. Typhimurium*, *P. aeruginosa*, *A. hydrophila* and *C. fimi* using the culture supernatant, and all indicator microoganisms tested (except for *S. Typhimurium*) using the intracellular extract. The antibacterial activity of LAB may be due to the production of organic acids, with a consequent reduction in pH, production of hydrogen peroxide, or bacteriocins (González et al., 2007). In the same way, Foulquié-Moreno et al. (2006) and Belgacem et al. (2010), reported that a considerable number of strains belonging to different *Enterococcus* species display many interesting biotechnological properties such as proteolytic, lipolytic, esterolytic and other enzymatic activities, citrate utilization, and bacteriocin production. These characteristics are relevant to their technological performance, and suitable strains may be selected for application in food fermentations or as probiotic.

In this study, the antioxidant effects were found in the culture supernatant of *E. durans* LAB18s when analyzed for $ABTS^{\bullet+}$ and DPPH methods, and when analyzed by TBARS method it was observed antioxidant ability with the culture supernatant and intracellular extract of *E. durans* LAB18s. According to Lin and Yen (1999), the intracellular extracts of some LAB have metal ion chelating ability, and reactive oxygen species scavenging ability. Indeed, intact cells of LAB were found to possess antioxidant activity *in vitro* (Ou et al., 2009). Nevertheless, using the intact cells as the delivery vehicles passing through the gastrointestinal tract, the intracellular constituents released from the LAB in gastrointestinal tract can be also antioxidative. Consumption of LAB containing foods or supplements may be recommended as healthy. It is well established that a wide variety of oxygen-centered free radicals and other reactive oxygen species are continuously produced in the human body and in food systems (Lobo et al., 2010). Besides the long history of consumption, which proves the safety of consuming of LAB, they have been reported to have health-promoting characteristics that make these microorganisms desirable for use in the production of various health and functional foods.

5. Conclusions

The results obtained in this study suggest that *E. durans* LAB18s is a resistant strain to pass through the gastrointestinal tract. The viability of this strain through the exposure rate and the combination of simulated gastric juice and bile salts, intestinal juice, bile and acid tolerance were also observed. The *E. durans* LAB18s exhibited some desirable probiotic properties *in vitro*, such as antimicrobial activity and antioxidant ability, which were evidenced in both culture supernatants and intracellular extracts. Further investigations may be warranted to elucidate its potential health benefit and its application as promising probiotic strain in the feed animal industry.

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5. CAPÍTULO III

Evaluation of resistance genes and virulence factors in a food isolated *Enterococcus durans* with potential probiotic effect

Evaluation of resistance genes and virulence factors in a food isolated *Enterococcus durans* with potential probiotic effect

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Abstract

Enterococci are inserted into the lactic acid bacteria (LAB) group, which are often considered to provide benefit to the host organism when consumed. However, these microorganisms have a potential as agent of infections, but little is known about its virulence. This study aimed to detect genes with potential virulence factors related with adhesion, aggregation, biofilm formation and resistance to vancomycin, likewise to evaluate the antibiotic susceptibility and adhesion capacity of a food isolated from “Minas Frescal” cheese, LAB18s, identified as *Enterococcus durans*. The results from this study showed that the strain *E. durans* LAB18s did not present any of the profiles of virulence factors when evaluated for adhesion collagen protein (*ace*), genes of aggregation substances (*agg* and *asa*), *bopA* (putative glycosyltransferase), *bopB* (beta-phosphoglucomutase), *bopC* (aldose 1-epimerase), and *bopD* (sugar-binding transcriptional regulator). Likewise, the strain *E. durans* LAB18s did not present resistance to the genes *vanA*, *vanB*, *vanC1* and *vanC2/*3, and exhibited sensibility to antibiotics, commonly used in animal feed, such as erythromycin, tetracycline, vancomycin, gentamicin and penicillin, as well as, to E-test®. *E. durans* LAB18s showed a strong capacity of biofilm formation. This strain also

exhibited satisfactory auto-aggregative and hydrophobicity features. The results indicate that this bacterium can be used in animal feed, do not presenting genes for resistance and virulence factor, and can bring many benefits with its consumption to animal health.

Keywords: enterococci; resistance genes; virulence factors; biofilms; safety.

1. Introduction

The genus *Enterococcus* belongs to a group of microorganisms known as lactic acid bacteria (LAB). They are ubiquitous bacteria, but their primary habitat is the intestine of health warm-blooded animals, a complex ecosystem in which a delicate balance exists between the intestinal microbiota and the host. In this system, *Enterococcus faecalis* and (to a lesser extent) *Enterococcus faecium* are the most dominant species (Cebrián et al., 2012). Enterococci may play beneficial roles in various traditional food products as they contribute to the ripening and aroma development of certain cheeses or fermented sausages (Franz et al., 2011).

Certain enterococcal strains are also successfully used as probiotics to improve human or animal health. These bacteria ingested in high numbers may achieve functional or probiotic effects especially for treatment of diseases such as irritable bowel syndrome, diarrhea or antibiotic associated diarrhea, or for health improvement such as lowering cholesterol levels or immune regulation (Franz et al., 2011). As the site of activity is the gastrointestinal system, these beneficial effects are brought about by the interaction of the enterococci with the gut microbial populations. Likewise, the use of enterococci in animal feed has similar goals, i.e. either to prevent disease by influencing the gastrointestinal micro-populations or stimulation of the immune system (Gaggìa et al., 2010; Franz et al., 2011).

On the other hand, other enterococcal strains are associated with nosocomial infections and cause human diseases such as endocarditis, bacteremia, and infections of the urinary tract, central nervous system, abdomen, and pelvis (Foulquié Moreno et al., 2006). Such pathogenic strains

often carry multiple antibiotic resistances and virulence factors such as adhesins and hemolysins. The role of some enterococci in human disease raises concerns for their safe use as either starters in food production or as probiotics. Studies on the incidence of virulence factors have shown that isolates from food can also contain virulence factors and antibiotic resistances (Franz et al., 2001; Trivedi et al., 2011). Generally, the occurrence of virulence determinants appears to be higher in *E. faecalis* strains than in *E. faecium* strains isolated from foods (Leavis et al., 2004; Franz et al., 2011; Cebrián et al., 2012).

The *Enterococcus* strain evaluated in this study, isolated from cheese, has being investigate as its capability for selenium bioaccumulation in biomass, potential probiotic, antimicrobial and antioxidant activity for possible application as probiotic enriched inoculum in feed animal. However, to guarantee the safe use of this strain as probiotic culture in animal feed, it is necessary to evaluate also the presence of potential virulence and resistance factors. Thus, the aim of this study was to detect genes involved in adhesion, aggregation, biofilm formation, resistance genes to vancomycin and susceptibility for antibiotics; also to evaluate the adhesion capacity by auto-aggregation and hydrophobicity.

2. Materials and methods

2.1. Microorganism and culture conditions

The strain *E. durans* LAB18s was isolated from Minas Frescal cheese (typical Brazilian soft cheese), and identified by phenotypic (Vitek® system) and molecular methods (16S rRNA gene sequence and species-specific *ddl* (D-Ala–D-Ala ligase) gene analysis) as *E. durans*. The strain was maintained in stock culture at -20°C in Brain Heart Infusion (BHI; Oxoid) containing 20% (v/v) glycerol. The bacterium was grown in BHI at 35°C and after, inoculated on Brain Heart Agar (BHA) plates and incubated at 35°C for 24 h for further evaluations.

2.2. DNA extraction

The strain was grown in BHI medium at 35°C for 24 h. Thereafter, plates were inoculated with BHA and, then, incubating at 35°C for 24 h to estimate the purity of the culture. Subsequently, colony forming units of bacterial cells were collected and then the DNA was extracted using the kit Promega Wizard Genomic DNA Purification Kit ™ (Cat # A1125).

2.3. Detection of virulence and resistance genes

The strain *E. durans* LAB18s was analyzed for the presence of virulence genes *ace* (adhesion collagen protein), *agg* (aggregation), *asa* (aggregation), *bopA*, *bopB*, *bopC* and *bopD* (biofilm formation). The polymerase chain reaction (PCR) was performed in a total volume of 25 µL containing: 2 µL of DNA template, 1.5 mM $MgCl_2$, 10 mM of each primer, 200 mM of each dNTP, 1 unit (U) of DNA Taq Polymerase and 1x reaction buffer.

The LAB was also evaluated for resistance genes *vanA*, *vanB*, v*anC*1 and *vanC2/3* by PCR. The PCR reaction was performed in a total volume of 25 µL containing: 2 µL of DNA template, 2.0 mM $MgCl_2$, 10 mM of each primer, 200 mM of each dNTP, 1 unit (U) of DNA Taq Polymerase and 1x reaction buffer.

The primer sequences are described in Table 1. The PCR conditions for all genes were denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing extension according to Table 1, followed by final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis with 1.5% agarose gels, stained with 5% (w/v) ethidium bromide solution and visualized under ultraviolet light.

Table 1. PCR primers and the annealing temperature used to detect the virulence genes.

Primer	Sequence (5'-3')	Product (bp)	*AT (ºC)	Reference
ace f	AAAGTAGAATTAGATCACAC	320	48	Duprè et al.
ace r	TCTATCACATTCGGTTGCG			(2003)
agg f	AAGAAAAAGTAGACCAAC	1553	48	Eaton e Gasson
agg r	AACGGCAAGACAAGTAAATA			(2001)
asa f	GATACAAAGCCAATGTGGTTCCT	101	48	Dunny et al.
asa r	TAAAGAGTCGCCACGTTTCACA			(1979)
bopA f	CAGCGACATGGACAGCCTAC	108	48	Vebø (2009)
bopA r	TTGCAGGACCGTCGAGTAAA			
bopB f	ATGACAGAATCCAAAACTGC	687	48	Cassenego et al.
bopB r	TTACGAAGGGGTTGATTCAC			(Unpublished results)
bopC f	TTATAGAAGGTTAAATTGAT	1010	48	Cassenego et al.
bopC r	ATGAAGGATAATCGTATCAC			(Unpublished results)
bopD f	GGCTTCCTCGTTGATGGCTTC	126	48	Hufnagel et al.
bopD r	ACGGCACGGAATTTGGGTAAAC			(2004)
v*anA* f	GGGAAAACGACAATTGC	732	50	Depardieu et al.
vanA r	GTACAATGCGGCCGTTA			(2004)
vanB f	ACGGAATGGGAAGCCGA	647	54	Depardieu et al.
vanB r	TGCACCCGATTTCGTTC			(2004)
vanC1 f	GGTATCAAGGAAACCTC	822	54	Dutka-Malen et al.
vanC1 r	CTTCCGCCATCATAGCT			(1995)
vanC2/3 f	CGGGGAAGATGGCAGTAT	848	54	Dutka-Malen et al.
vanC2/3 r	CGCAGGGACGGTGATTTT			(1995)

*AT - annealing temperature

2.4. Antibiotic susceptibility test

The antibiotic susceptibility testing was performed according to standard method disk diffusion recommended by Clinical and Laboratory Standards Institute (CLSI, 2008). The isolate was inoculated onto Mueller-Hinton (MH) agar plates and incubated at 35°C for 24 h. After growth, the colonies were resuspended in sterile saline solution (9 g/L NaCl) and spectrophotometrically adjusted to an OD_{600} 0.150 ± 0.02, which it corresponds to 0.5 McFarland scale.

Then, using swabs, plates with MH agar were inoculated with the standardized solution. Five commonly used antibiotics in animal feed were tested: erythromycin-15 (ERI), tetracycline-30 (TET), vancomycin-30 (VAN), gentamicin-120 (GEN) and penicillin-10 (PEN). The minimal inhibitory concentration (MIC) for vancomycin was determined using the E test® method according to the manufacturer's instructions. The diameter of inhibition zones were measured after incubation for 24 h at 35°C. The isolate was classified according to the CLSI criteria for susceptible, intermediate and resistant to the antibiotic susceptibility (CLSI, 2008). Data were expressed in millimeter (mm). The experiment was performed in triplicate in three independent experiments.

2.5. Evaluation of biofilm formation capability

The isolate was analyzed for its ability of biofilm formation according with Stepanovic et al. (2000). The strain was previously inoculated in a plate containing BHA and incubated at 35°C for 24 h. Microtitration plates were filled with 180 µL of sterile BHI. After overnight growth, microorganism colonies were resuspended in saline solution and spectrophotometrically adjusted at 0.150 ± 0.02 (OD_{600}). Then, 20 µL of solution were inoculated in each well containing 180 µL BHI. *Staphylococcus epidermidis* ATCC 25923 was used as a positive control, and a negative control was performed with BHI alone. The plates were covered and incubated at 35°C for 24 h. After growth, the cultures were aspirated with a multichannel pipette and the wells were washed three times with 200 µL saline solution. The micro-plate was inverted onto absorbent paper to dry and subsequently the samples were fixed with 150 µL of methanol (CH_3OH) for 20 min. After this time, methanol was discarded and the plates were maintained reversed overnight. The samples were stained with 150 µL crystal violet (5 g/L) for 15 min. After the plates were inverted and excess was removed under running water. Then, after a short drying period 150 µL ethanol (95% v/v) was added. The plates were maintained for 30 min and then the absorbance was measured with microplate reader (Anthos 2010 17550 Type 4894) at 450 nm.

Based on the OD produced by bacterial films, strains were classified into the following categories: no biofilm producers (0), weak (+), moderate (++) or strong biofilm producers (+++), as previously described by Stepanovic et al. (2000). Briefly, the cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows: OD ≤ ODc = no biofilm producer, ODc < OD ≤ (2 x ODc) = weak biofilm producer, (2 x ODc) < OD ≤ (4 x ODc) = moderate biofilm producer and (4 x ODc) < OD = strong biofilm producer. All tests were carried out in triplicate.

2.6. Determination of cell surface hydrophobicity

The hydrophobicity of cell surface was determined *in vitro* according to Rosenberg et al. (1980) with some modifications. Briefly, the isolate was inoculated in BHI and incubated at 35°C without agitation. After 24 h, the cells were collected by centrifugation at 10,000 x *g* for 10 min at 4°C, washed twice and resuspended in 10 mM phosphate buffered saline (PBS) pH 7.2, and the OD_{600} was spectrophotometrically adjusted to 0.800 ± 0.05 (A_0). An aliquot of 0.6 mL of either xylene or chloroform was added to tubes containing 3 mL of bacterial suspension. The solution was homogenized with vortex for 2 min. The tubes were allowed to stand at 35°C for 2 h for phase separation. After this period, the aqueous phase (A) was carefully removed and measured spectrophotometrically (OD_{600}). The cell surface hydrophobicity was calculated from three replicates using the following equation: $H\% = [(A_0 - A) / A_0) \times 100]$.

Two solvents were evaluated in this study. The xylene was used as an apolar solvent, and chloroform, which is considered an acid and monopolar solvent. In terms of bacterial adhesion, only xylene reflects the cell surface hydrophobicity. The values obtained with chloroform were considered as an electron acceptor characteristic of the bacteria (Bellon-Fontaine et al., 1996).

2.7. Auto-aggregation

The auto-aggregation was evaluated according to Juárez Tomás et al. (2005) with some modifications. The strain was grown in BHI at 35°C for 24 h.

After this, cells were sampled by centrifugation at 10,000 x *g* for 10 min at 4°C, washed twice and resuspended in 10 mM of PBS (pH 7.2), and the OD_{600} was spectrophotometrically adjusted to 0.600 ± 0.05 (A_0). The cell suspension was incubated at 35°C, and the ability to auto-aggregation was measured spectrophotometrically (OD_{600}) during 4 h (At) and after 24 h (At). The auto-aggregation was determined using the following equation: % AA = [(A_0 – A_t)/A_0) x 100].

3. Results

3.1. Analysis of presence of virulence genes

The presence of virulence genes was evaluated by PCR, and the presence of adhesion collagen protein gene (*ace*), *agg* and *asa* considered genes of aggregation substances were investigated (Figure 1).

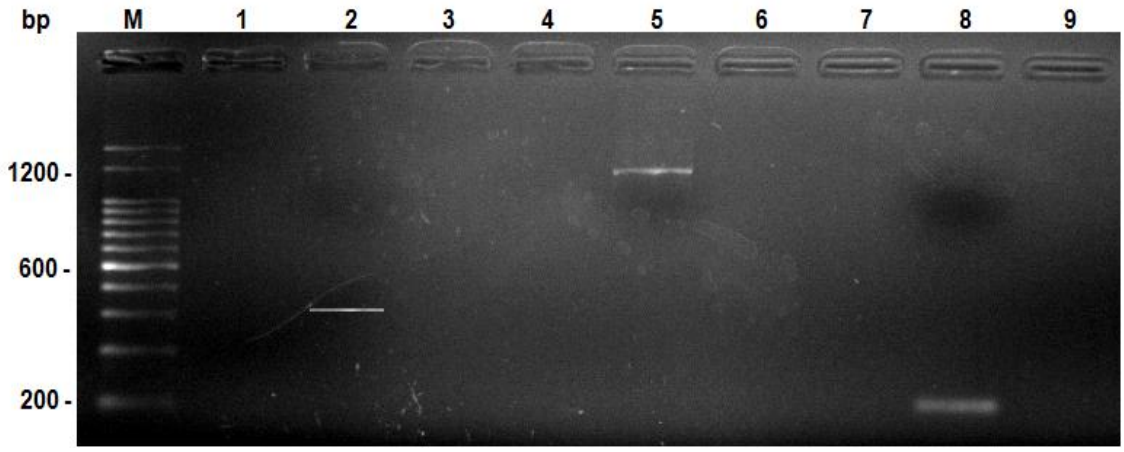

Figure 1. Amplification of virulence-associated genes: *ace*, *agg* and *asa*. The letter bp indicates bases pair; the letter M indicates the molecular size marker (100 bp DNA Ladder, Invitrogen) and the numbers are the respective samples: (1) the negative control of the *ace* gene; (2) the *positive control of the *ace* gene; (3) the absence of the *ace* gene in the *E. durans* LAB18s; (4) the negative control of the *agg* gene; (5) the positive control of the *agg* gene; (6) the absence of the *agg* gene in the *E. durans* LAB18s; (7) the negative control of

the *asa* gene; (8) the positive control of the *asa* gene; and (9) the absence of the *asa* gene in the *E. durans* LAB18s. * It was used as positive control to the evaluated genes strain vancomycin-resistant *Enterococcus faecalis* ID 2389.

The genes *bopA* (putative glycosyltransferase), *bopB* (beta-phosphoglucomutase), *bopC* (aldose 1-epimerase), and *bopD* (sugar-binding transcriptional regulator) involved in the metabolism of maltose and biofilm formation were also evaluated (Figure 2).

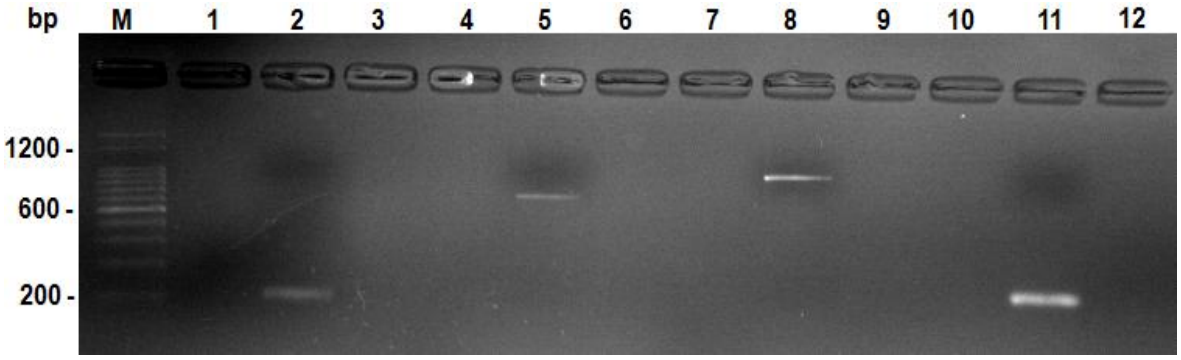

Figure 2. Amplification of virulence-associated genes: *bopA*, *bopB*, *bopC* and *bopD*. The letter bp indicates bases pair; the letter M indicates the molecular size marker (100 bp DNA Ladder, Invitrogen) and the numbers represent: (1) negative control of the *bopA* gene; (2) *positive control of the *bopA* gene; (3) the absence of the *bopA* gene in the *E. durans* LAB18s; (4) negative control of the *bopB* gene; (5) positive control of the *bopB* gene; (6) the absence of the *bopB* gene in the *E. durans* LAB18s; (7) negative control of the *bopC* gene; (8) positive control of the *bopC* gene; (9) the absence of *bopC* gene to *E. durans* LAB18s; (10) negative control *bopD* gene; (11) the positive control of the *bopD* gene; and (12) the absence of the *bopD* gene in the *E. durans* LAB18s. *It was used as positive control to the evaluated genes the strain vancomycin-resistant *Enterococcus faecalis* ID 2389.

The strain *E. durans* LAB18s did not present any of the profiles of virulence factors evaluated. Likewise, *E. durans* LAB18s was evaluated for

resistance genes *vanA*, *vanB*, *vanC1* and *vanC2/3*. It was showed in the Figure 3 that the *E. durans* LAB18s did not present any of these resistance genes.

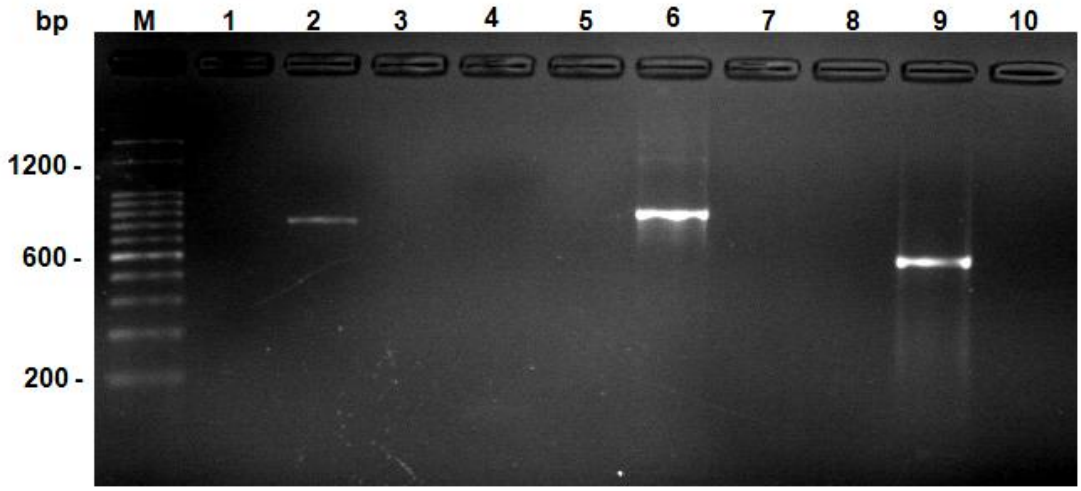

Figure 3. Amplification of resistence-associated genes by PCR: *vanA*, *vanB*, *vanC1* and *vanc2/3*. The letter bp indicates bases pair; the letter M indicates the molecular size marker (100 bp DNA Ladder, Invitrogen) and the numbers represent: (1) negative control of the *vanA* gene; (2) *positive control of the *vanA* gene; (3) the absence of the *vanA* gene in the *E. durans* LAB18s; (4) the absence of the *vanB* gene in the *E. durans* LAB18s; (5) negative control of the *vanC1* gene; (6) **positive control of the *vanC1* gene; (7) the absence of the *vanC1* gene in the *E. durans* LAB18s; (8) negative control of the *vanC2/3* gene; (9) the positive control of the *vanC2/3* gene; and (10) the absence of the *vanC2/3* gene in the *E. durans* LAB18s. *It was used as positive control to *vanA* the strain vancomycin-resistant *Enterococcus faecalis* ID 2389, and to **vanC1 and vanC2/3 it was used as positive control the *Enterococcus faecalis* strain CB356.

3.2. Analysis of antibiotic susceptibility

Analysis of the results obtained for antibiotic susceptibility test showed that the isolate exhibited high sensibility for all antibiotics tested, according to CLSI standards (Table 2). When the isolate *E. durans* LAB18s was analyzed

by E test®, a MIC ≤ 4 μg mL^{-1} (0.38 μg mL^{-1}) was observed indicating the susceptibility of the isolate to vancomycin.

Table 2. Analysis of antibiotic susceptibility of *E. durans* LAB18s and the classification according to CLSI. Values are expressed as mean ± standard error of three independent experiments.

Antibiotics	Classification of CLSI		
	Inhibition zone (mm)	Susceptible zone (mm)	*S – I – R
Erythromycin	23.3 ± 0.35	≥23	S
Tetracycline	20.5 ± 0.18	≥19	S
Vancomycin	19.0 ± 0.47	≥17	S
Gentamicin	21.5 ± 0.21	≥10	S
Penicillin	26.0 ± 0.47	≥15	S

* Interpretation standard of antimicrobial susceptibility tests with disc diffusion method of enterococci in accordance to CLSI standards: S – Susceptible; I – Intermediate; R – Resistant (CLSI, 2008).

3.3. Biofilm formation, hydrophobicity and auto-aggregation

The isolate exhibited a strong biofilm capacity of biofilm producer (+++). Interestingly, this factor was not associated with the presence of virulence-associated genes concerning of biofilm formation. This capacity could be related with the adhesion properties of probiotics.

The hydrophobicity of *E. durans* LAB18s was analyzed using two solvents: xylene, a nonpolar solvent; and chloroform, an acidic monopolar solvent. Table 3 presents the results of hydrophobicity and acid character of the cell wall expressed in percentage. Comparing the hydrophobicity and acid character, the strain showed a cell surface more acidic (chloroform - 66.87%) when compared with the cell surface non-polar characteristic using the xylene (31.13%).

Table 3. Hydrophobicity and auto-aggregation of *E. durans* LAB18s after incubation at 35°C. Data were expressed as mean percentage (%) ± standard error.

Time (h)	Hydrophobicity (%H)		Auto-aggregation
	Xylene	Chloroform	(%AA)
1	ne*	ne	27.41 ± 0.005
2	31.13 ± 0.009	66.87 ± 0.016	31.72 ± 0.005
3	ne	ne	32.55 ± 0.004
4	ne	ne	46.67 ± 0.012
24	ne	ne	79.23 ± 0.003

*ne – data not evaluated.

The percentage of auto-aggregation increased exponentially with the incubation period, obtaining a percentage of auto-aggregation of 27.41% at 1 h, 31.72% at 2 h, 32.55 % at 3 h, 46.67% at 4 h and 79.23% at 24 h of incubation. These data indicate that the strain has auto-aggregative and hydrophobicity features and these may be related with adherence to intestinal epithelium.

4. Discussion

Enterococci are present in numerous food and fermented dairy products (Ogier and Serror, 2008), and the species *E. faecalis*, *E. faecium*, and *E. durans* are commonly found in milk products (Franz et al., 1999). The presence of enterococci in foods has been known for a long time, but only recently they have been considered as potential probiotic agents (Perez-Pulido et al., 2006). Thus, obtaining precise information about these microorganisms, and to promote studies for their possible application in food or feed is mandatory to allow a safe use. The results from this study suggest that the strain *E. durans* LAB18s may be used in future animal testing, not providing undesirable effects to animal health and to feed industry.

Antibiotic susceptibility of *E. durans* LAB18s was evaluated with erythromycin, tetracycline, vancomycin, gentamicin and penicillin. The wide use

of tetracycline in animal husbandry activities is a possible explanation for the high level of tetracycline resistance frequently found among enterococci (Busani et al., 2004; Hayes et al., 2004). In contrast, the isolate *E. durans* LAB18s was sensitive for this antibiotic. Likewise, *E. durans* LAB18s was susceptible to erythromycin in according to classification CLSI. The resistance to erythromycin, a representative of the macrolide antibiotics, is a matter of concern, because of macrolides are common substitutes for patients with a penicillin allergy (Peters et al., 2003). Enterococci are considered intrinsically resistant to β-lactam antibiotics (Kak and Chow, 2002). In a previous study with clinical and food isolates, Lopes et al. (2005) stated that resistance of enterococci to β-lactam antibiotics seems to be associated with clinical strains and often inaccurately generalized in the literature to the genus. However, the results obtained in the present study are not in agreement with this generalization, since the isolate was susceptible to penicillin. Other authors obtained similar results (Ben Omar et al., 2004; McGowan-Spicer et al., 2008; Valenzuela et al., 2008). Similarly, the *E. durans* LAB18s exhibited susceptibility to gentamicin, nevertheless, investigations conducted by other authors found prevalence of gentamicin resistance among enterococci (Zouain and Araj, 2001; Riboldi et al., 2009). Vancomycin-resistant enterococci have emerged in the last decade as a frequent cause of nosocomial infections (Rice, 2001). In this study, the isolate *E. durans* LAB18s was not resistant to vancomycin, exhibiting sensibility in both tests (antibiogram and E test®). Other authors obtained similar results for enterococci isolates from food (Mannu et al., 2003; Peters et al., 2003; Messi et al., 2006).

Even doing the analyses of vancomycin susceptibility by antibiogram test and E test®, the *E. durans* LAB18s was evaluated for the presence of resistance genes. The genes *vanA*, *vanB*, *vanC1* and *vanC2/3* were investigated by PCR and the *E. durans* LAB18s did not show any of the tested genes. The assessment of resistance genes to vancomycin in strains with possible use in animal feed is of utmost importance, since this antibiotic is not metabolized by the animals and remains in the active form in the intestines (Yap et al., 2008), promoting vancomycin-resistant enterococci (VRE). In Europe, VRE (predominantly the *vanA* genotype) are frequently isolated from non-

hospitalized patients, animals and environmental sources, and they are rare in the hospital setting. This suggests that these bacteria come from a source in the community; they could be part of the normal animal microbiota and passed through the food chain. Enterococci of the *vanB* phenotype have not been yet documented in animals (Kobayashi et al., 2000). There are several reports on the influence of avoparcin in the emergence of VRE. Avoparcin, a glycopeptide antibiotic such as vancomycin, was widely used as a rapid growth promoter in broiler and swine in Europe and Korea in the late 20th century. Several studies have shown an association between the use of avoparcin and the occurrence of VRE in the community (Bonten et al., 2001; Jung et al., 2007). Data linking this community source of VRE to the extensive use of avoparcin, used as an animal feed supplement, results in a ban on avoparcin in Denmark and Germany in the mid-1990s. This has been followed by a European Union-wide ban (Kobayashi et al., 2000).

The substances produced by microorganisms that can cause damage to the host, such as toxins, are referred as virulence factors. Some characteristics determine the virulence of *Enterococcus* strains, among them the adherence to host tissue, invasion and abscess formation, modulation of inflammatory response, secretion of toxic products, intrinsic resistance to antibiotics and genes that confer resistance to antimicrobials. Depending on the type and combination of these factors, they become crucial for the strain pathogenicity (Mundy et al., 2000; Eaton and Gasson, 2001).

In *Enterococcus* species, genes conferring antibiotic resistance and other virulence factors, such as aggregation substance (*agg* and *asa*) and collagen adhesins (*ace*), can be acquired by genetic exchange. We analyzed the presence of *ace*, *agg* and *asa* by PCR amplification and none of these virulence genes were detected in the *E. durans* LAB18s. This factor can be important for human and animal health. Mundy et al. (2000) reported that aggregation substance is a pheromone-inducible surface protein of *E. faecalis* that promotes mating aggregate formation during bacterial conjugation. As an important component of the bacterial pheromone-responsive genetic exchange system, aggregation substance mediates efficient enterococci donor-recipient contact to facilitate plasmid transfer and also may lead to destruction of myocardial and

pulmonary tissues. *In vivo*, aggregation substance is known to be induced by pheromone signals and by serum, suggesting that aggregation substance-expressing cells likely form larger aggregates *in vivo* than cells not expressing it. Aggregation contributes to bacterial virulence in other systems, influencing phagocytosis and the subsequent fate of the organism. Likewise, aggregation substance increases the hydrophobicity of the enterococcal surface, which may induce localization of cholesterol to phagosomes and prevent or delay fusion with lysosomal vesicles.

Creti et al. (2006) identified an operon composed of four genes involved in biofilm formation in *E. faecalis*, which was named *bop* and appears to be regulated by the Fsr system through quorum-sensing. We also analyzed in this study the *bopA*, *bopB*, *bopC* and *bopD* virulence genes by PCR amplification; however, the *E. durans* LAB18s did not exhibit these genes. Bacteria in the form of biofilms are characterized by great genetic transfer capabilities. This statement associated with high incidence of virulence genes and the widespread ability of biofilm formation in both isolates from food and clinical sources, reinforce the concern devoted to this genus, since the virulence determinants can be transferred. Theses genes (*bopA*;*B*;*C*;*D*) were involved with biofilm formation, and biofilm production plays a major role in the pathogenesis of many clinically important pathogens (Donlan and Costerton, 2002). The occurrence of biofilm in enterococcal infections has frequently been reported (Mohamed and Huang, 2007; Liu et al., 2010; Daw et al., 2012); however, only a few studies have attempted to elucidate the underlying molecular mechanisms (Hufnagel et al., 2004).

Hufnagel et al. (2004) develop a study to gain a better understanding of the molecular mechanisms involved in the establishment of enterococcal biofilms and to clarify their role in pathogenicity. They reported that production of biofilm may be a virulence factor in enterococci that leads to prolonged bacteremia. The ability of the strain tested to produce biofilm *in vitro* is correlated with its persistence in the mouse bacteremia model *in vivo*. Possible mechanisms leading to increased bacterial colony counts in the blood of the biofilm enhanced mutant resistance to natural surfactants, and chemical biocides, resistance to phagocytes or better survival in macrophages, or

resistance to antibodies (Donlan and Costerton, 2002; Liu et al., 2010; Donlan, 2011; Daw et al., 2012). In the same way, the *in vitro* biofilm formation capability was analyzed in this study according with Stepanovic et al. (2000). The results obtained from this research showed that *E. durans* LAB18s exhibited a strong capacity of biofilm formation. However, this capacity was not associated with the virulence-associated genes. Probably this finding is related with the adhesion properties, a desirable characteristic of probiotic strains (Samot et al., 2011).

In this study, it was also evaluated the hydrofobicity and auto-aggregation capacity of *E. durans* LAB18s cells, and through of obtained results the strain showed satisfactorily adhesion properties. Hydrophobicity and auto-aggregation tests as surface properties were evaluated based on the principle that attachment in the epithelial surface is a necessary first step for the colonization of probiotic microorganisms, allowing the subsequent formation of biofilm (Ocaña Nader and Macías, 2002). The ability to adhere to epithelial cells and mucosal surfaces has been suggested as an important property of many bacterial strains used as probiotics (Collado et al., 2008). Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces. Several researchers have reported investigations on composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells and mucus. In most cases, aggregation ability is related to the cell adherence properties (Boris et al., 1997; Del Re et al., 2000; Ouwehand et al., 2002; Collado et al., 2005). The continuous shedding of intestinal epithelial cells and the peristaltic movement of food and water through the intestine, create a dynamic environment with considerable challenges for stable bacterial colonization of the gastrointestinal tract. Cell adhesion is a complex process involving contact between the bacterial cell membrane and interacting surfaces. Bacterial aggregation between microorganisms of the same strain (auto-aggregation) is considerable important in several ecological niches, especially in the gut, where probiotics are active (von Ossowski et al., 2010).

The hydrophobicity is directly related to the capacity of strains to adhere on dynamic or inert surfaces. It is related to the hydrophobic components

present in the outer membrane of the organism and it is believed that hydrophobic interactions play an important role in the adhesion of bacteria to epithelial cells. Through the test with solvents, it is possible to assess qualitatively the bacterial surface as either polar or non-polar, indicating the potential for adhesion to non-polar surfaces of the intestinal epithelium (Ocaña Nader and Macías, 2002; Vinderola and Reinheimer, 2003).

5. Conclusion

In summary, the strain *E. durans* LAB18s is a bacterium that can be considered safe for consumption, because it does not show virulence factors or resistance genes when tested for *ace*, *agg*, *asa*, *bopA*, *bopB*, *bopC*, *bopD*, *vanA*, *vanB*, *vanC1* and *vanC2/3*. Furthermore, the isolate was susceptible to the antibiotics tested, and also it showed adhesion and hydrophobicity capcity. This study is part of a major research, where *E. durans* LAB18s will be used as selenium-enriched culture for use in animal feed. The results of this study indicate that this bacterium can be used in animal feed, and can bring benefits that come from its consumption.

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6. CAPÍTULO IV

Identification and optimization of environmental conditions for production of selenium-enriched biomass by *Enterococcus*

Identification and optimization of environmental conditions for production of selenium-enriched biomass by *Enterococcus*

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Abstract

Selenium (Se) is an essential micronutrient for human and animal organisms. Currently it has been increased the interest to find an adequate source of dietary selenium supplementation. Thus, the aim of this study was to evaluate selenium bioaccumulation capacity of an *Enterococcus* strain. The isolate LAB18s was identified as *Enterococcus durans* by the Vitek® 2 system and analysis of 16S rRNA gene sequence (JX503528). After 24 h incubation, *E. durans* LAB18s bioaccumulated elevated Se(IV) concentrations with values between 2.60 mg g^{-1} and 176.97 mg g^{-1} in media containing initial concentrations at 15 mg L^{-1} and 240 mg L^{-1} sodium selenite, respectively. The isolate grew optimally and had high selenium bioaccumulation at initial pH 7.0. Optimum bioaccumulation of Se(IV) was at 30ºC. Time course studies showed that after 6 h of incubation, *E. durans* LAB18s displayed the highest bioaccumulation Se(IV) with the same behavior until 24 h. Analyzes from scanning electron microscopy (SEM) demonstrated the presence of filaments connecting the cells of *E. durans* LAB18s. It was demonstrated in this study that considerable amount of Se(IV) was absorbed by *E. durans* LAB18s. Therefore, the results obtained from selenium-enriched *E. durans* LAB18s presents a potential alternative source of organic selenium for animal feed.

Keywords: *Enterococcus*; probiotic; mineral bioremoval; selenium

1. Introduction

Selenium (Se(IV)) is found in plant foods mostly as selenomethionine and in animal tissues as selenocysteine and other unidentified forms. Some remote areas where people consume mainly plant-derived foods grown in selenium-deficient soil, especially in some regions of China, diets are often selenium supplemented (Burk, 2000; Zhang et al., 2009). Selenium together with vitamin E, is one of the basic essential nutritional elements whose function consists in the protection of cells and tissues from oxidation damage (Heindl et al., 2010). Sahin et al. (2002) reported that lower plasma concentrations of antioxidant vitamins such as vitamin C, E, folic acid, and minerals like zinc and selenium has been inversely correlated to increase oxidative damage in stressed poultry. According to Hartikainen (2005), an adequate intake of selenium for humans is needed to decrease the risk of myopathy, immunodeficiency, cardiovascular disease, cancer and other selenium deficiency syndromes. In animals, it was reported that selenium deficiency is linked to a number of diseases, which include white muscle disease and suppression of immunity (Rock et al., 2001).

Some authors suggest that organic selenium is an ideal additive because animals and humans absorb and retain it more than inorganic selenium. The main source of organic selenium is seafood and fish, whereas foods like cereals, meat, nuts, mushrooms and eggs can also increase the dietary selenium intake (Muñiz-Naveiro et al., 2005). Some researches showed that selenium-enriched lactic acid bacteria (LAB) can be considered as effective organic selenium supplement (Calomme et al., 1995a; Andreoni et al., 2000; Zhang et al., 2009). Enterococci that belongs to a LAB group are the majority in the intestinal microbiota of animals (Mountzouris et al., 2007), also it is part of the human intestinal microbiota (Cebrián et al., 2012), and are well known for their beneficial functions on health, such as control of gastrointestinal infections,

stimulation of the immune system, anti-ulcer activity, anti-tumor activity and antioxidative effects (Banwok et al., 2013).

The selenium-bioremoval by *Enterococcus* species was studied previously (Pieniz et al. 2011). Therefore, it is interesting to identify whether *Enterococcus* strain selected for this study could also be used as an effective source for selenium enrichment. If the *Enterococcus* could accumulate inorganic selenium from culture medium, selenium-enriched enterococci could be explored as a dietary selenium supplement source in food and animal feed. Thus, the aim of this study was to evaluate the selenium bioaccumulation in biomass of isolated LAB18s. The capacity of LAB18s strain to bioaccumulate and transform Se(IV) added to culture medium as well as the factors to affect selenium uptake and transformation in biomass were also evaluated.

2. Materials and methods

2.1 Bacteria and culture conditions

The isolate LAB18s (obtained from culture collection of the Laboratory of Applied Microbiology and Biochemistry of Foods - ICTA, Federal University of Rio Grande do Sul, Brazil) was isolated from “Minas Frescal” cheese, a typical Brazilian cheese. The isolate was cultured in Brain Heart Infusion (BHI) at 30ºC for 24 h without shaking. Five milliliters of BHI amended with 15 mg L^{-1} sodium selenite (Na_2SeO_3) were placed in 16 x 125 mm glass tubes with polypropylene caps, inoculated in three independent experiments with 100 µL inoculum of LAB 18s and gently mixed. Cultures were incubated at 30ºC for 24 h.

2.2 Automated phenotypic identification system (Vitek® 2)

The phenotypic identification by automated system (Vitek® 2) was realized in according Ligozzi et al. (2002). The culture was inoculated in Blood Agar (Difco) supplemented with 7% v/v of either sheep blood for 48 h of incubation at 30°C. The test panels (ID-GPC) contained 46 fluorimetric tests that included pH change tests and derivatives to detect aminopeptidases and –

osidases. Substrates used for detection of aminopeptidases are coupled with 7-amino-methylcoumarin (7AMC); substrates for the detection of -osidases are usually coupled with 4-methylumbelliferone (4MU). The 21 test substrates are as follows: 4MU-α-L-arabinofuranoside, 4MU-α-D-galactoside, 4MU-α-D-glucoside, 4MU-α-D-N-acetylneuraminic acid, 4MU-β-D-galactoside, 4MU-β-D-glucoside, 4MU-β-D-glucuronide, 4MU-β-D-mannoside, 4MU-*n*-acetyl-β-D-glucosaminide, 4MU-phosphate, alanine-7AMC, arginine-7AMC, aurease (butiloxicarbonyl-Val-Pro-Arg-AMC), histidine-7AMC, α-glutamic acid-7AMC, threonine-7AMC, lysine-7AMC, phenylalanine-7AMC, proline-7AMC, pyroglutamic acid-7AMC, and tyrosine-7AMC. Furthermore, the ID-GPC card includes 16 fermentation tests (for D-raffinose, amygdaline, arbutine, D-galactose, glycerol, D-glucose, L-arabinose, lactose, D-maltose, D-mannitol, N-acetylglucosamine, salicin, D-sorbitol, D-trehalose, D-melibiose, and D-xylose), two decarboxylase tests (for ornithine and arginine), and six miscellaneous tests (for urease, pyruvate, optochin, novobiocin, polymyxin B sulfate, and 6% NaCl). The card was automatically filled by a vacuum device, sealed and inserted into the Vitek 2® reader-incubator module (incubation temperature at 35°C), and subjected to a kinetic fluorescence measurement every 15 min. The results were interpreted by the ID-GPC database, and final results were obtained automatically. All cards used were automatically discarded into a waste container.

2.3 Molecular identification

2.3.1 Cultivation of isolate and DNA extraction

The isolate was plated by streaking on Brain Heart Infusion agar (BHA) plates and incubated at 30°C for 24 h for evaluation of culture purity. DNA was extracted from the cells using a Promega Wizard Genomic DNA Purification kit (Promega, Madison, WI). Briefly, cells from five representative colonies were re-suspended in 300 µL of nucleic acid lysis solution and incubated at 80ºC for 15 min and allowed to cool to room temperature. RNAse solution (1.5 µL) was added and incubated at 37ºC for 60 min. Protein precipitation solution (100 µL)

was added and the tubes incubated on ice for 5 min. Following centrifugation, the supernatant was transferred to a tube and 900 µL of ice cold 95% ethanol was added. The precipitate was recovered by centrifugation. Pellets were washed with 70% ethanol at ambient temperature and re-suspended in sterile distilled water.

2.3.2 Amplification of DNA by polymerase chain reaction

The 16S rRNA gene of LAB18s was amplified using bacterial universal primers corresponding to *E. coli* positions 27F (5`-AGATTTGATCMTGGCTCAG-3`) and 1492R (5`-TACGGYTACCTTGTTACGAC TT-3`) (Lane, 1991). The PCR reaction mixture consisted of 12.5 µL of PCR master mix (Promega, Madison, WI), genomic DNA template (0.5 µL), primer 27F (2.5 µL=12.5 pmol), primer 1492R (2.5 µL=12.5 pmol) and made up to 25 µL final volume with nuclease-free water. The 16S rRNA gene was amplified using a 35-cycle PCR (initial denaturation, 95ºC for 5 min; subsequent denaturation, 95ºC for 0.5 min; annealing temperature, 50ºC for 1 min; extension temperature, 72ºC for 1 min and final extension, 72ºC for 5 min). The PCR amplification products were analyzed by electrophoresis on a 1% agarose gel. Millipore Montage PCR filter units (Millipore, Billerica, MA) were used to remove primers salts, and unincorporated dNTPs according to the manufacturer's instructions except that an additional 400 µL of sterile nuclease free water was added to wash off residual PCR ingredients.

2.3.3 DNA sequencing

DNA polymerase-mediated amplification of templates in the presence of mixtures of dNTPs, fluorescently-labeled dideoxynucleotide triphosphates and primer 519r (5'-GWATTACCGCGGCKGCTG-3') using Sanger (BigDye) terminator kit (Applied Biosystems). Extension products were fractionated by capillary electrophoresis using Applied Biosystems 3730-XL DNA sequencing machine following the manufacturers' instructions.

2.3.4 Similarity of DNA sequence and phylogenetic analysis

The 16S sequences obtained were submitted to the BLAST search program of the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov) to search for homologous sequences. BioEdit (Hall 1999) was used to edit sequences and the phylogenetic tree was inferred from Clustal X alignments using the Neighbor-joining method (Saitou and Nei, 1987) in MEGA version 5.0 (Tamura et al., 2011). Genetic distance was calculated based on Kimura two-parameter model of nucleotide evolution (Kimura, 1980). The support of nodes was assessed with 1000 bootstrap replications (Felsenstein, 1985).

2.4 Amplification of *ddl* (D-Ala–D-Ala ligase) gene

The amplification of the specie-specific *ddl* gene fragment was performed by PCR, using the specie-specific *ddl* genes to *E. faecium* and to *E. faecalis* (Table 1). The PCR was realized in according to Depardieu et al. (2004), in a total volume of 25 µL containing: 50 ng of DNA template, 1.5 mM $MgCl_2$, 10 mM of each primer, 200 mM of each dNTP, 1 unit (U) of DNA Taq Polymerase and 1x reaction buffer. Amplification was carried out with the following thermal cycling profile: 3 min at 94°C and 30 cycles of amplification consisting of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, with 7 min at 72°C for the final extension. The PCR products were analyzed by gel electrophoresis with 1.5% agarose, stained with 5% solution of ethidium bromide and visualized under ultraviolet light.

Table 1. PCR primers used to the confirmation of *Enterococcus* specie.

Primer	Sequence (5'-3')	Gene	Product (bp)	Reference
DD13 f	CACCTGAAGAAACAGGC	*ddl (E. faecalis)*	475	Depardieu et al. (2004)
DD3-2 r	ATGGCTACTTCAATTTCACG	*ddl (E. faecalis)*		
FAC1-1 f	GAGTAAATCACTGAACGA	*ddl (E. faecium)*	1091	Depardieu et al. (2004)
FAC2-1 r	CGCTGATGGTATCGATTCAT	*ddl (E. faecium)*		

2.5 Analysis of bacterial biomass and determination of selenium content

Biomass development in culture was monitored by measuring optical density (OD_{600}) using sterile BHI broth as the blank. Samples were diluted 10 times in sterile BHI broth before the measurements. The selenium content in biomass development was determined according Zhang et al. (2009) with slight modification. Cell harvested by centrifugation (10.000 x g for 10 min) was digested in 6 mL of nitric acid. The samples were left for 10 h at room temperature and then heated to 90ºC for 1 h. The temperature was then elevated to 120ºC for 2 h. Thereafter 1 mL of perchloric acid was added and the temperature was elevated at 180ºC for 30 min. After cooling the vessels down at 50ºC, 5 mL of concentrated hydrochloric acid was added to reduce Se(VI) to Se(IV). Afterwards, the samples were heated at 120ºC for 30 min. Finally, the samples were transferred to volumetric flasks and made up to a final volume of 50 mL with deionized water. The selenium content in the sample was determined following digestion in concentrated nitric-perchloric acid by Optical Emission Spectrometry with Inductively Coupled Plasma (ICP-OES).

2.6 Effect of Se(IV) concentration on biomass

The effects of different concentrations of Se(IV) were determined using BHI medium amended with 15, 30, 60, 120 and 240 mg L^{-1} of Se(IV) using Na_2SeO_3. The sterile Se(IV) media were inoculated with 100 µL aliquots of *E.*

durans LAB18s (OD_{600}= 0.113, of cultures diluted 10 times) and incubated at 30ºC for 24 h. Inoculum preparation, Se(IV) analysis and biomass determination were as described under the previous sections.

2.7 Effect of temperature and pH on biomass

Temperature and pH effects on bacterial growth and Se(IV) bioaccumulation were examined using BHI medium amended with Na_2SeO_3 (15 mg L^{-1}). To investigate the effect of temperature, the cultures were incubated at 25, 30, 35, 40 and 45ºC for 24 h. In the experiment that examined the influence of pH, sterilized BHI medium amended with sodium selenite was adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 by addition of sterile 1M NaOH or 1M HCl. Inoculum was prepared by inoculating BHI containing 15 mg L^{-1} of sodium selenite with three loops of 24-h-old isolate *E. durans* LAB18s colonies in BHA. Cultures were incubated at 30ºC for 24 h. Cultures for pH studies were then inoculated with 100 µL of inoculum (OD_{600}= 0.105, diluted 10 times before measurement). The biomass was collected by centrifugation (10.000 x g for 10 min) and the content of selenium bioaccumulated in the biomass was determined following digestion in concentrated nitric-perchloric acid by ICP-OES.

2.8 Time course analysis of selenium biosorption

In time course studies of Se(IV) bioremoval, BHI medium was amended with 15 mg L^{-1} of Se(IV) using Na_2SeO_3. The medium was inoculated with 100 µL of LAB 18s (OD_{600}=0.117, of cultures diluted 10 times). Se(IV) bioaccumulation and biomass development were determined at 0, 2, 4, 8, 10 and 24 h of incubation at 30ºC. The biomass was collected by centrifugation (10.000 x g for 10 min) and the content of selenium bioaccumulated in the biomass was determined following digestion in concentrated nitric-perchloric acid by ICP-OES.

2.9 Scanning electron microscopy

The culture was inoculated in BHI medium and BHI amended with sodium selenite (15 mg L^{-1}) and incubated at 30ºC for 24 h without shaking. Then, the culture was collected by centrifugation (10.000 x g for 10 min) and immediately, immersed in a fixing solution containing 25% glutaraldehyde, 0.2 M phosphate buffer and distilled water. After 7 days in fixing solution, the samples were washed 3 times with wash solution containing 0.2 M phosphate buffer and distilled water in a 1:1 ratio, immersed for 30 min in each wash. Then, drying was performed on samples of increasing concentrations of acetone (30% for 10 min, 50% for 10 min, 70% for 10 min, 90% for 10 min, 90% for 20 min, 100% for 10 min and 100% for 20 min). After drying at room temperature, the samples were submitted to the Critical Point drying with the aid of liquid CO_2 (Critical Point Dryer - BlazersCPD030®). Then the samples were coated with gold (metallization) in the machine Sputter Coater – Blazers SCD050®. After metallization, the samples were observed in a scanning electron microscope (Jeol JSM-6060®).

3. Results

3.1 Characterization of isolate *E. durans* LAB18s

3.1.1 16S rRNA gene sequence

The isolate LAB 18s showed a significant ability to grow in airtight tubes, indicating as a facultative anaerobic micro-organism. GenBank Blast analysis of partial 16S rRNA gene sequence identified the isolate as *E. durans* (99% nucleotide sequence similarity). The phylogenetic tree derived from the 16S DNA data is shown in Fig. 1. Isolate *E. durans* LAB18s also was found to be closely related with *E. durans*, with 96% of branch support. Nucleotide sequence of isolate *E. durans* LAB18s (1293 bp) was submitted to the GenBank under accession number JX503528.

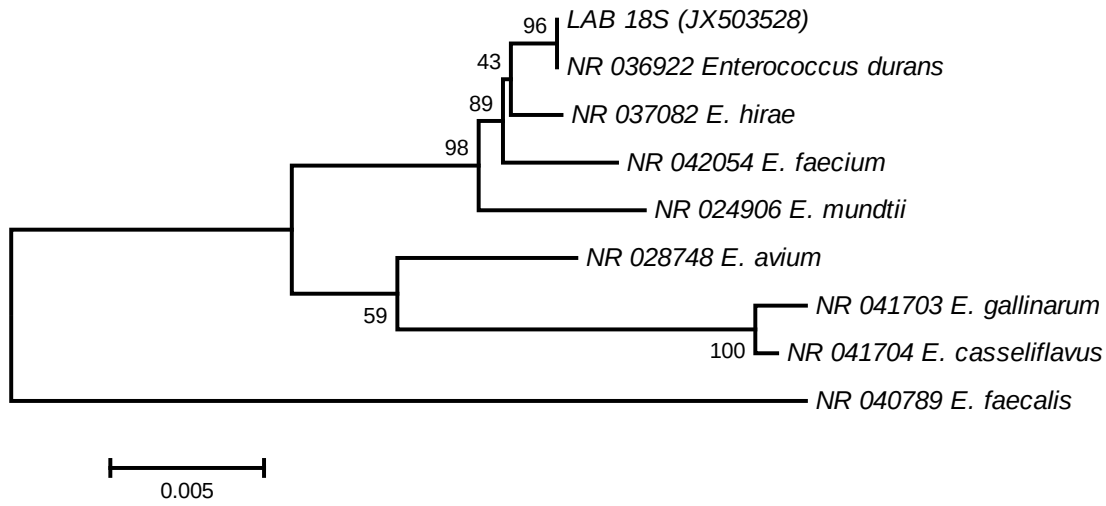

Figure 1. Phylogenetic tree showing evolutionary distance among *E. durans* LAB18s and other *Enterococcus* species based on 16s rRNA gene sequence (1293 bp). The bar represents the evolutionary distance value. Isolate LAB 18s was identified as *E. durans*, GenBank access number JX503528.

3.1.2 Specie-specific identification by *ddl* gene

The *E. durans* LAB18s was identified by molecular methods based on the analyses of 16S rRNA and by the amplification of a fragment of *ddl* gene using species-specific primers for *E. faecalis* and *E.faecium* (Figure 2). The PCR of *ddl* specific primers for *E. faecalis* and *E. faecium* confirmed that the isolate does not belong to these species since there is no amplification of *ddl* marker for *E. faecalis* (Figure 2A), and the *ddl* primers specific for *E. faecium* showed a fragment twice the expected size of 1200 bp, as shown by the positive control (Figure 2B).

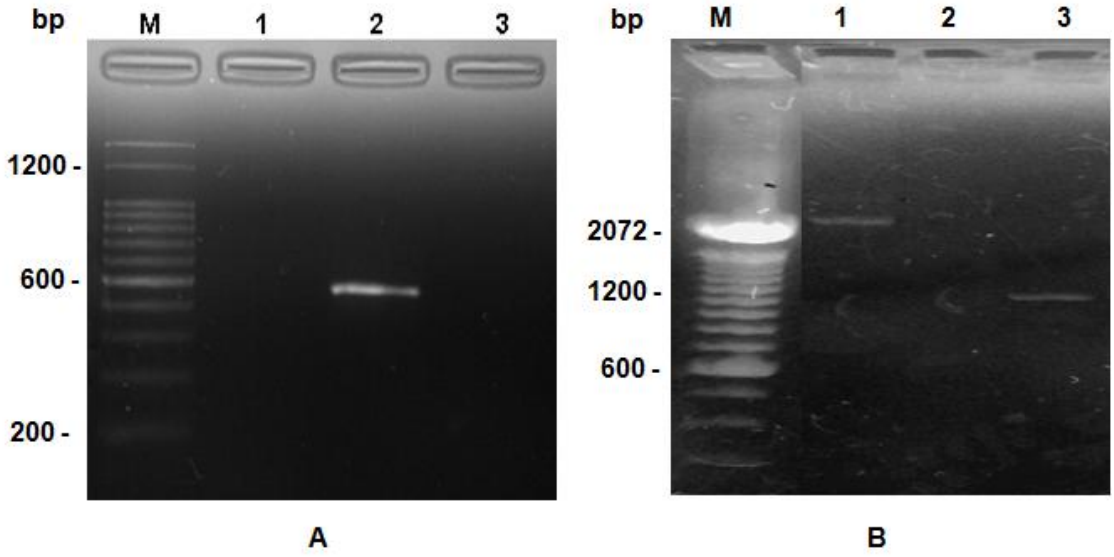

Figure 2. PCR amplification of the presence of genes *ddl* specific to *E. faecalis* (A) (Letter M indicates the molecular size marker (100 bp DNA Ladder, Invitrogen); number 1 indicates the negative control; number 2 indicates the positive control; and number 3 indicates the isolate *E. durans* LAB18s) and *ddl* specific to *E.faecium* (B) (Letter M indicates the molecular size marker (100 bp DNA Ladder, Invitrogen); number 1 indicates the sample containing *E. durans* LAB18s; number 2 indicates the negative control; and number 3 indicates the positive control).

3.1.3 Automated phenotypic identification system (Vitek® 2)

The results from Vitek® 2 system confirmed the specie as *E. durans*, corroborating with the analysis of sequencing 16S ribosomal RNA.

3.2 Effect of Se(IV) concentration on bioaccumulation

The influence of different Se(IV) concentrations on the growth and bioaccumulation of Se(IV) by *E. durans* LAB18s was evaluated in the concentration range of 0-240 mg L^{-1}. Se(IV) bioaccumulation increased as the concentration of inorganic selenium in the medium increases (Table 2). Values of Se(IV) bioaccumulation reached maximum values of 176.97 mg g^{-1} for the

concentration of 240 mg L^{-1} of Se(IV) after 24 h. Similarly, higher OD_{600} values were observed for cultures with rising Se(IV) concentration, increasing from 1.37 to 2.76 for the concentrations of 0 and 240 mg L^{-1}, respectively.

Table 2. Effect of Se(IV) concentration in the biomass production and in Se(IV) bioaccumulation after static incubation at 30ºC for 24 h.

Se (mg L^{-1})	**Biomass** (OD_{600})	**Se(IV) bioaccumulation** (mg g^{-1})
0	1.370±0.001*	00.01±0.030
15	1.536±0.003	2.60±0.164
30	1.671±0.001	5.39±0.180
60	1.951±0.011	12.62±0.276
120	2.413±0.042	59.73±0.303
240	2.760±0.070	176.97±11.768

*The values are average ± standard error.

3.3 Color modification

The Figure 3 shows the color modification with and without addition of 15 mg L^{-1} Se(IV), at initial time and after 24 h cultivation. At initial time the culture medium (A_{600nm} = 0.015) and culture medium containing 15 mg L^{-1} Se (IV) (A_{600nm} = 0.025) were not turbid and maintained the yellowish color (Figure 3A and 3B, respectively). While the isolate was inoculated in the medium, turbidity was observed, but did not modify the yellow color (A_{600nm} = 0.167; Figure 3C). On the other hand, when the isolate was inoculated in the medium enriched with 15 mg L^{-1} sodium selenite, the color was changed from yellow to reddish-brown color (A_{600nm} = 0.209; Figure 3D), showing that in this analysis was occurred the selenium reduction.

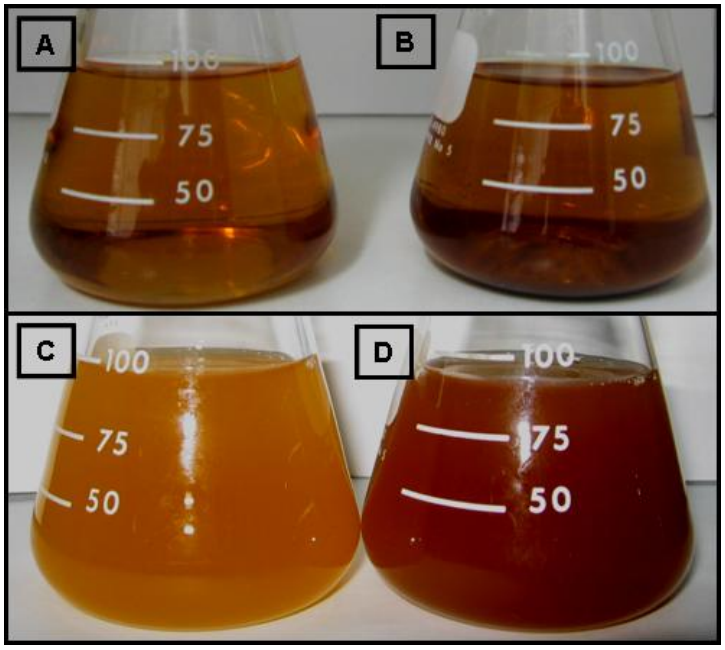

Figure 3. Color modification of the culture medium containing 15 mg L^{-1} of Se(IV). Culture medium (A,C) and culture medium containing 15 mg L^{-1} of Se(IV) (B,D). Culture medium at initial time (A,B) and after 24 h cultivation of *E. durans* LAB18s at 30^{o}C (C,D). For the spectrophotometric analysis the samples were diluted 10 times.

3.4 Scanning electron microscopy (SEM)

In the Figure 4 it was demostrated the morphology of the biomass of *E. durans* BAL18s and the biomass amended with sodium selenite (15 mg L^{-1}) by SEM. The SEM of biomassa of *E. durans* LAB 18s without selenium showed the cocci were morphologically arranged in pairs or not (Figure 4A), characteristic of this genus. It was observed that no major morphological changes in the *E. durans* LAB18s cells ocurred after selenium addition, however, it was observed the presence of filaments connecting the cells of *E. durans* LAB18s (Figure 4B). Although, the source of these filaments were not investigated.

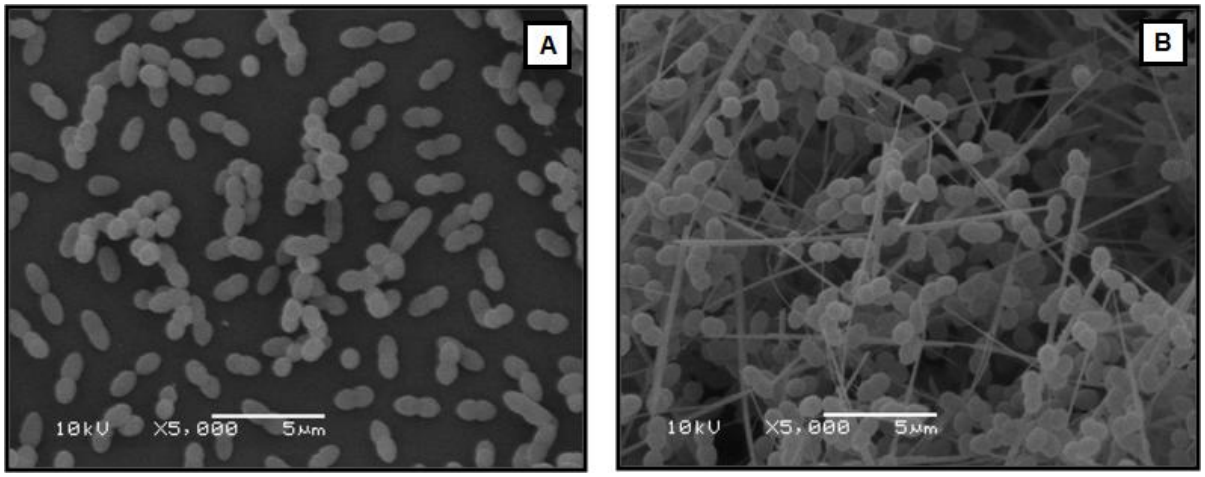

Figure 4. Scanning electron microscopy of *E. durans* LAB18s (10kv, 5,000x, bar = 5 µm). Bacterial biomass obtained after growth without selenium (A) and with 15 mg L^{-1} sodium selenite (B).

3.5 Effect of temperature on bioaccumulation

The effect of temperature on biomass production and Se(IV) bioaccumulation was evaluated (Figure 5). Temperature had similar effect on growth of the isolate *E. durans* BAL18s in the range from 20ºC to 45ºC. Biomass production was maximal by cultivation at 35ºC (Figure 5A). Significant decrease in growth of the isolate was observed at 45ºC. Similar results were found for Se(IV) bioaccumulation at 20-35^{o}C, with a significant decrease at temperatures of 40 and 45°C (Figure 5B). The maximum Se(IV) bioaccumulation was observed at 30°C.

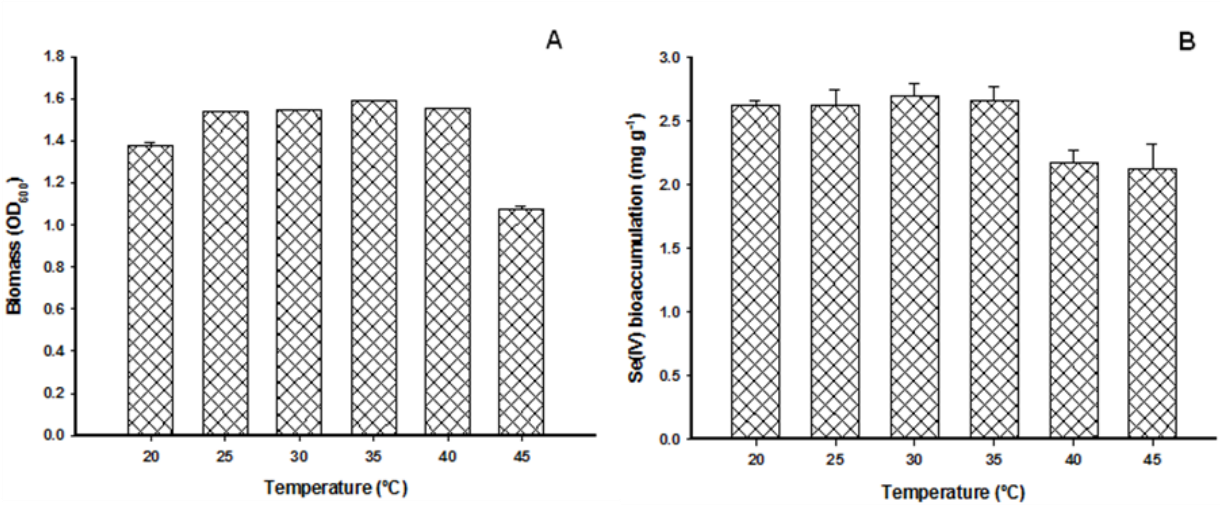

Figure 5. Effect of temperature on biomass production (OD_{600}) (A) and Se(IV) bioaccumulation (B) after 24 h in static incubation in BHI medium containing 15 mg L^{-1} sodium selenite. Error bars are standard errors of the means of triplicate independent experiments.

3.6 Effect of pH on bioaccumulation

The pH effect in development of biomass (OD_{600}) and Se(IV) bioaccumulation is showed in Figure 6. The isolate grew optimally at initial pH values ranging between 5.0 and 9.0. Biomass development was maximal at initial pH 7.0 (Figure 6A), although a similar increase was observed at pH 6.0 and 8.0. Similarly, the isolate *E. durans* BAL18s accumulated high concentration of Se(IV) in the biomass at pH 7.0 (Figure 6B). The *E. durans* BAL18s has an equivalent growth in all pHs evaluated.

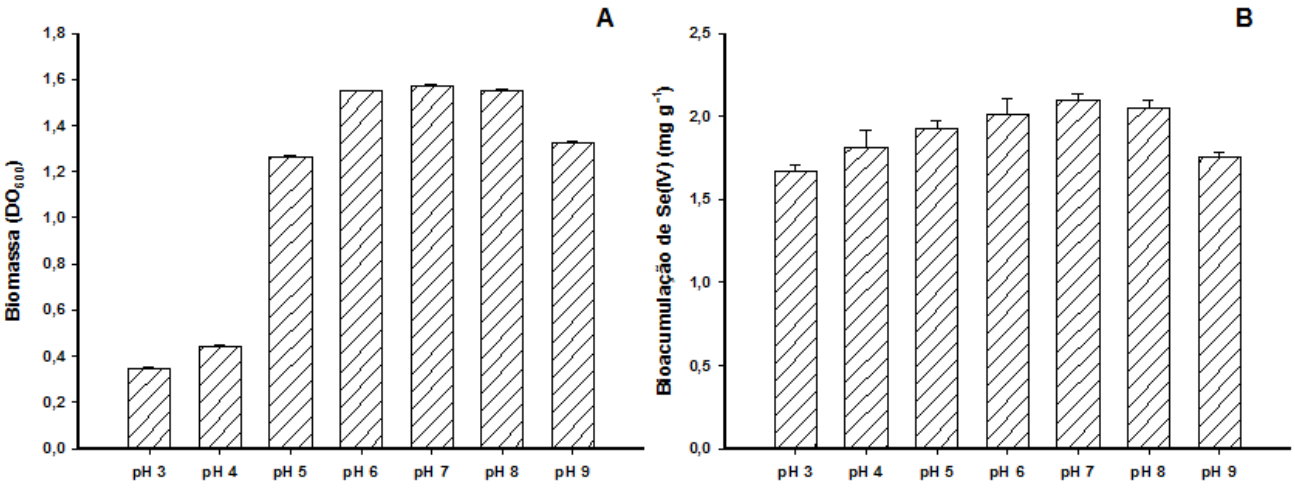

Figure 6. Effect of initial pH on biomass production (A) and Se(IV) bioaccumulation (B) in BHI medium containing 15 mg L^{-1} sodium selenite and incubated at 30ºC for 24 h without shaking. Error bars are standard errors of the means of triplicate independent experiments.

3.7 Growth profile

Time course of biomass development (OD_{600}) and Se(IV) bioaccumulation was evaluated in BHI medium and BHI containing 15 mg L^{-1} sodium selenite (Figure 7). The isolate *E. durans* BAL18s remained in the lag phase for 4 h and then grew exponentially and achieved the stationary phase within 8 h. This behavior was similar in both media with and without sodium selenite. The time course of Se(IV) bioaccumulation showed a similar growth profile (Figure 7). When it was analyzed the time course of the Se(IV) bioaccumulation, it was observed that the maximum peak of bioaccumulation was obtained at 6 h of incubation.

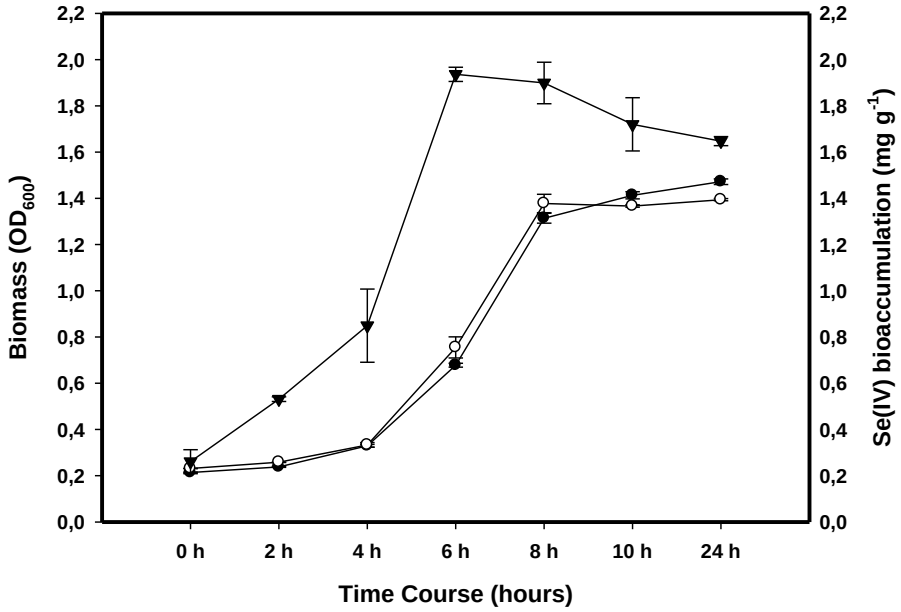

Figure 7. Time course of biomass production (OD_{600}) in BHI broth without (○) and with sodium selenite (●). *E. durans* LAB18s was maintained under static incubation at 30ºC for 24 h. (▼) Se(IV) bioaccumulation in biomass growing in medium containing 15 mg L^{-1} sodium selenite. Error bars are standard errors of the means of triplicate independent experiments.

4. Discussion

Selenium is an essential microelement for the normal growth and health of humans and animals, and there is a current increased interest in selenium supplements for human and animal nutrition. This interest has been especially regarded to the antioxidant properties of selenium and its role in the prevention of both carcinogenesis and coronary atherosclerosis, among other benefits (El-Bayoumy et al., 2006; Rayman, 2012). Many attempts have been developed to find a proper dietary source of selenium supplement, and selenium-enriched bacteria may be considered an interesting source of selenium. In this study, a food isolated *Enterococcus* was characterized for its capacity for selenium bioaccumulation.

The identification of isolate LAB18s was carried out using both phenotypic and molecular methods. For phenotypic identification, it was applied

automated identification system (Vitek® 2), and it was identified as *E. durans*. Vitek 2® system is a type of automated commercial systems and used as routine laboratory method. The new fluorescence-based technology of the Vitek 2® system is undoubtedly a positive contribution (Spanu et al., 2003). However, triggered by the limitations of phenotypic approaches and the increasingly important role played by molecular tools in the field of bacterial systematic, a range of DNA methods either based on fingerprinting or on sequencing has been used for the identification (Mohania et al., 2008). Hence, *E. durans* LAB18s was also identified by 16S rRNA gene sequence and phylogeny, and the results confirmed those obtained by Vitek 2® system, identifying the isolate as *E. durans*, with 96% confidence. To exclude the possibility of *E. durans* LAB18s to be *E. faecium* or *E. faecalis*, two closely related species that are potentially pathogenic, it was also performed the identification at the specie-level using the specie-specific *ddl* primers for these both species. The results confirm that the *E. durans* LAB18s belongs neither to the *E. faecalis* nor to the *E. faecium* species. Therefore these results taken together indicate that this strain belongs to the *E. durans* species. The correct identification of probiotic strains to the specie-level is essential for safety assessment as it allows a linkage to potentially relevant, species-related scientific and technological information, including data on growth conditions, metabolic characteristics and genomic information (Sanders et al., 2010).

In this study the isolate LAB18s was identified as the gender *Enterococcus*, specie *E. durans*. The genus *Enterococcus* belongs to the group of LAB and they are important in foods due to their involvement in food spoilage and fermentations, as well as their utilization as probiotics in humans and farm animals. These bacteria are ingested in high numbers to achieve functional or probiotic effects especially for treatment of diseases such as irritable bowel syndrome, diarrhea or antibiotic associated diarrhea, and or for health improvement such as lowering cholesterol levels or immune regulation (Franz et al., 2011). As the site of activity is the gastrointestinal system, these beneficial effects are brought about the interaction of the enterococci with the gut microbial populations, including pathogens in the case of disease, and with the gastrointestinal immune system. The use of enterococci in farm animals has

similar goals, i.e. either to prevent disease by influencing the gastrointestinal micro-population or stimulation of the immune system (Franz et al., 2011). Many aspects of the probiotics application in animal feed, especially in poultry, have been surveyed among their effects on productivity rates. Ozcan et al. (2003) demonstrated improvement in feed efficiency and carcass performance of broilers supplemented with *E. faecium* Cernelle 68, and it may contribute also to improve the quality of the animal's meat. *E. faecium* was reported to reduce the risk of acidosis when fed to dairy cows (Emmanuel et al., 2007). *E. faecium* produces also moderate amounts of lactic acid in the rumen. This could stimulate growth of lactic acid uses and stabilize ruminal pH (Nocek et al., 2002).

The isolate studied was evaluated and characterized in order to apply, in the future, in the feeding animals, especially in the diet of broilers as additive probiotic enriched. The literature emphasizes that the use of probiotic cultures may also improve benefits for both human and animal health if enriched with micronutrients like selenium. Studies suggested that selenium might enhance immunity, growth, reproductive performance, and the ability to resist disease (Harsini et al., 2012). Besides, selenium was also reported to have an antioxidant effect, and the importance of selenium as a microelement is mainly due to the vital functions of at least some selenoproteins (Ren et al., 2011). The majority of characterized selenoproteins are enzymes such as glutathione peroxidase (GSH-Px) and 5′-iodotyrosine deiodinase type I, most involved in redox reactions (McKenzie et al., 1998; Zhihua et al., 2011). On the other hand, an excess of inorganic selenium (e.g. Na_2SeO_3) can cause toxic reactions in living organisms. Attention has been given to organic selenium, due to good characteristics such as lower toxicity, better palatability, higher absorption and bioavailability, and less environmental pollution (Carlson et al., 2004).

Under appropriate conditions, some bacteria are capable of accumulating large amounts of trace elements such as selenium, zinc, copper, and incorporating them into organic compounds. Previous studies have shown that *Saccharomyces cerevisiae* is a good carrier of selenium biotransformation (Suhajda et al., 2000). Other microorganisms have also the ability to transform inorganic selenium into organic selenium such as *Bifidobacterium*,

Enterococcus and *Lactobacillus* (Zhang et al., 2009; Pieniz et al., 2011; Zhihua et al., 2011), and synthesize biomolecules containing selenium (Calomme et al., 1995a; Zhihua et al., 2011).

Considering the aspects of selenium-enriched probiotics reported in the literature, the analysis of selenium bioaccumulation in biomass of *E. durans* LAB18s was performed in this study. The results support that the isolate *E. durans* LAB18s incorporated high intracellular selenium concentrations, and the increased of bioaccumulation was correlating with the selenium concentration in the medium. Similar results were obtained by Pieniz et al. (2011) for selenium bioremoval by cells of *E. faecium* and *E. faecalis*. Those authors reported that bioremoval of Se(IV) increased with increasing concentrations from 10.0 to 60.0 mg L^{-1}.

The results of this study were also in agreement with others studies, which demonstrated that strains of *Saccharomyces* and *Lactobacillus* are capable of accumulating large amounts of trace elements such as selenium or zinc and incorporating them into organic compounds (Suhajda et al., 2000; Ponce de Leon et al., 2002; Stabnikova et al., 2008). Calomme et al. (1995b) have demonstrated that *Lactobacillus* are able to incorporate selenium as selenocysteine intracellular proteins. Zhang et al. (2009) reported that selenium-enriched *Bifidobacterium animalis* 01 could absorb 16.7-39.6% of inorganic selenium in the medium and transform it mostly into organic selenium, which accounted for 77.4-86.6% of absorbed selenium. Other studies reported that diets supplemented with selenium-enriched yeast, and sodium selenite, could improve the organization and whole-blood selenium content of growing-finishing pigs, and the effect of selenium-enriched yeast and/or lactic acid bacteria was better than that of sodium selenite (Mahan et al., 1999; Qin et al., 2007; Yang et al., 2009). This fact can be related to the form which the selenium is absorbed. The absorption of inorganic selenium (Na_2SeO_3) is performed by simple diffusion, and can have an antagonistic action to the absorption of other anions such as sulfates.

The absorption of organic selenium (selenium-enriched probiotics and selenomethionine - SeMet) occurs through active transport via the transportation system of neutral amino acids by sodium dependent channels,

which makes the process of absorption of selenium more effective than the inorganic sources. In the SeMet molecule, selenium replaces the sulfur in the molecule of methionine, being linked by covalent bonds. Thus, it does not undergo dissociation in the process of intestinal absorption. This characteristic distinguishes the organic selenium to chelated minerals, which are only associated to functional groups of proteins. Chelated minerals are transported to the border of the enterocytes, being released from the organic part of the molecule. In the post-absorptive metabolic, the selenium behaves as an amino acid, which differs completely from inorganic selenium metabolism (Suzuki, 2005).

It was observed in this study that the addition of 15 mg L^{-1} of Se(IV) in the culture medium containing the isolate changed the color from yellow to reddish-brown when compared with culture medium containing only the isolate without selenium. In addition, the color can also be influenced by the concentration of Se(IV) added. The higher concentration is a denser coloring medium. Similar results can be observed by Suhajda et al. (2000) which reported that the selenium concentration of 10.0 μg mL^{-1} in the medium, or higher, yielded a pink or reddish biomass. This can be related, since the selenium can accumulate in the bacteria as either organic or inorganic selenium, or certain circumstances the elemental selenium is formed, producing a red color on the bacterial biomass. Likewise, it was demonstrated the presence of filaments connecting the cells of *E. durans*, and through these results can be inferred that the filaments are produced by stimulation of Se(IV) added in the medium culture.

Temperature and pH are among the major environmental factors that affect growth. In this study it was observed that biomass production was maximal in Se(IV) culture at 35ºC, and significant decrease in growth of the isolate was observed at 45ºC. Since the maximal Se(IV) bioaccumulation was observed at 30°C, and significant decrease was observed at 40 and 45°C of temperature. The results from Pieniz et al. (2011) demonstrated that the species of *E. faecium* and *E. faecalis* showed the high growth at the temperature between 25ºC and 35ºC, and for Se(IV) bioremoval, the temperature had similar effect on growth in all evaluated temperatures. Rosso

et al. (1995) reported that the enterococci species growth at an extensive range of temperatures from 5 to 50ºC.

It was observed also in this study that the *E. durans* LAB18s amended with 15 mg L^{-1} has an equivalent growth at all pHs evaluated, however, it was verified the maximum peak of Se(IV) bioaccumulation at pH 7.0. Van den Berghe et al. (2006) reported that *E. faecalis* and *E. faecium* growth in a wide range of pH (4.6-9.9) with the optimum growth being 7.5.

Rapid growth is an important character for industrial microorganisms. The analysis of the time course studies on biomass development showed that *E. durans* LAB18s grew rapidly reaching a maximum growth in 8 h, and maximum Se(IV) bioaccumulation at 6 h. This result demonstrates that this bacterium has suitable growth and selenium accumulation rates, and this fact may have great importance for food or feed industry.

5. Conclusion

The research about the Se(IV) optimization in selenium-enriched *E. durans* LAB18s biomass demonstrated that this isolate has a potential Se(IV) bioaccumulation, likewise, it was found that the optimum temperature and pH for bioaccumulation of Se(IV) was at 30°C and 7, respectively. When it was analyzed the biomass by SEM it was found that the addition of Na_2SeO_3 did not change the morphology of the cells. Possibly, selenium-enriched *E. durans* LAB18s may represent an alternative source of organic selenium bioaccumulation through the biomass in animal feeding, as well as being an alternative micronutrient supplementation.

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7. CAPÍTULO V

Bioaccumulation study and speciation analysis of selenium enriched *Enterococcus durans*

Bioaccumulation study and speciation analysis of selenium enriched *Enterococcus durans*

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Abstract

Selenium is an essential component of all living organisms. Under appropriate conditions lactic acid bacteria (LAB) are capable of accumulating large amounts of trace elements, such as selenium, and incorporating them into organic compounds. In this study, the capacity of bioaccumulation of Se(IV) by *Enterococcus durans* LAB18s was evaluated. The distribution of organic selenium in selenium-enriched *E. durans* LAB18s biomass was analyzed in total protein, polysaccharides and nucleic acids. Likewise, the distribution of selenium in protein extracts obtained with different solvents (water, NaCl, ethanol and NaOH) and the determination of molecular weight and content of Se(IV) from fragments proteins contained in the biomass by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was evaluated. Selenium content in the samples was determined by Optical Emission Spectrometry Inductively Coupled Plasma (ICP-OES), and the cells were analyzed by scanning electron microscopy (SEM); scanning electron microscopy/energy dispersive spectrometry (SEM/EDS) and transmission electron microscopy (TEM). The results from this study revealed that *E. durans* LAB18s can be bioaccumulated concentration of Se(IV) in the cells. The highest

percentage of organic selenium was found in the fraction of total protein, followed by the fraction of polysaccharides and nucleic acids. When was analyzed the protein by different extractions (water, NaCl, ethanol and NaOH) it was observed in alkaline-soluble protein (NaOH) obtained the higher selenium content. In the analysis from SEM, TEM and SEM/EDS showed the morphology, the selenium particles bioaccumulated into the cells and the amounts of selenium present into the cells, respectively. Thus, the isolate *E. durans* LAB18s can be considered as a promising source probiotic supplement selenium-enriched.

1. Introduction

Lactic acid bacteria (LAB) are considered GRAS (generally recognized as safe) microorganisms and have long been used in the food industry to produce fermented foods and beverages (Xia et al., 2007). In addition, many reports show the usefulness of LAB as probiotics for human and animals, which have been associated to beneficial effects such as antimicrobial, immunomodulatory, anticarcinogenic, antidiarrheal, antiallergenic, and antioxidant activities (Saxelin et al., 2005; Singh et al., 2011). According to Suhajda et al. (2000) under appropriate conditions, probiotic microorganisms are capable of accumulating large amounts of trace elements such as selenium, zinc, and copper, and incorporating them into organic compounds. The capability of some LAB strains to concentrate selenium from the growth medium has also been reported (Calomme et al., 1995).

Selenium has received considerable attention as an essential micronutrient for animals and humans. It functions in the active site of a large number of selenium-dependent enzymes such as glutathione peroxidase and iodothrine 5′-deiodinase, which participate in the antioxidant protection of cells (Xia et al., 2007). Moreover, other selenium functions include regulating metabolism, improving immunity, enhancing reproductive performance, preventing cancer (Mater et al., 2005), postponing aging, detoxification from some heavy metals, and preventing some local epidemic diseases (Yang et al., 2009). In most countries, selenium supplementation in human and animal diets

is a first routine step toward good health (Kaushal and Bansal, 2007). Supplementation with sodium selenite as inorganic selenium in the diet has been limited in some countries because it has higher toxicity and potential to pollute the environment. Organic selenium is also retained more efficiently than are selenate and selenite in organisms and tissues. Hence, there is an increasing interest in the use of organic selenium in diets (Yang et al., 2009). The selenium-enriched with lactic acid bacteria has been considered as an organic form of selenium, and the selenium supplementation using microorganisms has received attention in the past decade (Suhajda et al., 2000; Xia et al., 2007).

Thus, the aim of this study was evaluate the capability of *E. durans* LAB18s to accumulate selenium and to characterize the species distribution of selenium in the selenium-enriched *E. durans* LAB18s. The biomass was obtained in after growth with different selenium concentrations and then nucleic acid, polysaccharide, and protein fractions were separated. Likewise, the cell proteins were also separated on the basis of their differential solubility and then the selenium content of all samples was analyzed. Finally, the distribution of selenium in the cells was performed by different electron microscopy methodologies.

2. Materials and Methods

2.1 Bacteria and culture conditions

LAB18s isolated from cheese "Minas Frescal" (typical Brazilian soft cheese) was used in this study. This strain was identified as *E. durans* by phenotypic method (Vitek® 2 system) and molecular methods (16S rRNA gene sequence and species-specific *ddl* (D-Ala–D-Ala ligase) gene analysis. The strain was maintained in stock culture in a freezer at -20°C in Brain Heart Infusion (BHI – Oxoid) containing 20% (v/v) of glycerol. The bacterium was grown in BHI at 35°C and after, inoculated on plates with Brain Heart Agar (BHA – Oxoid) and incubated at 35°C for 24 h for further evaluations.

2.2 Selenium-enriched biomass

The cultivation of *E. durans* LAB 18s was performed in BHI medium containing selenium as sodium selenite (Na_2SeO_3) incubated at 35°C for 24 h under anaerobic conditions. The investigation was performed using the following concentrations of sodium selenite: 0, 15, 30, 60 and 120 mg L^{-1}. Aliquots of 1 x 10^6 cells per mL^{-1} of bacteria were used for inoculation (Ponce de Leon et al., 2002). The biomass produced by *E. durans* LAB 18s was collected by centrifugation at 10.000 x g for 15 min at 4°C. To remove the inorganic selenium adsorbed to bacterial cells, the pellet was washed twice in 0.1 M Tris-HCl buffer pH 7.0 and centrifuged at 10.000 x g for 15 min at 4°C. The tubes containing the resulting biomass were placed in oven at 45ºC for 24 h to obtain dry mass, and then, the samples were stored at -20°C. To determine the incorporation of selenium in bacterial cells, samples were digested with nitric-perchloric acid, and analyzed by Optical Emission Spectrometry Inductively Coupled Plasma (ICP-OES) as detailed below.

The biomass obtained under these conditions (0, 15, 30, 60 and 120 mg L^{-1} of sodium selenite) was also used for the analysis of organic selenium after fractionation of nucleic acids, polysaccharides, and total protein. Protein fractions were also obtained by extraction with different solvents (water-soluble, salt-soluble, alcohol-soluble and alkaline-soluble).

2.3 Analysis of organic selenium in selenium-enriched *E. durans*

One gram of dried biomass of selenium-enriched *E. durans* LAB 18s grown with 0, 15, 30, 60 e 120 of mg L^{-1} sodium selenite was dialyzed (8000-12000 Da membrane) for 96 h against double-distilled water by changing the water every 12 h until no selenium was detected in the dialysing water. Thus, selenium compounds left in the sample were considered as organic selenium (Zhao et al., 2004).

2.4 Fractionation of nucleic acids from selenium-enriched *E. durans*

For the fractionation of nucleic acids, 1 g of enriched biomass was added to 30 mL of 120 g L^{-1} of NaCl, which was added in water bath at 95°C for 2 h (Zhao et al., 2004). Then, the supernatant was obtained by centrifugation at 10.000 x g for 10 min and the residue was washed twice in 10 mL of 120 g L^{-1} of NaCl. Following, the supernatants were combined and the protein was removed using the Sevag method (1:5 chloroform/butanol) (Qin et al. 2002). Subsequently, by adjusting the pH value of the supernatant to 2.5, the nucleic acids precipitated after 12 h at 4ºC. The resulting precipitate was recovered by centrifugation at 10,000 x g for 15 min at 4°C and stored until analyses.

2.5 Fractionation of polysaccharides from selenium-enriched *E. durans*

One gram of selenium-enriched biomass was stirred into 30 ml of 1.0 M NaOH at 60°C. After 4 h, the supernatant was obtained by centrifugation at 10.000 x g for 10 min and the residue was washed and re-precipitated twice with 10 ml of 1.0 M NaOH. The protein was extracted from the supernatant using the Sevag method (Qin et al., 2002) and then 95% ethanol was added to obtain a final concentration of 75% (v/v). After 12 h, the resultant precipitate was collected by centrifugation and dissolved in 10 mL of distilled water. This solution was filtered and dialyzed against distilled water at 4ºC three times to remove any other small molecules stored until analyses (Liu et al., 2003).

2.6 Fractionation of total proteins from selenium-enriched *E. durans*

For the extraction of total protein 1 g of enriched biomass was suspended in 30 mL of 0.25 M NaOH and incubated at 50°C for 4 h. The supernatant was obtained by centrifugation at 10.000 x g for 10 min and the residue was washed twice in 10 ml 0.25 M NaOH. Then, the supernatants were combined and made 80% saturated with ammonium sulfate at 4ºC. After 12 h, the resulting precipitate was collected by centrifugation (10.000 x g for 15 min at 4°C) and then dissolved in 10 ml of 50 mM Tris-HCl pH 8.0. This solution was dialyzed against 50 mM Tris-HCl pH 8.0 at 4ºC to remove any other small molecules and stored until analyses (Zhao et al., 2004).

Fractionation of proteins was carried out four times to obtain four different fractions. They were each analyzed independently. Fist of all, a water extraction, followed by an extraction with 0.5 M NaCl, and then an extraction with 75% (v/v) alcohol, finally, an extraction with 0.1 M NaOH was done (Chunhieng et al., 2004; Zhao et al., 2004).

2.7 Extraction of water-soluble protein

The extraction of water-soluble protein was performed using 1 g of enriched biomass, mixed in 30 mL of distilled water at 30°C. After 4 h, the supernatant was obtained by centrifugation at 10.000 x g for 10 min and the pH of the solution was adjusted to 4.5 by addition of 1 M HCl. Then the supernatant was made 80% saturated with ammonium sulfate. After 12 h at 4°C, the resultant precipitate was collected by centrifugation (10.000 x g for 15 min at 4°C) and then dissolved in 10 mL of 50 mM Tris-HCl pH 8.0. This solution was dialyzed against 50 mM Tris-HCl (pH 8.0) at 4ºC to remove any other small molecules and stored until analyses (Zhao et al., 2004).

2.8 Extraction of salt-soluble protein by NaCl

Extraction of salt-soluble protein was performed with the residue obtained above (extraction of water-soluble protein) which was mixed with 30 mL of 0.5 M NaCl. Four hours later, the supernatant was obtained by centrifugation at 10.000 x g for 10 min. The subsequently procedures were performing similarly as those used for the water-soluble protein extraction.

2.9 Extraction of alcohol-soluble protein by 75% ethanol

Extraction was carried out with the residue of the preceding operation, which was stirred into 30 ml of 75% (v/v) ethanol at room temperature. After 4 h, the supernatant was obtained by centrifugation at 10.000 x g for 10 min, being added to 20 ml of distilled water at 4ºC. The resultant precipitate was collected

by centrifugation and dissolved in 75% (v/v) ethanol. This solution was dialyzed against distilled water at 4ºC to remove any other small molecules.

2.10 Extraction of alkaline-soluble protein by NaOH

Extraction of soluble protein in alkaline medium was performing with the residue of the preceding operation, which was added 30 mL of 0.1 M NaOH. Other procedures were the same as those used for the water-soluble protein extraction.

2.11 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli (1970). For the protein separation the biomass enriched with 0, 15, 30, 60 e 120 mg L^{-1} of sodium selenite was suspended in 6 mL SDS reducing buffer (1 M Tris-HCl, pH 6.8; 50% glycerol; 10% SDS; 5% β-mercaptoethanol; and 1% bromophenol blue). Then the solution was boiled for 5 min and the supernatant was isolated by centrifugation at 6000 x g for 20 min at 4ºC. Proteins in the supernatant were concentrated in 4% polyacrylamide gel and electrophoretically separated in 12% gel at constant voltage of 120 V. To visualize the bands, the gel was then stained with Coomassie Brilliant Blue R-250 overnight and destained in a mixture with 15% trichloroacetic acid and 20% methanol for 1 h. To destained, was used a solution containing 10% acetic acid, 15% methanol, and 10% glycerol for 24 h. The selenium content of each segment visualized in the gel was analyzed by ICP-OES. The molecular mass of the different polypeptides was determined using standard molecular markers (BenchMark™ Protein Ladder - Invitrogen).

2.12 Determination of total selenium content by optical emission spectrometry inductively coupled plasma (ICP-OES)

The selenium concentration in the samples was determined by ICP-OES. The biomass was digested in 6 mL of nitric acid and left for 10 h at room temperature. Then, the temperature was elevated to 80ºC for 1 h, and subsequently at 120ºC for 2 h. Following, 1 mL of perchloric acid was added and the temperature was raised to 180ºC for 2 h. After cooling the tubes at 20ºC, the samples were transferred to volumetric flasks adding deionized water until complete a final volume of 50 mL. After these procedures, the determination of selenium content was determined by ICP-OES (Perkin-Elmer®).

2.14 Scanning electron microscopy (SEM)

E. durans LAB 18s was cultivated in BHI and BHI enriched with sodium selenite (15 mg L^{-1}) at 30ºC for 24 h without shaking. Then, the culture was collected by centrifugation (10.000 x g for 10 min) and immediately, immersed in a fixing solution containing 25% glutaraldehyde, 0.2 M phosphate buffer and distilled water. After 7 days, the samples were washed 3 times with 0.2 M phosphate buffer and distilled water (1:1), by immersion for 30 min in each wash. Then, drying was performed on samples with increasing concentrations of acetone (30% for 10 min, 50% for 10 min, 70% for 10 min, 90% for 10 min, 90% for 20 min, 100% for 10 min and 100% for 20 min). After drying at room temperature, the samples were submitted to critical point drying with the aid of liquid CO_2 (Critical Point Dryer - BlazersCPD030®). Then the samples were coated with gold in the Sputter Coater machine (Blazers SCD050®). After gold metallization, the samples were observed in a scanning electron microscope JEOL® JSM-6060.

2.15 Scanning electron microscopy/energy dispersive spectrometry (SEM/EDS)

The elemental analysis of the biomass was realized by SEM/EDS. Samples were prepared as the SEM procedure (according described above), and after the samples were coated with gold (metallization) the analyses were

performed using a Scanning Electron Microscope JEOL® JSM-5800 with a voltage of 20 kV.

2.16 Transmission electron microscopy (TEM)

A portion of 50 µL of bacterial cells from *E. durans* LAB18s was diluted in 0.1 M phosphate buffer pH 7.2 and fixed in the TEM fixing solution (1% glutaraldehyde in 0.1 M phosphate buffer) overnight. Cells were washed three times in phosphate buffer and exposed to 1% osmium tetraoxide for 45 min. After three additional washes with 0.1 M phosphate buffer, the cells were gradually dehydrated in solutions of increasing concentrations of ethanol (30% for 10 min, 50% for 10 min, 70% for 10 min, 90% for 10 min, 90% for 20 min, 100% for 10 min, and 100% for 20 min). Samples were infiltrated with a solution of ethanol and LR White resin (1:1) and washed three times in pure resin 15 minutes each. In the final wash, the sample was included in resin at 60°C for polymerization. The blocks containing the samples were cut on ultramicrotome equipped with a diamond knife into sections of 70 nm and sections mounted screens Copper (100 *mesh* coated with carbon film) suitable for microscopy. The sections were post-fixed with 1% uranyl acetate followed by lead citrate contrast. Samples were viewed in a transmission electron microscope JEM 1200 EXII high-resolution 120kV. Images were collected digitally on CCD camera attached to the microscope.

3. Results and discussion

3.1 Se(IV) bioaccumulation by *E. durans*

Significant amounts of Se(IV) were found in the biomass of *E. durans* LAB18s when sodium selenite was added to the culture medium (Table 1). High correlation was found between the content of Se(IV) bioaccumulated in biomass and the Se(IV) concentration removed in the culture medium. The Se(IV) concentration found in bacterial biomass was (mean ± standard error): 8449 ± 68; 15660 ± 82; 32383 ± 161; 61524 ± 653 µg g^{-1} dry weight when the added

concentration of sodium selenite in the culture medium was 15, 30, 60 and 120 mg L^{-1}, respectively. The ratio of selenium bioaccumulated was 2.70, 5.07, 19.80 and 29.11% respectively. From the data obtained it was observed that the bioaccumulation of Se(IV) increased gradually as increased the concentration in the medium.

According to Calomme et al. (1995), the selenium concentration in lyophilized cells of *Lactobacillus plantarum*, *L. delbrueckii* ssp. *bulgaricus* and *L. casei* ssp. *casei* enriched with 1 mg L^{-1} of sodium selenite were 375, 253 and 407 µg g^{-1}, respectively. Zhang et al. (2009) reported that the Se(IV) concentration accumulated in biomass of *Bifidobacterium animalis* 01, when 2.5, 5.0, 8.0 or 10 mg ml^{-1} of sodium selenite was added to the culture medium were respectively 528, 641, 898 and 1017 µg g^{-1}. Those authors also reported a decrease in the growth of *B. animalis* 01 in the presence of concentrations equal or greater than 10 µg mL^{-1} of Se(IV), when compared to non-enriched culture.

Table 1. Ability of Se(IV) bioaccumulation by *E. durans* LAB18s. Values represent the mean ± standard error of three independent experiments.

	Concentration of Se(IV)				
	0	**15**	**30**	**60**	**120**
	---- mg L^{-1} ---				
Se(IV) content in 100 mL of medium (µg L^{-1})	18 ± 1.1	3404 ± 0.7	4981 ± 23	9128 ± 48	17243 ± 36
Biomass in 100 mL of medium (mg g^{-1})	0.50 ± 0.1	11 ± 1.5	16 ± 3.5	56 ± 5.8	82 ± 14.6
Organic selenium content (µg g^{-1})	57 ± 0.9	8449 ± 68	15660 ± 82	32383 ± 161	61524 ± 653
***Accumulating rate (%)**		2.70	5.07	19.80	29.11

*Accumulating rate of selenium-enriched was calculated in according to the following equation: accumulating rate (%) = (organic selenium content in bacteria x biomass in 100 ml of medium / selenium content in 100 ml of medium) x 100.

3.2 Distribution of organic selenium in selenium-enriched *E. durans*

Percentage of organic selenium in *E. durans* LAB18s growing in media containing different selenium concentrations (15, 30, 60 and 120 mg L^{-1}) was 56.4%, 52.2%, 54.0%, and 51.3%, respectively. The results from this study indicated that *E. durans* LAB18s could take up respectively 2.70, 5.07, 19.8 and 29.1% of inorganic selenium in the medium and transform it mostly into organic selenium.

The percentage of total selenium found in the total protein, polysaccharides and nucleic acids, is showed in the Table 2.

Table 2. Distribution of organic selenium in selenium-enriched *E. durans* LAB18s. Values represent the mean ± standard error of three independent experiments.

Concentration of Se(IV) ---- mg L^{-1} ----	Se(IV) content	Total Proteins	Polysaccharide	Nucleic Acid
15	Se(IV) content (µg g^{-1})	4758 ± 114	2974 ± 82.1	41.52 ± 2.4
	% in organic selenium	56.31 ± 1.60	35.20 ± 2.15	0.49 ± 0.02
	% in total selenium	*31.72 ± 0.76	19.82 ± 0.55	0.27 ± 0.016
30	Se(IV) content (µg g^{-1})	7590 ± 112	5526 ± 66.5	30.29 ± 0.4
	% in organic selenium	48.47 ± 1.23	35.29 ± 2.30	0.19 ± 0.01
	% in total selenium	25.29 ± 0.37	18.41 ± 0.22	0.11 ± 0.00
60	Se(IV) content (µg g^{-1})	5331 ± 139	8152 ± 251.1	31.65 ± 2.1
	% in organic selenium	16.46 ± 0.91	25.17 ± 1.52	0.10 ± 0.02
	% in total selenium	8.88 ± 0.23	13.58 ± 0.41	0.05 ± 0.00
120	Se(IV) content (µg g^{-1})	14888 ± 1041	9143 ± 313.3	88.59 ± 8.8
	% in organic selenium	24.20 ± 0.72	14.86 ± 1.02	0.14 ± 0.05
	% in total selenium	12.40 ± 0.86	7.62 ± 0.26	0.07 ± 0.00

* The total selenium was calculated according to the following equation: total selenium (%) = selenium content (µg g^{-1}) / 1000 x 100 / added concentration.

The highest percentage of total selenium was observed in the total protein fraction (8.9 to 31.7%), following by polysaccharides (7.6 to 19.8%) and

nucleic acids (0.07 to 0.27%), respectively. Likewise, the percentage distribution of organic selenium was higher in the fraction of total protein, with percentages of bioaccumulation between 16.7 and 56.3%, followed by the fraction of polysaccharides with bioaccumulation percentages from 14.7 to 35.3%, and 0.10 to 0.49% in the fraction of nucleic acids.

In this work it was observed that most of the organic selenium was incorporated into the proteins. The data found in this study corroborate with the results found by other authors (Calomme et al, 1995; Suhajda et al, 2000; Zhao et al., 2004, Zhang et al., 2009) evaluating the incorporation of selenium by bacteria and yeasts. These authors have suggested in previous studies that selenium tends to be incorporated by protein compounds.

Interestingly, when percentages of bioaccumulation were correlated to the added concentration (15, 30, 60 and 120 mg L^{-1}), it was observed that the concentration of 15 mg L^{-1} was more efficient in terms of bioaccumulation organic selenium, with percentages of 56.3%, 35.2% and 0.49% for total proteins, polysaccharides and nucleic acids, respectively.

3.3 Distribution of selenium in protein extracts obtained with different solvents

The protein extracts obtained with sequential solubilization with different solvents (water, NaCl solution, ethanol and NaOH solution) were analyzed for selenium (Table 3). From the results it was observed that the extract of water-soluble protein contained a selenium concentration between 38 ± 0.32 and 137 ± 6.2 µg g^{-1}. Likewise, there was a low percentage of organic selenium bioaccumulated (0.55 to 0.92%).

In the extract of salt-soluble protein, the greatest selenium amount was obtained from the biomass produced at a concentration of 15 mg L^{-1} Se(IV) (68 ± 1.3 µg g^{-1}) and the lowest amount was from the medium containing 120 mg L^{-1} Se(IV) (48 ± 1.47 µg g^{-1}). This result was in opposition to those observed in the extract of water-soluble proteins. Concerning the percentage of selenium bioaccumulated it was observed a rate between 0.32 and 1.43%.

When the alcohol-soluble protein extract was analyzed, a low content of selenium (14 ± 2.1 to 17 ± 2.8 μg g^{-1}) was observed among the different samples. Concomitantly, there was a low percentage of organic selenium bioaccumulation (0.11 to 0.32%).

Table 3. Distribution of selenium in protein fractions of biomass enriched with different concentrations of Se(IV). Values represent the mean ± standard error of three independent experiments.

Concentration of Se(IV) ---- mg L^{-1} ----	Se(IV) content	Water-soluble Protein	Salt-soluble Protein	Alcohol-soluble Protein	Alkaline-soluble Protein
15	Se(IV) content (μg g-1)	38 ± 0.32	68 ± 1.3	14 ± 2.1	68 ± 4.2
	% of selenium in the total proteins	0.80 ± 0.01	1.43 ± 0.03	0.30 ± 0.02	1.43 ± 0.03
	% in total selenium	0.25 ± 0.02	0.45 ± 0.02	0.09 ± 0.01	0.45 ± 0.04
30	Se(IV) content (μg g-1)	42 ± 0.22	67 ± 2.6	16 ± 0.45	130 ± 3.6
	% of selenium in the total proteins	0.55 ± 0.02	0.89 ± 0.02	0.21 ± 0.01	1.72 ± 0.02
	% in total selenium	0.14 ± 0.01	0.22 ± 0.00	0.05 ± 0.00	0.43 ± 0.02
60	Se(IV) content (μg g-1)	45 ± 0.10	50 ± 3.5	17 ± 1.10	199 ± 7.3
	% of selenium in the total proteins	0.85 ± 0.09	0.94 ± 0.07	0.32 ± 0.02	3.74 ± 0.05
	% in total selenium	0.07 ± 0.00	0.08 ± 0.00	0.02 ± 0.00	0.33 ± 0.03
120	Se(IV) content (μg g-1)	137 ± 6.20	48 ± 1.47	17 ± 2.80	253 ± 12.10
	% of selenium in the total proteins	0.92 ± 0.07	0.32 ± 0.02	0.11 ± 0.04	1.70 ± 0.02
	% in total selenium	0.11 ± 0.00	0.04 ± 0.00	0.01 ± 0.00	0.21 ± 0.00

The alkaline-soluble protein extracts showed the higher selenium concentrations (68 ± 4.2 μg g^{-1} to 253 ± 12.1 μg g^{-1}) when compared with the

other extracts. Consequently, the highest percentages of organic selenium bioaccumulation (1.43 to 3.74%) were observed in these protein extracts. The biomass obtained from the medium containing 60 mg L^{-1} Se(IV) there was a showed the higher percentage of organic selenium bioaccumulation (3.74%).

According to the results, it can be inferred that the increasing order of protein extracts in relation to efficiency in selenium bioaccumulation was alkaline-soluble protein (68 ± 4.2 to 253 ± 12.10); water-soluble protein (38 ± 0.32 to 137 ± 6.20); salt-soluble protein (48 ± 1.47 to 68 ± 1.3) and alcohol-soluble protein (14 ± 2.1 to 17 ± 2.80). Thus, the alkaline-soluble proteins following by water-soluble proteins are more efficient in incorporating selenium in *E. durans* LAB18s biomass.

3.4 Determination of molecular mass and Se(IV) amount of proteins separated by SDS-PAGE

In this work, SDS-PAGE was carried out for the separation of proteins extracted from the biomass of *E. durans* LAB18s and the selenium-enriched *E. durans* LAB18s with 15, 30, 60 and 120 mg L^{-1} Se(IV) (Figure 1). The molecular mass of major protein bands was estimated by comparison with the molecular marker standards (20 - 220 kDa).

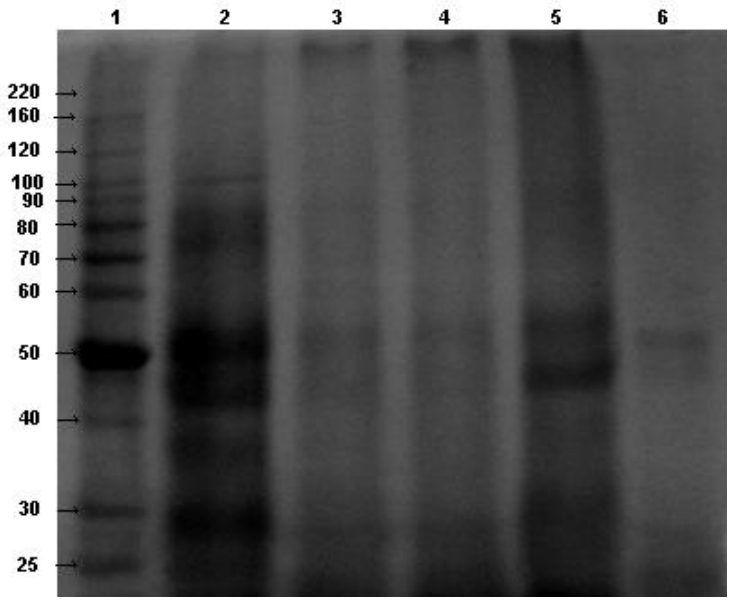

Figure 1. SDS-PAGE analysis of biomass produced by *E. durans* LAB18s and biomass enriched with 15, 30, 60 and 120 mg L^{-1} of Se(IV). The number 1 indicates the molecular marker standard (20 - 220 kDa – BenchMark™ Protein Ladder – Invitrogen); number 2 indicates the protein fragments obtained from bacterial biomass, and the numbers 3, 4, 5 and 6 indicates the protein fragments obtained at the concentrations 15, 30, 60 and 120 mg L^{-1} of Se(IV), respectively.

Analyzing the biomass produced by *E. durans* LAB18s (Figure 1; lane 2), the presence of six major protein bands was observed, with respective molecular masses of 25, 30, 45, 50, 80 and 100 kDa. When the proteins from biomass obtained from cultivation in medium containing 15, 30 and 60 mg L^{-1} of Se(IV) were analyzed, four distinct bands of 25, 30, 45 and 50 kDa were observed (Figure 1; lanes 3, 4 and 5).

The protein bands obtained by SDS-PAGE were cut out and digested in nitric-perchloric acid, and the selenium amount was determined by ICP-OES. Values between 5.85 and 8.95 µg g^{-1} of Se(IV) were associated to the protein bands (Table 4). When the protein extracts obtained from cultivation in 120 mg L^{-1} of sodium selenite were analyzed by SDS-PAGE, two specific bands of 45 and 50 kDa were observed, containing 11.20 and 11.34 µg g^{-1} Se(IV), respectively (Table 4).

Table 4. Selenium concentration obtained by protein fragments present in the biomass of selenium-enriched of *E. durans* LAB18s.

Concentration of Se(IV)	**Size protein band (kDa)**						
	25	**30**	**35**	**45**	**50**	**80**	**100**
--- mg L^{-1} ---	**--- µg g^{-1} ---**						
0	3.37	3.46	3.48	3.83	3.10	3.72	3.93
15	7.36	7.39	---	6.48	8.18	---	---
30	7.23	7.14	---	6.86	7.49	---	---
60	6.49	5.85	8.88	7.96	8.95	8.63	---
120	6.85	6.25	---	11.20	11.34	---	---

3.6 Scanning Electron Microscopy (SEM)

The SEM images of *E. durans* LAB18s and selenium-enriched *E. durans* LAB18s obtained in the concentration of 15 mg L^{-1} Se(IV) is shown in Figure 2. It can be inferred that no major morphological changes in the *E. durans* LAB18s cells occurred with the addition of selenium. Comparing the different treatments, it was observed that the sample with selenium-enriched *E. durans* LAB18s (Figure 2B) showed the presence of extracellular filament-binding, although the source of these filaments was not determined in this study.

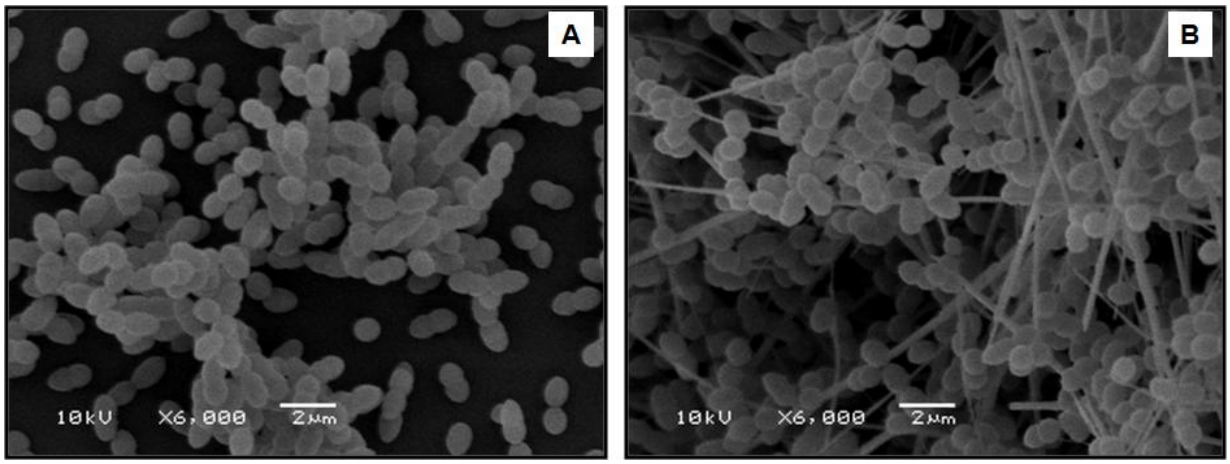

Figure 2. Scanning Electron Microscopy (10kV, x 6,000, 2µm) of biomass of *E. durans* LAB18s (A) and selenium-enriched of *E. durans* LAB18s (B) (15 mg L^{-1} of Se(IV)).

3.7 Transmission Electron Microscopy (TEM)

In order to investigate the intracellular accumulation of selenium, whole amounts of cells grown either in BHI medium or in BHI medium enriched with 15 mg L^{-1} of sodium selenite were viewed by TEM. The electron micrographs showed that the cells of *E. durans* LAB18s grown in BHI medium were straight and cocci-shaped, and contained no deposits (Figure 3).

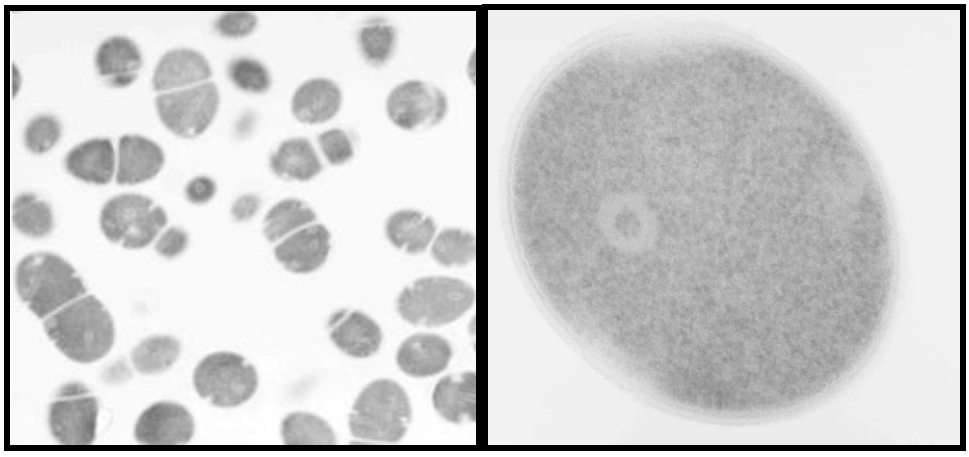

Figure 3. Transmission Electron Microscopy photographs of biomass of *E. durans* LAB18s (control) without of sodium selenite.

When grown in the presence of Se(IV), the cells did not exhibit altered morphology, but was observed some deposits of high density selenium particles dispersed over the cells (Figure 4).

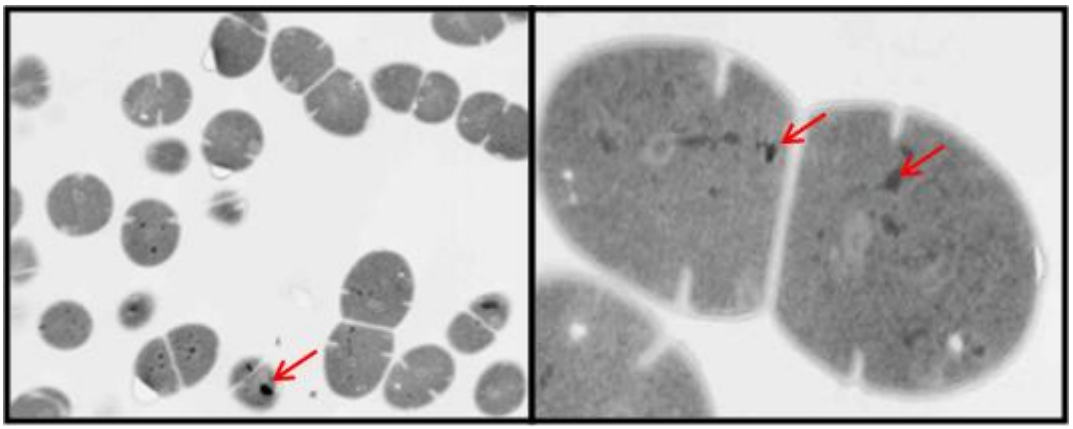

Figure 4. Transmission Electron Microscopy photographs of selenium-enriched *E. durans* LAB18s with 15 mg L^{-1} of sodium selenite.

3.8 Scanning electron microscopy/energy dispersive spectrometry (SEM/EDS)

The representavive spectrum of the biomass of *E. durans* LAB18s (Figure 5) and selenium-enriched *E. durans* LAB18s (Figure 6) was revealed by

elementar chromatogram from SEM/EDS and the quantitative elemental analysis of the essential elements including Carbon (C), Nitrogen (N), Oxygen (O), Sodium (Na), Potassium (K) and Selenium (Se), was described in the Table 5 (biomass *E. durans* LAB18s) and Table 6 (selenium enriched *E. durans* LAB18s).

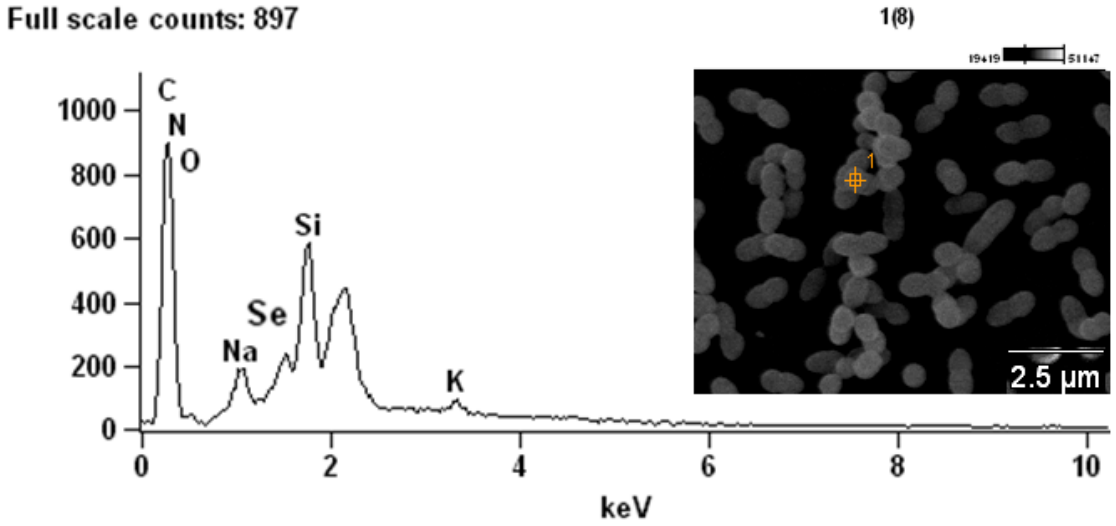

Figure 5. Representative SEM/EDS spectrum from biomass of *E. durans* LAB18s. The large Si (silicium) peak is from the glass used to support the cells. The energy range analyzed is from 0 to 10 keV. The dwelling time for the EDS analysis was 100s.

SEM/EDS is a widely applied elemental microanalysis method capable of identifying and quantifying all elements in the periodic table except Hydrogen (H), Helium (He), and Lithium (Li) (Newbury and Ritchie, 2012). While a SEM is useful for observing the morphology of mineral particles, the EDS systems commonly used on them can provide for quantitative chemical data (Paoletti et al., 2008).

As shown in Table 5, the biomass of *E. durans* LAB18s contained considerable compounds percentage (%) of K, following C, Zn, Na, O and N. However, Se was not detected. In the Table 6, it was demonstrated the presence of the elements as O, K, Zn, N, C, and despite the low content but detectable the selenium (0.49%), in the sample selenium-enriched *E. durans* LAB18s.

Table 5. Elemental analysis by SEM/EDS in biomass of *E. durans* LAB18s.

Net Counts								
	C-K	**N-K**	**O-K**	**Na-K**	***Si-K**	**K-K**	**Zn-K**	**Se-K**
1(8)_pt1	1780	424	1027	1638	51167	8304	1695	0.00
Weight %								
	C-K	**N-K**	**O-K**	**Na-K**	**Si-K**	**K-K**	**Zn-K**	**Se-K**
1(8)_pt1	40.44	16.96	11.10	2.04	19.55	5.15	4.76	0.00
Atom %								
	C-K	**N-K**	**O-K**	**Na-K**	**Si-K**	**K-K**	**Zn-K**	**Se-K**
1(8)_pt1	53.91	19.39	11.11	1.10	11.21	2.11	1.17	0.00
Compound %								
	C-K	**N-K**	**O-K**	**Na-K**	**Si-K**	**K-K**	**Zn-K**	**Se-K**
1(8)_pt1	40.44	16.96	11.10	2.04	19.55	5.15	4.76	0.00

*Si (Silicis) detected in analysi were from the glass used to hold the specimens.

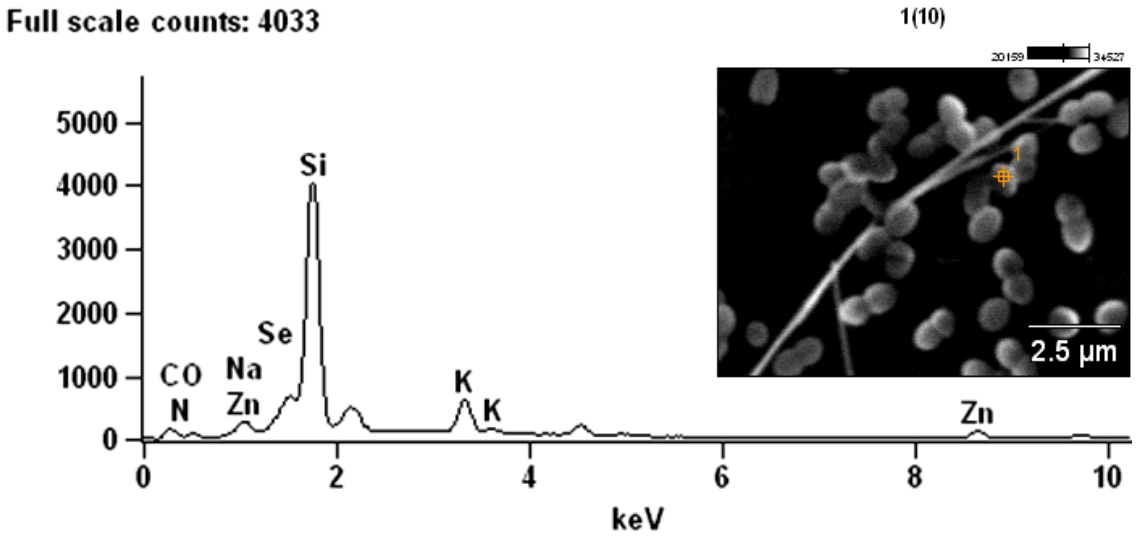

Figure 6. Representative EDS spectrum of deposits found in culture containing 15 mg L^{-1} of sodium selenite. The large Si peak is from the Silicis glass used to support the cells. The energy range analyzed is from 0 to 10 keV. The dwelling time for the EDS analysis was 100s.

Table 6. Elemental analysis by SEM/EDS of selenium enriched *E. durans* LAB18s.

Net Counts								
	C-K	***N-K***	***O-K***	***Na-K***	**** Si-K***	***K-K***	***Zn-K***	***Se-K***
1(10)_pt1	288	497	958	2102	55655	8910	2500	38
Weight %								
	C-K	***N-K***	***O-K***	***Na-K***	***Si-K***	***K-K***	***Zn-K***	***Se-K***
1(10)_pt1	13.65	21.18	12.77	2.25	32.22	7.95	9.49	0.49
Atom %								
	C-K	***N-K***	***O-K***	***Na-K***	***Si-K***	***K-K***	***Zn-K***	***Se-K***
1(10)_pt1	22.60	30.07	15.87	1.24	23.17	4.04	2.89	0.12
Compound %								
	C-K	***N-K***	***O-K***	***Na-K***	***Si-K***	***K-K***	***Zn-K***	***Se-K***
1(10)_pt1	13.65	21.18	12.77	2.25	32.22	7.95	9.49	0.49

*Si (Silicis) detected in analysi were from the glass used to hold the specimens.

The results from SEM/EDS support the results obtained in the SEM and TEM analyses. We observed in the Figure 2B, the morphology when added Se(IV) in the biomass of isolate, similarly, was observed in the Figure 4, selenium particles were bioaccumulated into the cells of *E. durans* LAB18s. Hence, in the SEM/EDS analysis showed that convinced amounts of selenium were presented into the cells.

4. Conclusions

In conclusion, it can be inferred that selenium can be concentrated in biomass by *E. durans* LAB18s in an organic form when sodium selenite is added to culture medium. The results from this study revealed that *E. durans* LAB18s can be bioaccumulated great concentration of Se(IV), and it was observed that by increasing the concentration increased gradually the Se(IV) bioaccumulation. The highest percentage of organic selenium was found in the fraction of total protein, followed by the fraction of polysaccharides and nucleic

acids. Interestingly, when it was analyzed the percentage of organic selenium among different concentrations and different fractions evaluated (total protein, polysaccharide and nucleic acid), it was demonstrated that the concentration of 15 mg L^{-1} of Se(IV) was most efficient in terms of bioaccumulation of organic selenium. When was analyzed the protein by different extractions (water, NaCl, alcohol and NaOH) it was observed in alkaline-soluble protein (NaOH) obtained the higher selenium content. In the analysis from SEM, TEM and SEM/EDS showed the morphology, the selenium particles bioaccumulated into the cells and the amounts of selenium present into the cells, respectively. These results possibly confirm the results described above regarding the bioaccumulation of selenium by *E. durans* LAB18s. Some authors suggest that selenium tends to be incorporated by protein compounds, the likewise, it was observed in this study that selenium it was bioaccumulated in more concentration in the fraction protein. Microbial protein products can be produced with nonpathogenic bacteria, yeasts, filamentous fungi, and microalgae. They are good protein supplements to cereal diets for animals and humans, as well as excellent natural resources of B vitamins. Therefore, the isolate *E. durans* LAB18s can be considered as a promising source probiotic supplement enriched with micronutrients and could constitute a health feed that could be interesting to consume with moderation for animal.

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8. CONCLUSÕES GERAIS

Os resultados obtidos neste estudo demonstraram que o isolado *E. durans* LAB18s demonstrou capacidade de sobrevivência em condições ácidas e em todas as concentrações de sais biliares testadas (de 0,1% à 1,5%). Demonstrou ainda capacidade de sobrevivência na presença do suco gástrico simulado contendo pepsina (pH 3,0) e na presença do suco intestinal simulado contendo pancreatina (pH 8, com ou sem adição de sais biliares). Apresentou da mesma forma, viabilidade quando exposto aos pHs 3,0 e 4,0, e quando exposta ao pH2 observou-se uma contagem reduzida na viabilidade das células logo na primeira hora de exposição.

O isolado *E. durans* LAB18s apresentou amplo espectro de atividade antimicrobiana contra bactérias patogênicas como *L. monocytogenes*; *E. coli*; *B. cereus*; *S. aureus*; *S. Typhimurium*; *S. Enteritidis*; *P. aeruginosa; A. hydrophila* e *C. fimi*, sendo observados halos inibitórios quando utilizando o sobrenadante bruto frente aos micro-organismos *L. monocytogenes*, *E. coli*, *S. Typhimurium*, *P. aeruginosa*, *A. hydrophila* e *C. fimi* e, quando utilizado o extrato intracelular verificou-se halos de inibição em todos os micro-organismos avaliados, com exceção para o micro-organismo indicador S*. Typhimurium*. Da mesma forma, a capacidade antioxidante foi observada no sobrenadante bruto de *E. durans* LAB18s quando analisados os métodos $ABTS^{\bullet+}$ e DPPH, e quando analisado o método de TBARS, observou-se que a capacidade

antioxidante foi exibida por ambos os extratos, sobrenadante bruto e extrato intracelular.

Além disso, o isolado *E. durans* LAB18s foi analisado quanto à presença de genes de virulência (*ace*, *agg*, *asa*, *bopA*, *bopB*, *bopC* e *bopD*) e genes de resistência a vancomicina (*vanA*, *vanB*, *vanC1* e *vanC2 /3*) através da amplificação por PCR, sendo que o isolado não apresentou nenhum dos genes avaliados. A susceptibilidade aos antimicrobianos eritromicina, tetraciclina, vancomicina, gentamicina e penicilina, também foi avaliada e, os resultados demonstraram que o isolado *E. durans* LAB18s foi sensível para todos os antimicrobianos testados, de acordo com a classificação da CLSI. O isolado demonstrou ainda capacidade de adesão e hidrofobicidade, bem como forte capacidade para a formação de biofilme, sendo que esta característica não foi associada com os genes de virulência envolvidos na formação de biofilmes (*bopA*, *bopB*, *bopC* e *bopD*).

Outro fator significantivo observado neste trabalho indicou que o selênio pode ser bioacumulado em concentrações consideráveis na biomassa de *E. durans* LAB18s na forma orgânica quando o Na_2SeO_3 é adicionado ao meio de cultivo. Ao realizar a análise da especiação de Se(IV) observou-se que a maior parte do selênio orgânico foi incorporado nas proteínas totais (56,31 %; 48,47 %; 16,46 % e 24,20 % nas respectivas concentrações de 15, 30, 60 e 120 mg L^{-1}). Além disso, as análises microscópicas de MEV, MET e MEV/EDS corroboraram com os resultados obtidos em termos de bioacumulação de selênio na biomassa de *E. durans* LAB18s demonstrando que não houve alteração morfológica nas células quando adicionado Se(IV), porém foi observada a presença de filamentos de ligação (MEV); foram observados depósitos visíveis de Se(IV) na biomassa bacteriana (MET) e, da mesma forma, foi observada uma pequena quantidade, porém significativa de Se(IV) (0,49%) na biomassa enriquecida (MEV/EDS).

Assim, pode-se inferir que os resultados em relação à bioacumulação de selênio por *E. durans* LAB18s são de grande interessante e, desse modo, a biomassa enriquecida pode representar uma fonte alternativa de selênio orgânico para a alimentação animal. Além disso, este enriquecimento pode contribuir para uma melhor biodisponibilidade de selênio no organismo, sendo

ainda considerada uma fonte de baixo custo, característica esta que representa um fator muito importante para a indústria alimentícia. Estudos adicionais deverão ser realizados para elucidar o efeito benéfico quanto à aplicação do isolado *E. durans* LAB18s com potencial probiótico enriquecido em animais. Por fim, os resultados deste estudo indicam que este micro-organismo possivelmente poderá ser utilizado na alimentação animal, trazendo benefícios advindos do seu consumo, sendo considerada uma bactéria promissora na indústria de alimentação animal.