Letícia de Castro Oliveira

Análises ômicas e funcionais de duas bactérias potencialmente probióticas revelam novas bacteriocinas de *Lactobacillus rhamnosus* L156.4 e proteínas com potencial imunomodulador de *Lactococcus lactis* NCDO 2118

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Tese apresentada como requisito parcial para a obtenção do grau de **Doutor** pelo **Programa Interunidades de Pós-Graduação em Bioinformática**, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais.

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Coorientador: Prof. Dr. Vasco Ariston de Carvalho Azevedo

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2018

"*A vida é um processo fluente e em alguns lugares do caminho coisas desagradáveis ocorrerão. Podem deixar cicatrizes, mas a vida continua a fluir. É como a água fluente, que ao estagnar-se, torna-se podre; não pare! Continue bravamente... porque cada experiência nos ensina uma lição*".

Bruce Lee

Dedico este trabalho aos meus colegas e amigos, que infelizmente descobri tantos, estarem passando por provações duras nos momentos em que a vida mais lhes tem exigido. Fiquem firmes e confiem!

AGRADECIMENTOS

Gostaria de agradecer primeiramente a Deus e à minha família; com meu retorno à Uberaba como aluna visitante de doutorado, tive a chance de tê-los próximos, me apoiando em todos os momentos, principalmente nos que pareciam ser intransponíveis. Graças a vocês tive a firmeza e garra para continuar de pé e não sucumbir às tempestades que surgiram nesses anos.

Agradeço aos amigos e colegas do LGCM e do Laboratório de Imunologia da UFTM. Fui muito bem recebida e acolhida por vocês. Acabamos nos tornando uma família e aos poucos encontrei muito de mim em vocês e de vocês em mim, e isso me fez sentir em casa.

Agradeço a cada um dos membros da banca pelo aceite em contribuir e lapidar este trabalho.

Agradeço aos professores Virmondes, Carlo, Helioswilton e Marcos, por todo apoio constante nos momentos de grande dificuldade.

Agradeço ao Vasco, por ter sido um amigo e um pai, principalmente nesse último ano. Sou muito feliz por ter tido a sorte de trabalhar e conviver um pouco com você durante meu mestrado e doutorado. Agradeço à Sheila, Márcia Natália e Fernanda pelo companheirismo e amizade nos momentos certos e incertos!

Agradeço imensamente ao meu orientador e companheiro, Siomar. Este trabalho é fruto de uma vitória silenciosa que já alcançamos muito antes juntos. Agradeço e sou feliz por não termos desistido!

SUMÁRIO

LISTA DE ABREVIÇÕES

BL	bactérias láticas
GABA	ácido gama-aminobutírico
GEI	ilha genômica
GIT	trato gastrointestinal
GRAS	*generally recognized as safe*
MI	ilha metabólica
MSI	região de fatores metabólicos e simbióticos
RBS	sítio de ligação ao ribossomo
ScFvs	*Single-chain variable fragment*
SI	ilha simbiótica
SP	peptídeo sinal
Usp45	*unknown secreted protein of* 45 kDa
XIES	sistema de expressão por indução de xilose

LISTA DE FIGURAS

LISTA DE TABELAS

RESUMO

As bactérias do gênero *Lactobacillus* e *Lactococcus* se destacam no grupo das bactérias lácticas, tanto por já serem utilizadas na indústria alimentícia há anos, como por apresentarem novas linhagens caracterizadas como probióticas. Utilizando análises *in silico* e *in vitro* buscou-se conhecer genes e proteínas envolvidos no efeito probiótico de *Lactobacillus rhamnosus* L156.4 e *Lactococcus lactis* subsp. *lactis* NCDO 2118. As análises focaram nos estudos da atividade antagonista; análises preliminares mimetizando o trato gastrointestinal *in vitro*, além da predição de genes realacionados à probiose, respectivamente. Nos estudos com *L. rhamnosus* L156.4, o seu espectro antagonista foi avaliado, onde foram preditos genes e proteínas relacionadas às bacteriocinas, além da caracterização da atividade das células e do sobrenadante. Por meio destas análises foi observado o amplo espectro antagonista desta linhagem, mesmo no uso apenas do sobrenadante, que não apresentou inibição apenas contra espécies gram-negativas utilizadas no estudo. Sendo este o primeiro relato de um *Lactobacillus* isolado de fezes de camundongos NIH, esta linhagem ganha destaque por apresentar uma característica significativa probiótica. No estudo com a linhagem NCDO 2118, outros 15 genomas completos disponíveis no NCBI foram utilizados para realização das análises de genômica comparativa. Estas análises destacaram a grande semelhança entre as linhagens *L. lactis* NCDO 2118 e *L. lactis* KF147, as quais compartilham uma mesma ilha genômica caracterizada como metabólica e simbiótica. Na avaliação das características probióticas, NCDO 2118 apresentou certa sobrevivência (48%) ao meio gástrico, características adesivas (52%), além da presença de bacteriocinas e proteínas relacionadas. As análises *in silico* não predisseram nenhum gene relacionado à resistência nem regiões adquiridas por transferência horizontal ou mesmo a presença de ilhas de resistência à antibióticos. A abordagem de proteômica veio acrescentar possíveis dados que possam estar ligados diretamente à capacidade anti-inflamatória e imunomodulatória de *L. lactis* NCDO 2118, entretanto, uma análise aprofundada se faz necessária para chegarmos a uma conclusão. Assim sendo, os resultados mostraram possibilidades que ainda precisam ser estudadas de forma integrada, caminhando para outras abordagens ômicas, como a transcriptômica, por exemplo. Por meio dela, poderemos expor esta linhagem em estudo à condições específicas, avaliando assim, a expressão dos genes mediante situações específicas.

Palavras-chave: *Lactobacillus rhamnosus* L156.4, *Lactococcus lactis* NCDO 2118, bactérias do ácido lático, ômicas, probiótico.

ABSTRACT

The bacteria of the genus *Lactobacillus* and *Lactococcus* stand out in the group of lactic acid bacteria, both because they have been used in the food industry for years, and because they present new strains characterized as probiotic. Using *in silico* and *in vitro* analyzes, we searched for genes and proteins involved in the probiotic effect of *Lactobacillus rhamnosus* L156.4 and *Lactococcus lactis* subsp. *lactis* NCDO 2118. The analyzes focused on studies of antagonistic activity; preliminary analyzes mimicking the gastrointestinal tract *in vitro*, in addition to the prediction of genes related to probiosis, respectively. In the studies with *L. rhamnosus* L156.4, its antagonistic spectrum was evaluated, where genes and proteins related to bacteriocins were predicted, in addition to the characterization of cell and supernatant activity. By means of these analyzes the broad antagonistic spectrum of this strain was observed, even in the use of the supernatant alone, which did not show inhibition only against gram-negative species used in the study. Being the first report of a *Lactobacillus* isolated from feces of NIH mice, this strain stands out for presenting a significant probiotic characteristic. In the study with the *L. lactis* NCDO 2118 lineage, another 15 complete genomes available in the NCBI were used to perform comparative genomic analyzes. These analyses highlighted the great similarity between *L. lactis* NCDO 2118 and *L. lactis* KF147 strains, which share the same genomic island characterized as metabolic and symbiotic. In the evaluation of the probiotic characteristics, NCDO 2118 presented a certain survival (48%) to the gastric environment, adhesive characteristics (52%), besides the presence of bacteriocins and related proteins. *In silico* analyses did not predict any gene related to resistance nor regions acquired by horizontal gene transfer or even the presence of islands of resistance to antibiotics. The proteomics approach has added potential data that may be directly linked to the anti-inflammatory and immunomodulatory capacity of *L. lactis* NCDO 2118, however, an in-depth analysis is necessary to reach a conclusion. Thus, the results showed possibilities that still need to be studied in an integrated way, moving towards other omic approaches, such as transcriptomics, for example. Through it, we can expose this strain under study to specific conditions, thus evaluating the expression of genes through specific situations.

Keywords: *Lactobacillus rhamnosus* L156.4, *Lactococcus lactis* NCDO 2118, lactic acid bacteria, omics, probiotic.

I. APRESENTAÇÃO

I.1 Colaboradores

Este trabalho foi realizado nos: Laboratório de Genética Celular e Molecular (LGCM), Laboratório de Doenças de Animais Aquáticos (Aquavet) da Universidade Federal de Minas Gerais (UFMG) e Laboratório de Imunologia do Instituto de Ciências Biológicas e Naturais (ICBN) da Universidade Federal do Triângulo Mineiro (UFTM) em uma colaboração entre os seguintes pesquisadores:

Prof. Dr. Henrique Cesar Pereira Figueiredo - Aquavet;

Prof. Dr. Siomar de Castro Soares - UFTM;

Prof. Dr. Vasco Ariston de Carvalho Azevedo – LGCM/UFMG

Este trabalho teve o auxílio das seguintes instituições: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig) e Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

II. PREFÁCIO

II.1 As bactérias láticas

II.1.1 Definição

As bactérias láticas (BL) fazem parte de um grupo bastante heterogêneo de bactérias gram-positivas, não esporuladas, que alberga espécies dos gêneros: *Streptococcus, Lactococcus, Leuconostoc, Pediococcus e Lactobacillus.* Revisões quanto à taxonomia destas bactérias foram feitas propondo vários gêneros novos ao grupo das BL como: *Carnobacterium, Aerococcus, Dolosigranulum, Alloiococcus, Enterococcus, Globicatella, Oenococcus, Tetragenococcus, Weissella e Vagococcus* (Khalid, 2011).

O termo BL as define principalmente com relação à sua capacidade metabólica comum; salvo poucas exceções, elas são responsáveis pela conversão de açúcares (principalmente glicose) em ácido láctico através de vias metabólicas específicas. É possível observar outras características compartilhadas entre estas bactérias como: (i) serem anaeróbicas facultativas; (ii) não produtoras de catalase; e, (iii) serem imóveis. Além disso, existe uma proximidade filogenética entre as bactérias deste grupo que pode ser observada na **Figura 1** (Pfeiler & Klaenhammer, 2007).

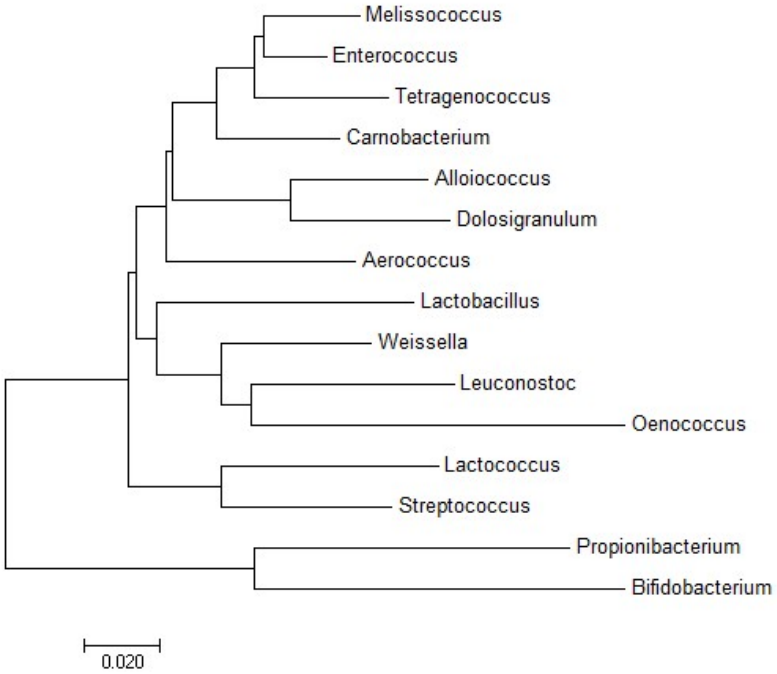

Figura 2 Filogenética das bactérias láticas. A história evolutiva foi inferida usando o método Neighbor-Joining method (Saitou & Nei, 1987). A árvore ideal com a soma do comprimento dos ramos =

0.99806005 é apresentada. A árvore é desenhada em escala, com os tamanhos dos ramos nas mesmas unidades das distâncias utilizadas para inferior a árvore filogenética. As distâncias evolucionárias foram computadas usando o método de Máxima Versossimilhança Composta (Tamura *et al*., 2004) e estão nas unidades de substituição de numerous de base por sítio. A análise envolveu 15 sequências nucleotídicas. As posições dos codons incluídos foram 1º+2º+3º+não-codificante. Todas as posições faltando dados ou com gaps foram eliminadas. Havia um total de 1479 posições no dataset final. As análises evolucionárias foram conduzidas com o MEGA7 (Kumar *et al*., 2015).

II.1.2 Importância industrial e biotecnológica

As BL manipuladas nas indústrias de laticínios vivem em um nicho diferente, definidos por razões tecnológicas, tais como o crescimento e produção rápida de ácido láctico no leite (Bolotin *et al.*, 2001).

A maioria das BL adquire energia através da transformação de açúcares, principalmente da glicose em ácido láctico (via homofermentativa, ou homolática) e/ou ácido láctico e outros produtos (via heterofermentativa ou mista) (Carr *et al.*, 2002). De um modo geral, estes organismos estão associados ao preparo de alimentos, na preservação de carnes, grãos e vegetais (Bolotin *et al.*, 2001).

Dessa forma, estudos relacionados às análises de sequência do genoma das BL despertam grande interesse da indústria alimentícia. Além disso, devido à sua utilização ao longo dos anos na manutenção e preservação de alimentos, as espécies do gênero *Lactococcus* são consideradas bactérias seguras ou "GRAS" (*Generally Recognized As Safe*), mesmo seu grupo apresentando algumas poucas espécies patogênicas. No grupo das bactérias láticas, a *Lactococcus lactis* é uma das espécies melhor caracterizada e que figura como um microrganismo modelo para estudos deste grupo (Bolotin *et al.*, 2001).

II.1.3 Novas utilizações das bactérias láticas

As BL também possuem um grande potencial de utilização na produção de biomoléculas. Proteínas de interesse biotecnológico e médico, como antígenos, citocinas (Marinho *et al.*, 2010), enzimas (Du *et al.*, 2010) e alérgenos **(Tabela 1)**, já foram produzidos por meio de engenharia genética pelas BL (Bermudez *et al.*, 2004; Nouaille *et al.*, 2003).

No grupo das BL, a espécie *Lactococcus lactis* tem sido intensivamente utilizada na produção de proteínas heterólogas (Mierau & Kleerebezem, 2005). Isto se deve ao fato de que poucas proteínas são conhecidas como secretadas por esta espécie e apenas uma, Usp45 (*Unknown Secreted Protein of* 45 kDa) é secretada em quantidades detectáveis por gel corado com *coomassie*; outro motivo refere-se ao fato de que as proteínas secretadas são propensas, na maior parte do tempo, à degradação extracelular (Lee *et al.*, 2000; Wu *et al.*, 1991; Wu *et*

al., 1998) e as linhagens laboratoriais de *L. lactis* não produzem nenhuma protease extracelular (Nouaille *et al.*, 2003).

Se tratando da linhagem em estudo, *L. lactis* NCDO 2118 é uma robusta linhagem isolada de ervilha que possui a capacidade de crescer em meios menos complexos que a maioria das linhagens lácteas, permitindo seu uso em sistemas de produção de menor porte (Miyoshi *et al.*, 2004).

Tabela 2 Aplicação atual das bactérias láticas. Várias moléculas têm sido produzidas nas BL e muitos modelos já foram utilizados (Wells & Mercenier, 2008). ScFvs = Single-chain variable fragment.

PRODUTO	MODELO	APLICAÇÃO
DNA	camundongos	Vacinas
Peptídeo	camundongos	HIV-1, fusão de inibidores, colite
ScFvs	camundongos	Anti-infectante
Antígenos	camundongos e galinhas	Vacinas
Citocinas	camundongos e humanos	Doença inflamatória intestinal e colite, adjuvantes de vacina
Enzimas	camundongos	Terapia para colite e deficiência de enzimas
Alérgenos	camundongos	Terapia e prevenção de alergia

Em 1999, Langella & Loir desenvolveram ferramentas para a secreção de proteínas heterólogas onde relataram o aumento de expressão do gene *nuc* (nuclease), uma proteína repórter, por meio da clonagem de várias cópias do vetor e de um promotor deste gene de *Lactococcus*. Com este trabalho, foi relatado a possibilidade de *L. lactis* ser uma forte candidata para desenvolvimento de vacinas vivas como veículos de entrega (Langella & Loir, 1999).

Já Miyoshi e colaboradores desevolveram um sistema de expressão e endereçamento protéico utilizando *L. lactis.* Este sistema, nomeado XIES (*Xylose-Inducible Expression System*), utiliza-se do promotor P*xylt* (gene da xilose permease) e dos elementos genéticos: sítio de ligação ao ribossomo (RBS) e sequência codificadora do peptídeo sinal (SP) da proteína Usp45 de *L. lactis.* XIES foi utilizado com sucesso na linhagem *L. lactis* NCDO 2118, onde demonstrou ser capaz de: (i) produzir e secretar, por um período mais longo, elevados níveis da proteína modelo Nuc (nuclease B) de *Staphylococcus aureus*, na presença de xilose, (ii) endereçar o produto final para o citoplasma ou meio extracelular e (iii) permitir ativar e desativar a expressão gênica por meio da adição de xilose ou glicose, respectivamente. Ademais, este sistema é mais vantajoso que os atuais sistemas de expressão

por ser de mais fácil manipulação, menos dispendioso e principalmente, por ser mais seguro para o uso humano e animal (Miyoshi *et al.*, 2004).

A linhagem *L. lactis* NCDO 2118 foi descrita como produtora do ácido gama-aminobutírio - GABA (Mazzoli *et al.*, 2010), um neurotransmissor capaz de diminuir a pressão sanguínea de pacientes levemente hipertensos (Inoue *et al.*, 2003), além de induzir um efeito tranquilizante e diurético (Jakobs *et al.*, 1993; Wong *et al.*, 2003), prevenir diabetes (Adeghate & Ponery, 2002), e reduzir a resposta inflamatória de artrite reumatóide em modelo murino (Tian *et al.*, 2011).

Devido a estas características e alta capacidade industrial de *Lactococcus lactis* como principais componentes na produção de produtos lácteos, é grande o interesse relacionado a esta espécie (Siezen *et al.*, 2011). Dentre os vários papéis benéficos das bactérias probióticas no trato gastrointestinal (GIT), podem-se destacar: a inibição no crescimento de *H. pylori* (Ushiyama *et al.*, 2003), redução dos biomarcadores de câncer do colo retal (Rafter *et al.*, 2007), diminuição dos níveis de colesterol no sangue (Ataie-Jafari *et al.*, 2009), diminuição dos riscos de diarréia associada a antibióticos (Gao *et al.*, 2010), dentre outros.

Se tratando da linhagem *L. lactis* NCDO 2118, a atenção estará voltada para o seu potencial probiótico relatado recentemente na literatura por Luerce e colaboradores (Luerce *et al.*, 2014).

II.2 A linhagem *Lactococcus lactis* subsp. *lactis* NCDO 2118

Os projetos desenvolvidos no Laboratório de Genética Celular e Molecular (LGCM) com a linhagem *Lactococcus lactis* subsp. *lactis* NCDO 2118 (aqui, *L. lactis* NCDO 2118), foram possíveis em virtude da colaboração entre Brasil e França. Esta linhagem veio de uma das coleções da Unidade de Genética Microbiana do *Institut Nacional de La Recherche Agronomique* (INRA), em Jouy-en-Josas na França, e hoje, faz parte da bacterioteca do LGCM. *L. lactis* NCDO 2118 é um isolado de ervilha congelada e vem sendo utilizada na rotina laboratorial do LGCM para produção de proteínas heterólogas. Esta linhagem foi descrita por Mazzoli e colaboradores, em 2010, como produtora do ácido gama-aminobutírio (GABA) (Mazzoli *et al*., 2010), conhecido por agir positivamente na saúde humana. O GABA é um neurotransmissor distribuído de forma abundante no sistema nervoso central dos vertebrados e é capaz de diminuir a pressão sanguínea de pacientes levemente hipertensos (Inoue *et al*., 2003), induzir um efeito tranquilizante e diurético (Jakobs *et al*., 1993; W*o*ng *et al*., 2003), prevenir diabetes (Adeghate & Ponery, 2002), além de reduzir a resposta inflamatória de artrite reumatóide em modelo murino (Tian *et al*., 2011).

Dos trabalhos realizados com esta linhagem, merecem destaque os projetos desenvolvidos pelo grupo LGCM. Em um deles, Miyoshi e colaboradores desevolveram um sistema de expressão e endereçamento proteico utilizando *L. lactis* NCDO 2118. Este, se mostrou eficaz e duradouro quanto à produção e secreção dos níveis da proteína nuclease B de *Staphylococcus aureus*, por meio de um promotor P*xylt* (gene da xilose permease) e da proteína Usp45 (Miyoshi *et al*., 2004). Em outro trabalho, Luerce e colaboradores demonstraram a atividade antiinflamatória e imunomodulatória de *L. lactis* NCDO 2118 por meio da avaliação de três linhagens de *L. lactis in vitro*, com o uso de células epiteliais intestinais (Luerce *et al*., 2014).

Compreendendo a importância prática das BL no processo fermentativo e em outras áreas de aplicação: bioprocessamento, agricultura, alimentos e, atualmente, na medicina, este grupo de bactérias tem sido o objeto de várias pesquisas considerando seu uso no comércio durante décadas. Auxiliando estes estudos, grandes esforços foram e estão sendo feitos para determinar as sequências genômicas das espécies e linhagens representativas das BL (Klaenhammer *et al*., 2002).

III. ESTRUTURA DO MANUSCRITO E CONTRIBUIÇÃO DOS AUTORES

Esta tese está dividida em Introdução e 2 (dois) capítulos, baseados em: um artigo de revisão e dois artigos originais, respectivamente:

a. A Introdução, apresentada como artigo de revisão, objetivou levantar alguns estudos importantes relacionados à utilização das mais diversas abordagens ômicas (estrutural e funcional) na caracterização de organismos probióticos. Neste trabalho, eu escrevi o manuscrito com suporte científico, coordenação e revisão dos coautores.
b. O primeiro capítulo, apresentado como artigo original, foi composto por análises *in silico* e *in vitro*, objetivando conhecer melhor uma das caracterícias probióticas da linhagem *Lactobacillus rhamnosus* L156.4 - sua atividade antimicrobiana. Uma busca *in silico* nos trouxe a identificação de bacteriocinas, uma delas confirmada por meio de testes *in vitro*. Por meio de experimentos, foi observada também a caracterização desta bacteriocina, quanto à temperatura e pH, por exemplo, além de também, ter sido identificada em eletroforese em gel. Toda parte *in vitro* deste trabalho foi realizado por colaboradoresdo Departamento de Microbiologia da UFMG. A parte *in silico,* do seqüenciamento e montagem da linhagem foi realizado com colaboração do Laboratório de Microbiologia da Universidade CEUMA, de São Luís, e eu desenvolvi a filogenia, identificação das bacteriocinas e depósito das sequências.
c. O segundo capítulo, apresentado como artigo original, foi composto por análises *in silico* e *in vitro*, analisando a probiose da linhagem *Lactococcus lactis* NCDO 2118. Foram realizadas comparações genômicas entre a linhagem de interesse e outros 15 genomas completos disponíveis do banco de dados do NCBI, predições de ilhas genômicas, fagos e bacteriocinas. Nas análises *in vitro* foram avaliados os estresses ao ácido gástrico e ao biliar, resistência a antibióticos, capacidade de adesão, atividade antagonista e também, quanto ao proteoma. Neste trabalho, desenvolvi as análises *in silico* com colaboração , na obtenção das micrografias para análise de parede celular. Nas análises *in vitro*, obtive o apoio de colaboradores, que geraram os dados brutos do proteoma e os demais experimentos de caracterização da probiose da linhagem *L. lactis* NCDO 2118.

IV. INTRODUÇÃO

IV.1 Omics of probiotic bacteria: what features should I search for?

Probiotic bacteria - omics approach

Letícia de Castro Oliveira, Mariana Oliveira, Alissa de Sarom, Leandro de Jesus Benevides, Carlo José Freire de Oliveira, Henrique Cesar Figueiredo, Vasco Ariston de Carvalho Azevedo and Siomar de Castro Soares

Esta mini-review foi submetida para "*Plos Computational Biology*". Nela, foi realizado um levantamento breve a respeito da história dos probióticos, destacando as análises *in silico* de determinadas espécies, principalmente do grupo das bactérias lácticas, que apresentam abilidade anti-inflamatória e imunomodulatória. Estas análises foram destacadas principalmente pelo fato de terem sido realizadas por meio de abordagens ômicas, brevemente descritas destacando a sua importância no auxílio e na agilidade de se caracterizar a probiose destes organismos.

Omics of probiotic bacteria: what features should I search for?

Probiotic bacteria - omics approach

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Abstract

Probiotics are live microorganisms extensively used, for more than a century, in pharmaceutical and medicinal industry due to their bioactive properties. Recently, the attention has focused on these probiotic features, and other abilities that some old and new species of this group have presented. In general, desired features of probiotics include resistance to acid and bile salts, antimicrobial and antioxidant activity, the ability to avoid dysbiosis, capacity of inducing immune system development and, immunomodulatory and anti-inflammatory properties. The vast majority of probiotic species already known have more than one of these biological properties. The advent of next-generation sequencers has propelled the genomic area, which can now be used in the search for probiotic features in a large range of probiotic species, specially bacteria. In this context, functional genomics analyses may help in deepening inside these big data, correlating the findings with comparative genomics analyses, in the search for some direct application. Proteomics and Transcriptomics methodologies are now elucidating important informations about the proteins and transcripts differentially expressed during specific conditions that mimic host environments during health and disease, for example. In addition, new research approaches have been created based on probiotics, such as metabiotics and metagenomics analyses of host microbiota. In summary, there is a huge interest in probiotic bacteria and the structural and functional genomics analyses have the potential to help researches in the area.

Keywords: omics approach, probiotics, metabiotics, genomics, culturomics.

Introduction

The consumption of microorganisms with the ability to prevent the putrefaction process has been made by humans during centuries, through fermented food. There is a disseminated idea that the fermentation process emerged due to contamination and appropriate climate and environment, resulting in the widely distributed fermented products, such as *kefir, leben* and *koumiss* (1). In addition to its safety for consumption aspects, fermented food had great cultural importance, highlighted by the citation of some of these products on the Holy Bible and sacred books of Hinduism (1–3). However, it was only after the XIX century that fermentation process and the probiotic concepts were deeply studied by Louis Pasteur and Élie Metchnikoff (4), the fathers of microbiology and innate immunology, respectively. Later on, *Bacillus bulgaricus*

(currently known as *Lactobacillus delbrukeckii* subsp. *bulgaricus*) was recovered from human feces and it was shown to reduce putrefaction toxins and help in colitis treatment (4).

In 1930, a Japanese physicist isolated a species from human feces that survived the gastrointestinal tract (GIT), known as *Lactobacillus casei* Shirota, which was later used to develop the fermented milk *Yakult* (3). Besides the use of these probiotics in the production of fermented milk, other lactic acid bacteria (LAB) are also used in the preservation of vegetables, grains and meat (5). More interestingly, the probiotics are crossing barriers from the functional food market to the pharmaceutical and therapeutic ones. This expansion is directly correlated with the advances in the scientific and regulatory aspects of LAB related probiotic and the study of their protein delivery mechanisms (6,7).

Nowadays, probiotics are widely studied to be used in the treatment of functional gastrointestinal disordes (FGIDs) including irritable bowel syndrome, Crohn's disease and ulcerative colitis. This happens for several reasons but the one of greater significance is that there are few options of pharmaceutical treatments for FGIDs and those indicated treatments present low efficacy and serious side effects (8). FGIDs are very common and are believed to be the cause or consequence of changes in gastrointestinal microbiota (9). This information awakens for the application of different properties of probiotics as an excellent alternative for the treatment of these diseases.

Moreover, the use of probiotics has also shown to be a favorable strategy not only against FGID but to a wide range of disorders, because they may reinforce the gut barrier function, conferring clinical benefits at distant sites on an immunomodulatory basis (10). Some studies have shown the beneficial effects of probiotics in modulating inflammatory and autoimmune diseases, such as against rheumatoid arthritis (11), type I diabetes (12), multiplesclerosis (13), atopic dermatitis (14), and myasthenia gravis (15). Moreover, probiotics have benefitial effects in the treatment of cancer, neurodegenerative diseases, Metabolic syndrome and psychiatric illnesses, among other pathologies. Also, some evidences suggest the reduction of ventilator-associated pneumonia in intensive care units patients receiving mechanical ventilation (10). Despite the advantages, one of the challenges involving probiotics is how to select a certain strain with potent immune modulating properties (15).

In view of this, there is a growing interest in the study of probiotic bacteria through structural and functional genomics for the discovery of probiotic-related features. Because of the development of NGS, the bacterial whole-genome sequencing has become a low cost and suitable approach for a rapidly and accurate screening of potential probiotic candidates for the treatment of each disorder (16,17). This approach allows researchers to detect and discard candidate strains that have potential risk factors, like the presence of antibiotic resistance or virulence

genes. It also facilitates the analysis and description of functional mechanisms, avoiding the difficulties of isolating and growing the microorganisms (18). This new approach using NGS techniques to screen potential probiotic candidates makes it very important to better understand the genomic features that could be analyzed.

Probiotic related features

Firstly, the simplified definition of probiotic bacteria is originally related to live cultures that help in the maintenance of a healthy and balanced intestinal microbiota (19). This definition has gained new approaches so that probiotics currently have many functions and demonstrate different mechanisms of action. Specifically for the GIT, probiotics have been elucidated through the fusion of structural and functional genomics techniques. Inside this context, three main features deserve attention in the mechanisms of probiotic action: (i) survival through GIT passage (bile salts and gastric acidity) (20), (ii) competitive exclusion and antimicrobial activity, like microcin and hydrogen peroxide production (21) and (iii) modulation of the immune system of the host GIT (4) (Figure 1). Those mechanisms will be further described in the next sections.

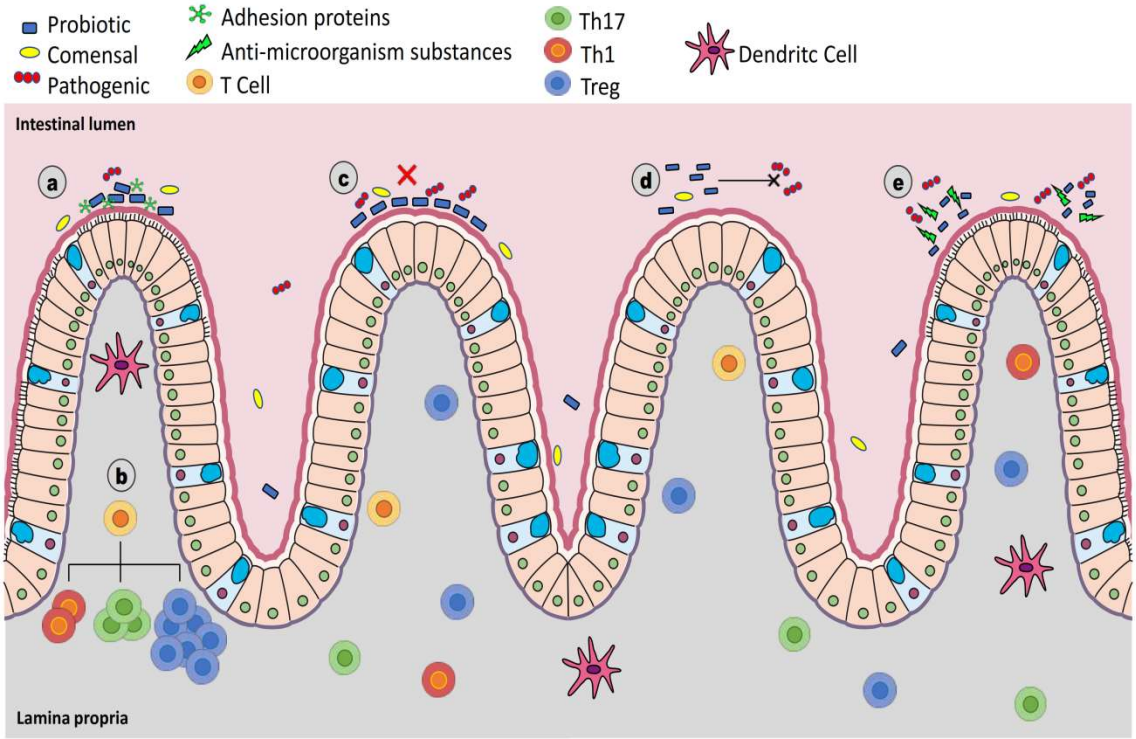

Figure 1. After surviving the gastric juice and bile salts, the probiotic organism can act through specific mechanisms: (**a**) adhesion and colonization, (**b**) modulation of the immune system, (**c**) enhancement of epitelial barrier, (**d**) competitive exclusion, (**e**) production of anti-microorganism substances

Different bacterial genus and species are used as probiotics, for instance: *Lactobacillus casei* Shirota, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus* La5, *Lactobacillus plantarum* 299v, *Lactobacillus fermentum* KLD, *Lactobacillus reuteri* SD2112, *Bifidobacterium breve, Bifidobacterium longum* BB536*, Bifidobacterium lactis* Bb12*, Propionibacterium freudenreichii* JS*, Bacillus subtilis, Bacillus cereus* toyoi*, Escherichia coli* Nissle 1917 and *Enterococcus faecium* SF68. All of these species present therapeutic applications in prevention and treatment of intestinal disorders, such as diarrhea in newborns (22).

The first widely known scientific report about probiotic bacteria dates back to 1907 and demonstrated the correlation between the ingestion of LAB and the increase of longevity of Bulgarians and other populations (23). Fermented foods produced with the use of many bacteria of the *Lactobacillus* genus have been widely employed as therapeutics for the prevention or treatment of diseases due to their beneficial properties, such as relief of the lactose intolerance symptoms and a decrease of diarrhea by rotavirus (22).

Recently, most of the studies and utilization of probiotics are related to the LAB, especially *Lactobacillus* isolated from GIT. When it comes to the administration of probiotic bacteria, there is a determined amount of bacteria necessary to exert a desired feature and consequent host response, which may vary according to the strain, usage,and formulation and it is generally recommended at least 10^7 microorganisms per milliliter daily (24).

Bacterial safety aspects

In the course of time, it is possible to observe changes in the content and in the order of genetic information of the organisms due to genomic plasticity and the evolutionary pressure, which will ultimately act in the bacteria leading it to fix or lose the genomic modifications (25).

The genomic plasticity is the dynamic property of DNA that arises from genetic conversion and point mutations, rearrangements (through translocation and inversion, for example), deletion and insertion of genetic material from other organisms (plasmids, transposons, bacteriophages, among others). These mechanisms alter the bacterial lifestyle, contributing to its adaptation to different environments and influencing in evolution (26).

Phage regions are important in studies of the genomic plasticity of probiotic bacteria because they are used in fermented products for human consumption. Phages are obligate parasites and most of them have a multiplication cycle that culminates in cellular lysis, where hundreds of viral particles are released, ready to infect nearby cells (27). Moreover, phages are widely distributed over the world, where it is possible to find up to 10^8 phages in just a drop of water from the ocean (28).

All industrial or biotechnological processes that require bacterial use in the production of food products or molecules could be interrupted in some moment because of the presence of virulent phages. They are a primary cause of failure in the fermentative process during the industrial transformation of milk (29).

The first description of phages infecting dairy starter dates from 1935 and, since then, important improvements have been made, particularly in ecology, phage genomics and resistance to environmental factors (30). The literature also reports phage regions in species of *Lactococcus* genus, such as *Lactococcus lactis* (31). However, even with all the advances in the area, phage contamination still damage the products and reduce the productivity (32). Phages may present several places of origin, therefore, it is very important to study all potential sources of contamination and their action on the production of dairy products, damaging the fermentation process (29).

Another feature that characterizes the acquirement of genomic material is the presence of genomic islands (GEIs). GEIs may be classified as pathogenicity islands (PAIs), metabolic islands (MIs), symbiotic islands (SIs) and resistant islands (RIs). They are large regions transferred through horizontal gene transfer, harboring a significant amount of genes (encoding similar functions and operons) with the potential to take the bacteria to evolve by leaps (25)

Probiotic bacteria have to be analyzed searching for PAIs and RIs, which contain a high concentration of virulence factors and antibiotic resistance genes, respectively, and could be transferred to other organisms, implicating in the safety aspects of the bacteria. Desirable probiotic bacteria should only contain natural resistance, with no trace of virulence factors or antibiotic resistance genes in unstable regions, like GEIs, phages, and plasmids (33). Besides, there are some specific points that characterize the probiotic action within the host GIT that will be discussed in the next section.

Gastric juice and bile salts resistance

One of the main attributes of a probiotic microorganism is its ability to survive the GIT environment. A study with comparative genomics analysed the niche-based stress-responsive genes of two *Lactobacillus helveticus* strains: MTCC 5463 (a potential probiotic) and DPC4571 (a cheese start), in which, the first one harbor many genes involved in stress response. In addition, the potential probiotic strain presents a higher number of genes related to heat, osmotic, cold and oxidative stress resistance compared to DPC 4571 (34).

The functional genomics studies have been complementing and elucidating some questions related with stress response. Using transcriptomics and proteomics, a study with the probiotic *Lactobacillus rhamnosus* GG analysed the effects of bile stress and demonstrated that 316

transcripts changed in level expression and 42 proteins (intracellular and surface-exposed), were differentially abundant. The authors associated the changes observed with the adaptation process of *L. rhamnosus* GG (35). Performing the same omics to *Bifidobacterium longum* BBMN68, the expression level of 236 transcripts changed significantly and 44 proteins were differently abundant. The hypothesis related with the modification of cell membrane composition (cyclopropane fatty acid increases and transmembrane proteins decreases) was confirmed with surface hydrophobicity assay (36).

Bacterial pathogens exclusion mechanisms

The competitive exclusion of pathogens is another criterion to select a probiotic bacterium. Among the mechanisms of action, there are, for instance, the enhancement of the epithelial barrier, production of anti-microorganism substances, competitive exclusion of bacterial pathogens, increased adhesion to intestinal mucosa and modulation of the immune system (37).

Adhesion-related genes

In addition to the survival through the GIT, the adhesion to the intestinal epithelium is another factor that may contribute to the probiotic activity, through exclusion mechanisms. The interaction between microbe and host occurs via adhesion-related proteins that recognize and bind to specific receptor regions of the host cell, activating the innate response, promoting the invasion or bacterial colonization. Adhesion may be intermediated by pili or fimbriae extending out from the bacterial cell wall or Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) (38).

Preliminary *in vitro* studies using intestinal epithelial cells revealed multiple probiotic *Lactobacillus* producing adhesions (39, 40) and *Bifidobacterium* spp. was shown to adhere to the human intestinal mucus (41). The access to the genomic sequence data combined with genomic techniques helped to elucidate the adhesion mediators. Most of these are secreted or bound to the cell wall in a sortase-dependent way, aiming to interact with the intestinal epithelium (42,43). Studying 43 *Lactobacillus* strains, Harriset al used the Cluster of Orthologous Groups (COG) to identify at least one sortase A gene for each. Seven genomes among them have an extra sortase A, of which, five have a sortase C gene and a putative pilus operon (44).

In *L. acidophilus* NCFM, an *in silico* search on the genome allowed the identification of five cell surface adhesion proteins, including: one fibronectin binding protein (FbpA), one S-layer protein (SlpA), one mucin binding protein (Mub) and two homologous R28 proteins involved in *Streptococcus* adhesion (45).

Through mutational analyses, FbpA, SlpA, and Mub were shown to contribute to the adhesion to Caco-2 epithelial cells. Similarly, one stress response protein and one aggregation promoting factor (both surface proteins) were later found in other studies, which are also responsible to contribute in the adherence to Caco-2 cells (46,47).

Comparative genomics was used with two *L. rhamnosus* strains and revealed the presence of genomic islands, where one of them, predicted in *L. rhamnosus* GG, harbours genes coding for three secreted proteins, sortase-dependent pili, encoded by *spaCBA* operon that was later confirmed through experimental analyses as pili encoding genes (48). Functional annotation was used to characterize the probiotic potential of *Bacillus coagulans* HS243, in which 11 genes were predicted as adhesion-related proteins, among them: enolase, fibronectin binding protein and flagellar hook associated proteins (49).

The adhesion mechanism is an important property to select a probiotic strain and using *in silico* analyses it is possible to determine more details of the adhesion sites, such as mucin and binding to fibronectin (18)

Antimicrobial peptides

The LAB action in the conservation of food is due to both medium acidification (pH 4.5 a 3.5) and the production of numerous bacterial agents, such as organic compounds and bacteriocins (50). Bacteriocins are bacterial produced peptides, which act against other microorganisms and to which the producer has specific immunity mechanisms (51).

The first work reporting the mechanism of action of bacteriocin mediated inhibition reported the discovery of antagonists among *Escherichia coli* strains (52). Although the use of bacteriocins has been formally proposed later (53), it is probable that humans are already benefitting from bacteriocin production for ~8,000 years since the first production of cheese and fermented food (C51).

Bacteriocins were first classified in 1993 (54) and, since then, some suggestions about their classification have been proposed (51). Bacteriocins are divided into classes I, II, III and IV. Class I harbors lantibiotics or thermostable peptides with a molecular weight below 5 kDa produced by gram-positive bacteria and present atypical amino acids, such as lanthionine (Lan), metilanthionine (MeLan) and others (55). The class II bacteriocins are represented by non-lanthionine bacteriocins, they are thermostable and have 10 kDa

molecular weight, slightly heavier than class I. Due to differences in the structure of class II bacteriocins, they are divided into subclasses: pediocin (IIa), lactacin F (IIb), enterocin (IIc) and lactococcin A (IId) (51, 55). Using comparative genomics to characterize the potential probiotic feature of *Lactobacillus plantarum* ZJ316, a study showed that this strain is an important

producer of bacteriocins, since it is capable of producing at least two classes of bacteriocins, IIb and IIc (56).

Bacteriocins weighting more than 30 kDa are classified in class III. They are thermolabile and are mainly produced by gram-positive bacteria (57). Class III bacteriocins are also divided into subclasses, where a group is represented by bacteriolytic enzymes (bacteriolisins), which acts lysing sensible strains, and the non-lytic group of antimicrobial proteins, represented by lysostaphin and enterolysin A (51,55). Through comparative genomics, a potential probiotic strain of *Lactococcus lactis* was analysed for the presence of bacteriocins. Using an annotated and curated genome, the strain NCDO 2118 presented one bacteriocin for each of three classes, in which, two were not previously predicted in the genome sequence (class I and III) (58).

Finally, class IV includes bacteriocins that require the presence of a portion of carbohydrates or lipids in their molecule to have a complete activity (59). Compared to the use of antibiotics in infection treatments, bacteriocins are more target-specific, have low or no toxicity to eukaryotic cells, and are active against antibiotic-resistant strains. However, there is still a lack of evaluation about the affect of the gut microbiota general composition and the probiotic effects in healthy animals (60).

Besides the gut, skin and other mucosal tissues are in direct contact with the external aggressive agents and are thus continuously exposed to huge numbers of pathogenic microorganisms.Thus, to fight against these pathogens, the epithelial/mucosal surface, along with the microbiota, induces a diversity of mecanisms of action that directly kill or inhibit the growth of these microorganisms (63,62,61). Only to strengthen, these bacteria of the microbiota also produce bacteriocins and these molecules are also essential for the host protection in health and disease.

Immune development and function

Coevolution between microbes and mammals, including humans, brought many mutual benefits and, depeding on the concentration and microenviroment of these microbes, specially gut bacteria, they are related to prevention of many human diseases. Taking into consideration the benefits of this coevolution for humans it is possible to highlight the ability of the microbiota to assist in the development of the human immune system (64). One of the clearest examples of this role is that germ-free animals, since the early life, have great damage to the development of the immune system in the gut (65). In other words, animals depleted of gut microbiota have smaller Peyer's patches, fewer antimicrobial peptides, antibodies and B cells, as well as other immunedeficiencies (66). Still, immune development induced by gut microbiota is associated with the host protection against inflammatory disorders (67) and infectious diseases (68).

Besides its role in the formation of the immune system, probiotics are important because they present potent immunomodulatory and anti-inflammatory activities (shown in Figure 1), acting on the prevention and treatment of inflammatory and autoimmune diseases. Because of these properties, there are several dairy products available in the market for consumption and the most widespread are fermented milk (69).

Among these activities, an important strategy of mammals to maintain the homeostasis of the intestinal environment is to minimize the contact among lumen microorganisms and the surface of intestinal epithelial cells (IEC). Different types of pattern recognition receptors are expressed by IEC such asToll-like receptors (TLR), NOD-like (Nucleotide oligomerization domain) receptors and G protein-coupled receptors (GPCR), that recognize microbial compounds (MAMPS, *microbe-associated molecular patterns) and the response of modulating cells* (70).

Evaluating the probiotic potential of *Lactobacillus jensenii* TL2937 in pigs via extracellular proteome, six proteins related with potential immunogenic properties were found, like: chaperonic protease ClpB, Rpf protein (possess a G5 protein family domain – present in various extracellular petidases, responsible for cleaving human IgA) (71,72).

Omics applied to probiotic bacteria

Genomics

The first completely sequenced genome of the LAB group was *Lactococcus lactis* subsp. *Lactis* IL1403 strain, published in 2001. This study revealed biosynthetic pathways, phages and part of components that participate in aerobic metabolism (6). In 2002, a program intended for the mass sequencing of LAB genomes was announced by Lactic Acid Bacteria Genome Sequencing Consortium (73). Currently, more than 100 *Lactococcus* genomes are available on the NCBI database (National Center for Biotechnology Information - http://www.ncbi.nlm.nih.gov/genome/genomes/156), from which 36 are complete genomes.

LAB present small genomes with approximately 2Mb in length and 2000 genes, respectively, and which may range from 1600 to 3000 genes in different species. This variation results from LAB evolution through gene loss, duplication,and acquisition (74).

Bolotin et al showed that dairy streptococcus have undergone a reductive evolution, where a divergence occurred between them and pathogenic streptococcus. The most remarkable example occurred in *Streptococcus thermophilus*, where it diverged from other species of

Streptococcus through the loss of virulence factors, such as those involved in adhesion and antibiotic resistance (75).

Many studies highlight the importance of genomic sequencing in the discovery of new features related to the LAB, such as the identification of several genes encoding proteolytic enzymes (which participates on cheese maturation) in *Lactobacillus helveticus* (76). The sequencing of the first *Lactobacillus* species: *Lactobacillus plantarum* WCFS1 (77), *Lactobacillus johnsonii* NC533 (79,78) and *Lactobacillus acidophilus* NCFM (80), revealed some interesting characteristics, such as, lifestyle adaptation islands; lack of biosynthesis pathways; and the presence of unique structures named potential autonomic units (PAU), respectively.

The bioinformatics approach assisted in identifying the citrate catabolic pathway in *Lactobacillus casei* (81) and other studies have identified genes responsible for decarboxylation of branched-chain alpha-ketoacid of *Lactococcus lactis* (82, 83). Genomic sequencing has also played a role in the elucidation of LAB probiotic effects, for instance: in the study of antimicrobial compounds and immunomodulatory mechanisms of *Lactobacillus reuteri* (84), the comparative analysis of pilus associated genes and metabolic pathways in *Lactobacillus rhamnosus* and *Lactobacillus casei* (85) and, the identification of adhesion associated proteins (*cwaA*) in *Lactobacillus plantarum* (86).

To identify a probiotic organism, many experiments *in vitro* and *in vivo* must be performed and it takes a considerable time. The omics approach came to speed up these studies enabling the identification of potential probiotic microbes. A recent study with 269 species of the families of Lactobacillaceae and Leuconostocaceae was performed. Using phylogenetic approach, 29 ribosomal proteins and housekeeping genes were analysed and it was possible to demonstrate that *Lactobacillus* genus has different subclades, opening the possibility of reclassification of lactobacilli. The group highlighted the importance of this sub-division that allows accurate molecular markers that will prevent some issues, like the misidentification of probiotic strains, for example (87).

Finally, besides the genomic approach (Figure 2), other omics have been providing analysis of divergence and evolution of the most varied species over time (88). Through omics approach, it is possible, for example, to correlate protein data with the survival inside the host during stress conditions or secreted proteins that may exert a specific role in probiotic effects of certain strains, through analysis of bacterial-host interaction.

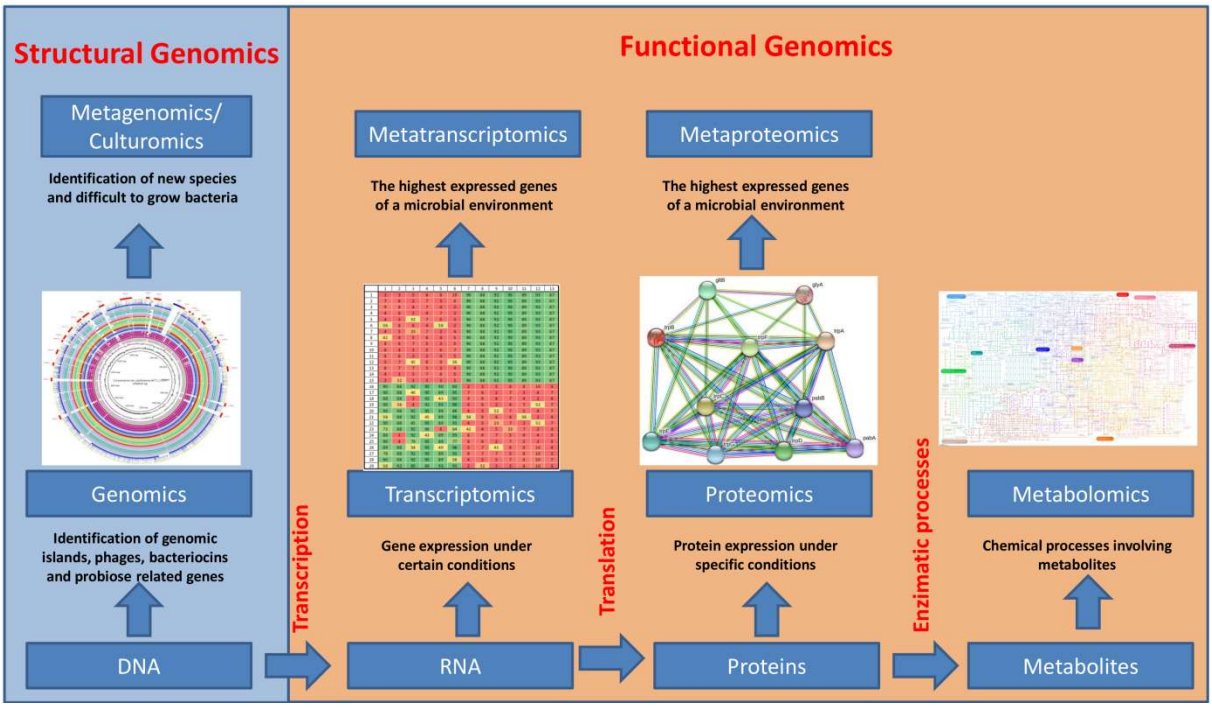

Figure 2. The goals of omics approach.

Metagenomics

Through metagenomics (Figure 2) analyses, it is possible to access physiological and genetic information about uncultured organisms, such as the human GIT microbiota, through the sequencing of 16S rRNA genes (89). This type of tool gives important genetic information for uncultured organisms, creating novel hypotheses of microbial function. Initially, the field consisted of cloning DNA from environmental sources, followed by functional expression screening (89,90).

One pioneer study in this area is about the large-scale metagenomics projects in Sargasso sea, in which a massive microbial population was characterized through 1.045 billion of base pairs from the seawater samples. This amount of sequences provided important information on the diversity, gene content and the relative abundance of the organisms (91).

The development of metagenomics, mainly with the advent of next-generation sequencing technologies (NGS), and the creation of the International Human Microbiome have both boosted the field and opened a new door in the analyses of bacterial host interactions. The culture free methodology used by NGS technologies expanded the analyses of microbial composition and may now be used not only to predict new probiotics from the comparison of the microbiota from healthy and diseased individuals, but may also be used to analyze the composition of the microbiota before and after administration of a given probiotic bacteria (92).

Studying the microbiota of mice genetically obese and their lean littermates, Turnbaugh et al demonstrated through metagenomics analyses that the obesity was associated with the abundance of two groups of bacteria: Bacteriodetes and Firmicutes (93).

Nobutani et al studied two groups of patients with IBS: for the first group they administrated *Lactobacillus gasseri* and to the second one, a placebo. In this analyses, they identified 87 genera, where 13 genera presented differences in bacterial occupation when both placebo and CP2305 group were compared, where *Dorea, Enterococcus,* and Dialiste genera were decreased in the CP2305 group (94).

Culturomics

Some studies have risen the importance of culturomics approach (Figure 2) for probiotic analyses (Dubourg et al., 2014).Culturomics approach consists in the use of multiple culture conditions followed by matrix-assisted laser desorption/ionization-time of flight and 16 rRNA for the identification of less representative species in the sample (96, 97). This technique has presented significant results on the identification of new organisms, generally not found through metagenomics technique (95,98).

Seeking to determine a larger amount of organisms, the culturomics may identify populations with a culture concentration lower than 10^3 - 10^4 bacteria (Lagier et al., 2012). Culturomics rebirths with the studies of environmental microbiologists. For instance, in 2007, Bollman et al created a new method of isolation that was able to obtain almost 40% of the cells present in a specific marine environment (100).

The first study of the microbial composition of the gut microbiota using culturomics dates from 2012. Lagier et al traced 212 different culture conditions and used mass spectrometry and 16S rRNA amplification and sequencing to test the colonies found. Among so many data, the culturomics analyses of microbiome resulted in 31 new species in addition to more than 100 species never described in the literature (99).

Culturomics and metagenomics leverage the potential of identification of new species. Together, they complement each other allowing a greater knowledge and understanding of new and/or difficult to grow bacteria. A database was created in order to group the several prokaryotic species associated with human beings (commensals or pathogens), highlighting the importance of culturomics and metagenomics. From the 2172 species listed, Hugon et al classified in 12 different fila, most of them as Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (101). More recently, it was reported that the amount of aforementioned species increased, totalizing in 2776 species due to culturomics techniques that facilitated the identification of new bacterial species (102).

Stool samples of malnourished and healthy patients from Senegal and Niger were analysed using culturomics and metagenomics. Besides finding an important decrease diversity and an enrichment of potentially pathogenic bacteria, they could identify some probiotic bacteria only in healthy children. Even more, new species could be identified, such as species from Propionibacteriaceae and Bacillaceae families (103).

Functional genomics

Transcriptomics

The area of transcriptomics (Figure 2) may elucidate how genes are involved in specific conditions. Meulen et al identified 375 novel regulatory mRNAs in *L. lactis* MG1363 involved in stress response and metabolic processes, such as internal promoters, operon structures and novel ORFs (111). Using probiotic preparations containing *L. acidophilus, L. casei* and *L. rhamnosus*, an *in vivo* experiment was performed with volunteers to analyze the gene-regulatory networks and pathways in the human mucosa. A significant variation was observed between the transcriptomics results from volunteers, but clarified that there are other reasons to define a probiotic response, such as the resident microbiota, diet, genetic background and lifestyle (112). Funtional genomics may also contribute to refine some phylogenetic studies related to the existence of high homology among some bacteria, like *Lactobacillus acidophilus.* Using core and transcriptomic data it was possible to identify small ORFs highly conserved and transcribed in various species of this group, highlighting the new possibilities to characterize and present new probiotics to the market (113). Studying the transcription profile of genes associated with adhesion and stress response of the probiotic *L. acidophilus* NCFM, Weiss and Jespersen used specific conditions to mimic GI tract *in vitro*. During gastric digestion, the genes enconding GroEL, ClpP and DnaK had an important up-regulation. The genes enconding mucin-binding and fibronectin-binding proteins were up-regulated in incubation process (duodenal juice and bile) (114).

Proteomics

Proteomics area (Figure 2) allows the study of the expression of a big range of proteins from a specific organism. A proteomic analysis comparing a wild strain of *Lactobacillus plantarum* with a mutant one under physiological and heat stress conditions showed an induction of proteins related with re-folding of proteins under cellular damage elucidating the importance of CtsR regulon control in lactic acid bacteria (115). Another study using proteomics comparison with three *L. plantarum* strains confirmed the bile resistance characteristics of *L. plantarum* 299 V, already known as a probiotic. The analyses were made using strains with different levels of bile

resistance and helped to understand how these strains modulate their metabolism to survive in stressed environments (116).

Studying a long-chain carbohydrate known as prebiotic, called inulin, it was observed that *L. plantarum* was able to use this compound and it was identified an operon (*fosRABCDXE*) for inulin metabolism in this genome (117).

Proteomic analyses of *Bifidobacterium longum*, isolated from stool, was performed to evaluate the protein expression under the effect of bile salts. Using different quantities of exposure to bile, it was possible to identify 34 different proteins differentially regulated, amongst them: general stress response chaperones and some enzymes of pyruvate and glycolysis catabolism (118).

Metabiotics and metabolomics

Other omic area recently used is metabolomics (Figure 2). Through this approach it is possible to determine and quantify the metabolites present intracellularly (104). There are some metabolites that promote health, named Metabiotics. They are metabolites from the structural components, metabolites or signaling molecules of probiotic bacteria, such as lactic acid, short chain fatty acids (SCFAs), linoleic acid, some glycoproteins/peptides and potentially carcinogenic metabolites. Metabiotics have beneficial bioactive substances that act in the host-specific physiological functions, regulatory, metabolic and/or behavior reactions (105, 106). Among these, SCFAs are the most studied, being a source of energy for colonocytes and the modulators of various metabolic activities (106).

Metabiotics-producing bacteria include not only the well known probiotic species from *Lactobacillus*, *Escherichia,* and *Enterococcus*, but also other strains from the human dominant intestinal phyla (Bacteroides, Firmicutes, Proteobacteria, Actinobacteria, and Archae) for nutrition and medical aims (106).

Probiotics produce several bioactive substances with beneficial effects in the GIT diseases, which help in homeostasis and competitive exclusion of pathogens (107). More interestingly, the multifunctional SCFA acetate plays an important role in epithelial cell division, ileal motility and others (108).

The most studied metabiotic is butyrate, produced by *Faecalibacterium prausnitzii* and *Eubacterium rectale* in the gut (109), which has the potential to differentiate between cancer and normal cells to exert epigenetic effects and inhibit the growth of cancer cells (110). Butyrate has been associated with the induction of apoptosis in colon cancer cells due to its ability to convert procaspase 3 to active caspase 3 (110).

Some technologies are extremely useful for metabolomics area, the most current and integrated method related with separation and detection process are liquid chromatography (LC: high-performance, HPCL and ultra performance, UPLC) and mass spectrometry (MS). HPLC tecnique is able to separate multiple compounds according with stationary phase and UPLC results is similar to HPLC, however this technology has more capacity, resolution, sensitivity and higher speed (104).

Conclusions

Probiotic bacteria have been used by humans for a long time in the maturation of cheese and the production of fermented food. However, their importance has been only recently highlighted with the study of their safety aspects, exclusion mechanisms, survival through the host GIT and production of immunomodulatory and anti-inflammatory proteins. With the advent of NGS technologies for structural and functional genomics coupled with whole proteomics analyses using mass spectrometry, there are several new possibilities from probiotic identification using metagenomics of GIT microbiota to the comparison of microbial changes under disease and after probiotic administration. We can highlight the importance of auxiliariy techniques like culturomics on the identification of bacteria not detected in metagenomics, for example. In addition both approaches may be used to elucidate some misindentification of probiotic strains. Genomic studies may also be used in the analyses of genome plasticity between probiotic and non-probiotic related strains, for the identification of genes related to each one of the probiotic features. Finally, transcriptomics and proteomics may help in the identification of differentially expressed genes in probiotic and non-probiotic species for the later elucidation of metabolic pathways and protein-protein interactions analyses. Future improvements in the area may involve the identification of probiotic-pathogenic and bacterial-host protein-protein interactions in a more wide system biology perspective. The omics approach brought new paths and forms to analyse more deeply some characteristics of future potential probiotic bacteria, broaden the understanding of its different ways to interact with the gut microbiota of the host and this was only possible with integrative omics approach.

Author contributions

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V. OBJETIVOS

V.1 Objetivo geral

Identificar, através da genômica comparativa e análises *in vitro*, possíveis genes que estariam relacionados à capacidade probiótica e imunomodulatória das espécies *Lactobacillus rhamnosus* 156.4 e *L. lactis* NCDO 2118.

V.1.1 Objetivos específicos

- Analisar a filogenia e sintenia gênica dos genomas de *L. rhamnosus* 156.4 e *L. lactis* NCDO 2118, além dos genomas completos já depositados no banco de dados do NCBI;
- Predizer as ilhas genômicas entre as espécies de *Lactococcus* e vias metabólicas exclusivas e regiões de fagos de *L. lactis* NCDO 2118;
- Avaliar*in vitro*, a susceptibilidade de *L. lactis* NCDO 2118 em relação ao estresse ácido, resistência a bile e capacidade de adesão;
- Utilizar análises *in silico* e *in vitro* para predizer e investigar bacteriocinas das duas linhagens de interesse, além de genes de resistentência a antibióticos presentes em *L. lactis* NCDO 2118;
- Predizer *in silico*, proteínas secretadas, possivelmente relacionadas à atividade anti-inflamatória e imunomodulatória de *L. lactis* NCDO 2118.

VI. ARTIGOS RESULTANTES

VI.1 – Capítulo I

In silico prediction, *in vitro* antibacterial spectrum, and physicochemical properties of a putative bacteriocin produced by *Lactobacillus rhamnosus* strain L156.4

Letícia de C. Oliveira†, Aline M. M. Silveira†, Andréa de S. Monteiro†, Vera L. dos Santos, Jacques R. Nicoli, Vasco A. de C. Azevedo, Siomar de C. Soares, Marcus V. Dias-Souza and Regina M. D. Nardi

†Autores contribuíram igualmente neste trabalho.

Buscando identificar possíveis bacteriocinas em *Lactobacillus rhamnosus* L156.4, testes *in silico* e *in vitro* foram realizados, objetivando elucidar uma possível característica inicial probiótica. *L. rhamnosus* L156.4, isolada de fezes de camundongos NIH, foi identificada por meio de espectrometria de massas (MALDI-TOF) e 16S rRNA. O genoma foi sequenciado, montado e anotado, partir daí foi realizado a predição de bacteriocinas e inferência filogenética. Avaliando o espectro antagonista de forma detalhada, 23 linhagens patogências foram utilizadas; células do sobrenadante foram utilizadas para avaliação da atividade antagonista diante de diferentes temperaturas, pH e sensibilidade à enzimas proteolíticas. Com o BAGEL, foram identificados genes relacionados à síntese de bacteriocinas que apresentaram homologia e identidade com *Lactobacillus rhamnosus* GG e *Lactobacillus rhamnosus* HN001. Nos ensaios *in vitro* foi possível confirmar a presença da bacteriocina predita *in silico*, além de observar uma atividade antimicrobiana significativa e avaliação satisfatória em relação aos aspectos físicoquímicos. Foi identificada atividade contra *Lactobacillus fermentum* ATCC 9338 e a presença de bandas em gel, correspondendo às zonas de inibição bacteriana testadas. Tais resultados, nos levam a apostar em novas análises para conhecer melhor esta linhagem quanto ao seu potencial probiótico.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 May 2017
doi: 10.3389/fmicb.2017.00876

In silico Prediction, *in vitro* Antibacterial Spectrum, and Physicochemical Properties of a Putative Bacteriocin Produced by *Lactobacillus rhamnosus* Strain L156.4

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OPEN ACCESS

Edited by:
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Reviewed by:
Atte Von Wright, University of Eastern Finland, Finland
Vincenzina Fusco, Consiglio Nazionale Delle Ricerche (CNR), Italy
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 12 October 2016
Accepted: 01 May 2017
Published: 19 May 2017

Citation:
Oliveira LdC, Silveira AMM, Monteiro AdS, dos Santos VL, Nicoli JR, Azevedo VAdC, Soares SdC, Dias-Souza MV and Nardi RMD (2017) In silico Prediction, in vitro Antibacterial Spectrum, and Physicochemical Properties of a Putative Bacteriocin Produced by Lactobacillus rhamnosus Strain L156.4. Front. Microbiol. 8:876. doi: 10.3389/fmicb.2017.00876

A bacteriocinogenic Lactobacillus rhamnosus L156.4 strain isolated from the feces of NIH mice was identified by 16S rRNA gene sequencing and MALDI-TOF mass spectrometry. The entire genome was sequenced using Illumina, annotated in the PGAAP, and RAST servers, and deposited. Conserved genes associated with bacteriocin synthesis were predicted using BAGEL3, leading to the identification of an open reading frame (ORF) that shows homology with the L. rhamnosus GG (ATCC 53103) prebacteriocin gene. The encoded protein contains a conserved protein motif associated a structural gene of the Enterocin A superfamily. We found ORFs related to the prebacteriocin, immunity protein, ABC transporter proteins, and regulatory genes with 100% identity to those of L. rhamnosus HN001. In this study, we provide evidence of a putative bacteriocin produced by L. rhamnosus L156.4 that was further confirmed by in vitro assays. The antibacterial activity of the substances produced by this strain was evaluated using the deferred agar-spot and spot-on-the lawn assays, and a wide antimicrobial activity spectrum against human and foodborne pathogens was observed. The physicochemical characterization of the putative bacteriocin indicated that it was sensitive to proteolytic enzymes, heat stable and maintained its antibacterial activity in a pH ranging from 3 to 9. The activity against Lactobacillus fermentum, which was used as an indicator strain, was detected during bacterial logarithmic growth phase, and a positive correlation was confirmed between bacterial growth and production of the putative bacteriocin. After a partial purification from cell-free supernatant by salt precipitation, the putative bacteriocin migrated as a diffuse band of approximately 1.0–3.0 kDa by SDS-PAGE. Additional studies are being conducted to explore its use in the food industry for controlling bacterial growth and for probiotic applications.

Keywords: *Lactobacillus rhamnosus, in silico* prediction, bacteriocin, BAGEL, genomic and physicochemical characterization

INTRODUCTION

Lactobacillus rhamnosus is a facultative heterofermentative lactic acid bacterium (LAB) that is closely related to Lactobacillus casei and Lactobacillus zeae and encompasses a genetically diverse group of strains with a high frequency of discriminative core genome polymorphisms and a remarkable accessory genome, or variome distribution (Ceapa et al., 2015). This species has strain-specific genetic and metabolic characteristics that explain its occurrence in a variety of ecological niches (Douillard et al., 2013; Ceapa et al., 2015). Bacteriocinogenic L. rhamnosus strains have been isolated from human feces (Gorbach, 1996; Gill et al., 2000; Cukrowska et al., 2009; Dimitrijević et al., 2009; Aguilar-Uscanga et al., 2013), vaginal microbiota (Li et al., 2005), fermented beverages (Todorov and Dicks, 2005), grape peels (Sarika et al., 2010), milk samples (Srinivasan et al., 2013), and cheese (Jeong and Moon, 2015). This species is generally recognized as safe (GRAS) and has been widely explored as a probiotic in animal production (Weese and Anderson, 2002) human health (Gill et al., 2000; Cukrowska et al., 2009; Douillard et al., 2013; Szajewska and Kołodziej, 2015) and as a biopreservative in food systems (Cotter et al., 2005; Douillard et al., 2013).

Bacteriocins are a heterogeneous group of ribosomally synthesized peptides or proteins that have a narrow or broad antibacterial spectrum of activities against the same species or species that are phylogenetically related to the bacteriocin producer (Klaenhammer, 1993). Bacteriocin-producing strains are immune to their own bacteriocins due to the production of an immunity protein. The currently accepted system for classifying bacteriocins is based on whether they are post-translationally modified (class I) or are unmodified/minimally modified (class II) (Cotter et al., 2005, 2013).

Although the production of bacteriocins by LAB has been widely explored, few studies have been conducted using L. rhamnosus strains, which should be further explored due to their technological potential in human and veterinary medicines, and for food quality and safety. Since the purification of these molecules requires laborious procedures, the utilization of classical methods for new bacteriocins is cumbersome. Currently, genome mining approaches that explore both DNA and peptide databases enable prospection studies of bacteriocins in silico (van Heel et al., 2013). The present study reports the in silico prediction of bacteriocin genes in L. rhamnosus L156.4. Additionally, we performed in vitro assays to determine the antibacterial spectrum of a putative bacteriocin and made a partial physicochemical characterization.

MATERIALS AND METHODS

Isolation and Characterization of the Strain L156.4

Strain L156.4 was isolated from the feces of NIH mice (Taconic, Germantown, USA) and was initially identified as L. rhamnosus by Gram staining, catalase test, and carbohydrates fermentation pattern as determined by the API50CHL kit (bioMérieux, Marcy l'Etoile, France). The strain L156.4 was stored at −80°C in Man Rogosa Sharpe broth (MRS, Difco Laboratories Inc., Detroit, MI, USA) supplemented with 15% glycerol. Prior to the experiments, L. rhamnosus L156.4 was propagated twice in MRS broth for 18 h at 37°C in an anaerobic chamber (Forma Scientific Company, Marietta, OH, USA) containing an atmosphere of N_2 (85%), H_2 (10%), and CO_2 (5%).

Identification of Strain L156.4 by MALDI-TOF Mass Spectrometry

The identification of the new strain L156.4 was performed using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry. The strain was cultured overnight on MRS agar at 37°C in anaerobic conditions. For the analysis, individual samples colonies were scraped up using a sterile plastic loop and then applied as a thin film onto a 24-spot steel plate (Bruker Daltonics, Bremen, Germany). After being air-dried, the sample was co-crystallized with 1 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid matrix (HCCA; Bruker Daltonics, Bremen, Germany) in 50% acetonitrile/2.5% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA). Mass spectra were acquired in reflector-positive mode on a MicroFlex LT system tabletop instrument (Bruker Daltonics) using the manufacturer's default settings. Captured spectra were analyzed using the MALDI Biotyper automation control and Bruker Biotyper 2.0 software (Bruker Daltonics, Bremen, Germany). The identification criteria used in our analysis were as follows: a score ≥2.000 indicated a species level identification, a score of 1.700 to 1.999 indicated identification at the genus level, and a score <1.700 was interpreted as not identified. Escherichia coli ATCC 8739 was used as a positive control.

Next Generation Sequencing of Genomic DNA and Data Analysis

The genomic DNA of L. rhamnosus L156.4 was extracted using a Gentra Puregene Cell kit (Qiagen, Hilden, Germany) and then was sequenced with the Illumina MiSeq Reagent kit V2 500 (http://www.illumina.com/products/miseq_reagent_kit_v2.html), using a paired end 250 prepared with the Nextera DNA Library Preparation Kit (http://www.illumina.com/products/nextera_dna_library_prep_kit.html) according to the manufacturer's recommendations. The de novo genome assembly was performed using the A5 pipeline (Tritt et al., 2012).

In order to infer the phylogenetic relationships of strain L156.4, the 16S rRNA gene was predicted using the software RNAmmer (Lagesen et al., 2007). The resulting 16S rDNA sequence was then searched for on NCBI using BLASTn against the 16S ribosomal RNA sequences database and the best BLAST hits were retrieved in addition to the 16S sequences from various Lactobacillus spp. The 16S rDNA sequence from Lactococcus lactis NCDO 2118 was used to root the tree (Oliveira et al., 2014). The software Muscle (Edgar, 2004) was used to generate a multiple sequence alignment and the output file was added on Splits Tree (Huson and Bryant, 2006) to create a phylogenetic tree using the Neighbor-Joining method (Saitou and Nei, 1987).

Genome Annotation, Deposition, and *in silico* Bacteriocin Prediction

We annotated the L. rhamnosus L156.4 draft genome with the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) (Angiuoli et al., 2008) and Rapid Annotation Subsystem using Technology (RAST) (Aziz et al., 2008).

We used BAGEL3 (BActeriocin GEnome mining tooL), a bacteriocin search software, to predict genes related to bacteriocin synthesis, such as prebacteriocins, immunity proteins, ABC transporters, and regulation genes. The input file was the genome sequence of L. rhamnosus L156.4 in.fna format (van Heel et al., 2013). Afterwards, the predicted bacteriocin was submitted to a BLASTp search against the Uniprot database (http://www.uniprot.org/). In addition, conserved genes associated with bacteriocin synthesis were retrieved using the Rapid Annotation Subsystem using Technology (RAST) server (Aziz et al., 2008). The region identified in BAGEL3 and the proteins related to the predicted bacteriocin were detected and manually curated in Artemis to confirm their prediction (Rutherford et al., 2000). A search for the Pediocin-box sequence was conducted using the PFAM database. The motif YGNGVXC was used in the alignment of the predicted bacteriocin with the sequence of class IIa peptides registered on PFAM (Punta et al., 2012).

Furthermore, we made two comparisons of whole genomes using Mauve and the Artemis Comparison Tool (ACT) software in order to distinguish L. rhamnosus L.156.4 from L. rhamnosus GG (ATCC 53103) (Darling et al., 2010; Carver et al., 2005).

Determination of the Antagonistic Activity

L. rhamnosus L156.4 was screened for its antagonistic activity using the deferred agar-spot assay and the spot-on-the lawn assay (Tagg et al., 1976). For the deferred agar-spot assay, a 5 µl sample of an 18 h MRS broth culture was spotted onto the surface of MRS agar and was incubated for 24 h at 37˚C under anaerobic conditions. The cells were killed by exposure to chloroform for 30 min, and the residual chloroform was allowed to evaporate. Then, an MRS agar plate was overlaid with 3.5 ml of soft agar (0.75%) of Brain Heart Infusion (BHI) or MRS previously inoculated with indicator strains at a final concentration of 10^6 CFU/ml (**Table 2**). Plates were then incubated for 24 h at 37˚C under aerobic or anaerobic conditions according to the requirement of the indicator bacteria. The antagonistic activity was evidenced by the presence of a growth inhibition zone around the spot.

For the spot-on-the lawn assay, a total volume of 100 ml of an 18 h culture L. rhamnosus L156.4 in MRS broth was centrifuged at 7,500 g (4˚C) for 15 min and the supernatant was sterilized by filtration through a 0.22-µm pore size PVDF filter (Millipore Corp., Bedford, MA, USA). An aliquot of this cell-free supernatant (CFS) was neutralized with 1 M NaOH and used as follows: a volume of 3.5 ml of MRS soft agar (0.75%) was inoculated with the strains mentioned in **Table 2** at a final concentration of 10^6 CFU/ml. This mixture was overlaid onto MRS agar and 10 µl of the CSF was spotted directly onto this lawn. Sterile BHI or MRS media were used as a negative control. The inhibition zone was evaluated after incubation at 37˚C, for 24 h in an anaerobic chamber or in aerobic conditions.

Effect of Temperature, pH, H_2O_2, and Proteolytic Enzymes on the CFS Antagonistic Activity

The antagonistic activity of the CFS obtained in the previous step was assessed after exposure to different pH-values, high temperatures, or in the presence of catalase and proteolytic enzymes. Aliquots of 5 ml of CFS had pH-values adjusted in a range from 3 to 9 using either sterile 1 M HCl or 1 M NaOH. Fresh MRS broth adjusted to the same pH-values was used as a control. To investigate the temperature effect on the antagonistic activity of the bacteriocin, CFS aliquots were exposed at 60, 80, and 100˚C for 30 min, or at 121˚C for 15 min. The samples were then allowed to cool to room temperature before being tested. The sensitivity of the antagonistic substance to enzymatic degradation by catalase and proteolytic enzymes was evaluated using catalase (E.C.1.11.1.6) at pH 7.0 (50 mM potassium phosphate buffer), trypsin (E.C.3.4.21.4, type II), α-chymotrypsin (E.C.3.4.21.1, type II), and proteinase K (E.C. 3.4.21.64) at pH 7.5 (100 mM Tris-HCl buffer), and using pepsin (E.C.3.4.23.1) at pH 3.0 (50 mM glycine buffer added at 20 mM $CaCl_2$) (all enzymes were from Sigma Chemical Co., St Louis, MO, USA). Aliquots of the CFS at different pH-values were incubated (1:1 v/v) with enzyme solutions (1 mg/ml) and their respective controls at 37˚C for 2 h.

After the previously mentioned treatments, the remaining antibacterial activity of the CFS was determined by spot-on-the lawn assay using the sensitive strain Lactobacillus fermentum ATCC 9338 at a final concentration of 10^6 CFU/ml. This mixture was overlaid onto MRS agar, then 10 µl of each treated CFS or the respective controls were spotted directly onto the lawns. The presence of an inhibition zone was evaluated after incubation at 37˚C for 24 h in an anaerobic chamber.

Evaluation of Antibacterial Activity during *L. rhamnosus* l156.4 Growth

L. rhamnosus L156.4 was used to inoculate 700 ml of 1% LAPTg (v/v) (Raibaud et al., 1963) and was incubated at 37˚C under anaerobic conditions. Samples were removed at different time intervals for determinations of pH (model B474, Micronal, S.A., Brazil), antibacterial activity, and optical density (OD) at 600 nm, using a spectrophotometer (Biosystems Ltda, PR, Brazil). The bacterial growth was also evaluated by cell counting (CFU/ml) from aliquots of 10-fold serial dilutions in sterilized phosphate-buffered saline (PBS, pH 7.5) plated on LAPTg agar and incubated in an anaerobic chamber at 37˚C for 24 h. The antibacterial activity was quantified by spotting aliquots (10 µl) of serial 2-fold dilutions of centrifuged and filtered culture medium in ultrapure water on a lawn of L. fermentum ATCC 9338. Arbitrary units (AU) of antagonistic activity were defined as the reciprocal of the highest serial dilution that displayed an inhibition zone and was expressed per milliliters of culture media (Tagg et al., 1976). This assay was performed in duplicate. Pearson's correlation coefficient was used to investigate

the correlations between growth and the putative bacteriocin production. Values of $p < 0.05$ were considered statistically significant.

Partial Purification of the Putative Bacteriocin by Precipitation

L. rhamnosus L156.4 was cultivated in LAPTg broth (100 ml) for 18 h in an anaerobic chamber. The CFS, obtained as described in section Effect of Temperature, pH, H_2O_2 and proteolytic enzymes on the CFS antagonistic, activity was precipitated in an ice bath with ammonium sulfate to 40% saturation, and then centrifuged (12,500 g, 30 min, 4˚C). The pellet was resuspended in 5 ml of ammonium acetate buffer (50 mM, pH 5.0), and desalted against ultrapure Milli-Q water using a 1 kDa cut-off dialysis membrane (Spectrum Inc., CA, USA). The desalted CFS (DCFS) and the same volume of CFS were freeze-dried. Then, the powder was dissolved in 50 µl of ultra-pure water, and the inhibitory activity of this fraction was determined by a spot-on-the lawn assay using L. fermentum ATCC 9338 as the indicator strain (Tagg et al., 1976).

Direct Detection of the Putative Bacteriocin on Gels

In order to estimate the molecular mass of the bacteriocin, we estimated the position of the inhibitory zone of CFS and DCFS in the gel. Aliquots of CFS and DCFS were subjected to Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) as described by Schägger and Von Jagow (1987) using a 16.5% gel. After electrophoresis at 60 mA for 3 h, the gel was cut into two vertical sections. Half of the gel was stained with Coomassie Brilliant blue R250 (Bio-Rad, Hercules, CA, USA) and the other half was fixed for 2 h in a 20% 2-propanol/10% acetic acid solution, and then was extensively washed with regularly replaced sterile water for 6 periods of 30 min. To detect the bacteriostatic region, the gel was overlaid with LAPTg soft agar (0.75%), seeded with L. fermentum ATCC 9338 as the indicator strain. After an overnight incubation at 37˚C, the gel was examined for the presence of inhibition zones. The molecular mass of the bacteriocin was estimated by a relative mobility method, comparing the migration pattern of the bacteriocin to a mixture of protein markers (ultra-low molecular weight marker M3546, Sigma-130 Aldrich, St. Louis, MO, USA; Bhunia et al., 1987).

RESULTS

Microbial Identification and Phylogenetic Tree

The identity of L. rhamnosus L156.4 was determined by both MALDI-TOF MS analyses and DNA sequencing. By comparing the 16S rDNA sequences (accession number KX644947) with other L. rhamnosus strains deposited in GenBank, the identification of the strain was confirmed as L. rhamnosus, with an identity threshold >98%. Phylogenetic inferences confirmed the identification of the L156.4 strain as L. rhamnosus, which was most closely related to the L. rhamnosus JCM1136 and NBRC3425 strains (**Figure 1**).

Genomic Characterization and Prediction of the Putative Bacteriocin

The Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession MTJY00000000. The version described in this paper is version MTJY01000000. We predicted the presence of an ORF (AOI_1; orf010: locus tag BWR10_11520) using BAGEL3 and PGAAP. This region presented 100% identity with the prebacteriocin of L. rhamnosus strain GG (ATCC 53103) sequence WP_005686837.1 with an E-value of 3.9e-05 and 113 amino acids (**Table 1**). The scanning of this sequence for conserved motifs, as described at http://pfam.sanger.ac.uk, indicated the presence of a putative conserved domain belonging to the Enterocin A superfamily (pfam08951). The Enterocin A domain predicted in orf010 is incomplete, and it corresponds to amino acids 12–66 of the full sequence. A pediocin-like YGNGVXC motif, characteristic of class IIa bacteriocins, was not found in the genome of L. rhamnosus 156.4.

Furthermore, genes that encode components required for bacteriocin synthesis, regulation and hypothetical proteins were detected in the genome of L. rhamnosus L156.4 and are shown in **Figure 2**. All predicted locus tags were manually annotated in order to check and confirm the predicted information. The accession numbers of the coding sequences are shown at **Table 1**.

Considering the similarities of the bacteriocins of L. rhamnosus L156.4 and L. rhamnosus GG (ATCC 53103), we conducted a comparative analysis of the whole genomes of these strains. The results showed that the related region (cluster) of the predicted bacteriocin of L. rhamnosus L156.4 is not identical to the one identified in L. rhamnosus GG (ATCC 53103). Using both approaches (Mauve and ACT), it is possible to see some differences in the genomes regarding regions of deletions and insertions (**Supplementary Figures S1**, **S2**).

Spectrum of Antibacterial Activity of Antagonistic Substances

The deferred agar-spot assay showed the inhibitory activity of antimicrobial substances produced by L. rhamnosus L156.4 against enteropathogenic E. coli, Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes and other gram-positive and gram-negative bacteria, but not against Lactobacillus acidophilus, Lactobacillus brevis and Lactobacillus rhamnosus. Additionally, the spot-on-the-lawn assay was performed using the neutralized CFS, and no inhibition zone was observed after this treatment for all gram-negative indicator strains, suggesting the inhibitory effect was due in part to the action of organic acids (**Table 2**).

Physicochemical Characterization of Antagonistic Substances

The effect of temperature, pH, H_2O_2 and proteolytic enzymes on the antibacterial activity of CSF was evaluated. The inhibitory activity of CSF against L. fermentum ATCC 9338 was maintained at pH-values ranging from 3 to 9 and was not altered by heat

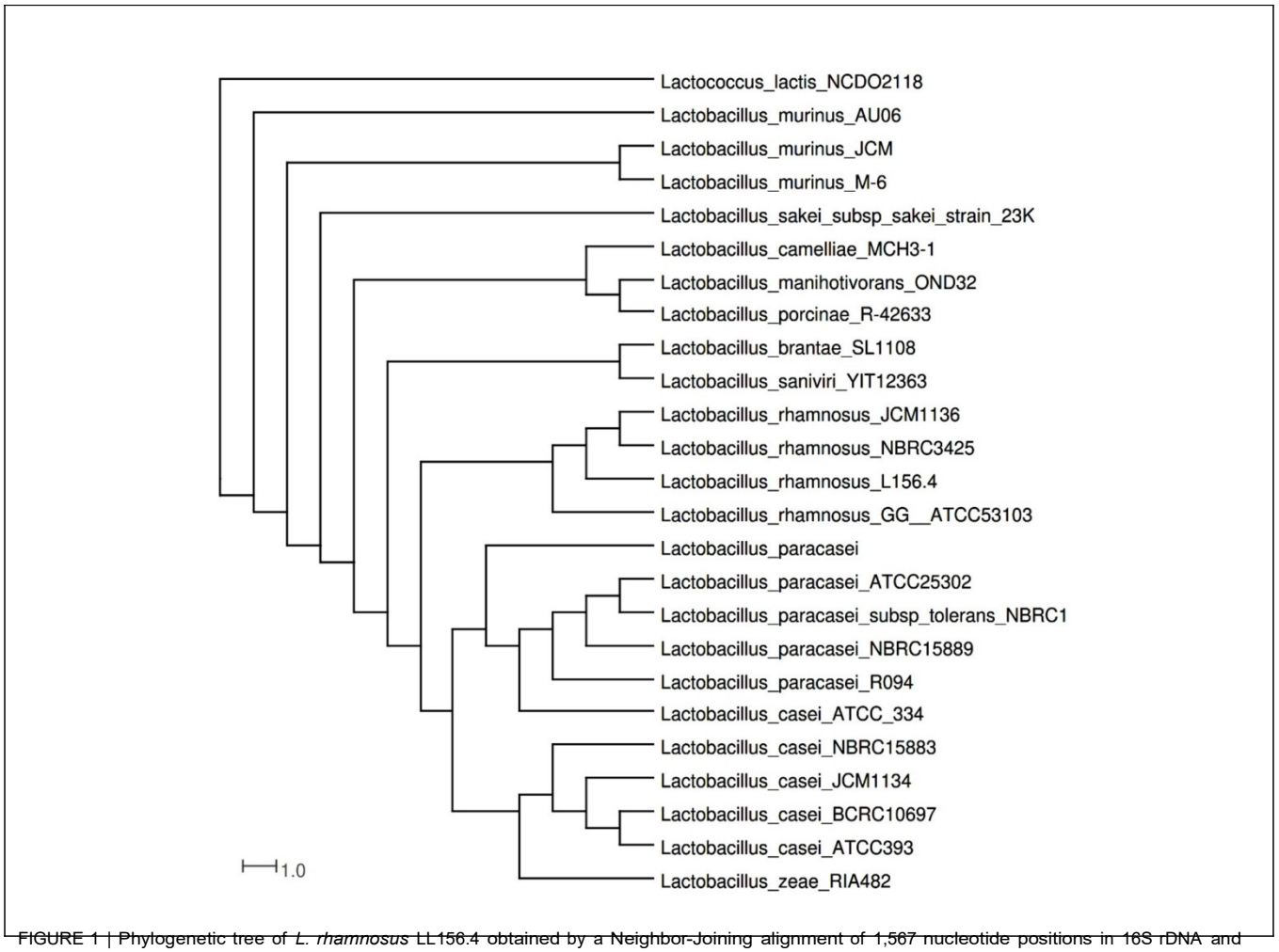

FIGURE 1 | Phylogenetic tree of *L. rhamnosus* LL156.4 obtained by a Neighbor-Joining alignment of 1,567 nucleotide positions in 16S rDNA and compared to 234 *Lactobacillus* spp. sequences. L. rhamnosus strain LL156.4 was positioned among L. rhamnosus NBRC3425 and L. rhamnosus JCM1136.

treatment after 30 min at 60, 80, 100, or 121˚C. Moreover, the inhibitory activity was observed after catalase treatment, but not after being treated with proteolytic enzymes (**Table 3**).

Production of Putative Bacteriocin in LAPTg Medium

Figure 3 shows the growth curve of L. rhamnosus L156.4 in LAPTg broth. The increase in bacterial counting, as determined by CFU counting and optical density, was accompanied by pH decrease from 6.8 to 4.0 after 24 h. The production of the antibacterial substances started after 4 h of incubation and occurred during logarithmic growth phase, reaching a maximum value of 3,200 AU/ml after 12 h and was constant for up to 24 h. In addition, a positive correlation was observed between the variables, indicating that the production of substances is associated with bacterial growth ($r^2 = 0.94$, $p < 0.05$ for log CFU/ml and $r^2 = 0.98$, $p < 0.05$ for OD at 600 nm).

Partial Purification of the Putative Bacteriocin by Salt Precipitation

The putative bacteriocin in the CFS was partially purified by a 40% salt saturation precipitation, and its antibacterial activity against L. fermentum remained stable after desalting with a 1 kDa cut-off membrane. The supernatant showed no antibacterial activity after removing precipitated proteins.

SDS-PAGE Analysis and Direct Detection of the Putative Bacteriocin on Gels

The examination of the protein profile by Tricine SDS-PAGE stained with Coomassie Blue (**Figure 4A**, lanes 2, and 3), revealed a diffuse band of approximately 1.0–3.0 kDa for both CFS and DCFS (**Figure 4A**, lanes 2, and 3), which coincided with a single zone of bacterial inhibition for both CFS and DCFS (**Figure 4B**, lanes 4, and 5). The results also showed an increase of the band and inhibition zone size corresponding to the active compound in the DCFS when compared to the CFS, for both Coomassie Blue staining and inhibitory activity.

DISCUSSION

Data mining of genomic and metagenomic sequences has been an important strategy for the identification of bacteriocin producers. This is a promising approach since many features of bacteriocin gene clusters, and especially bacteriocin modification genes,

TABLE 1 | *In silico* prediction of the functions of proteins associated with bacteriocin synthesis from *Lactobacillus rhamnosus* L156.4.

Locus tag	Predicted protein function (PGAAP)	Length (aa)	Protein deposit number	Protein accession number (BLASTp)/homology screening
BWR10_11430	Bacteriocin ABC transporter permease	459	KY355786	WP_031547267.1/L. rhamnosus HN001
BWR10_11435	Bacteriocin cleavage/export ABC transporter	730	KY355785	WP_005686870.1/L. rhamnosus HN001
BWR10_11440	ATP-binding protein	431	KY355784	WP_005686867.1/L. rhamnosus HN001
BWR10_11445	DNA-binding response regulator	258	KY355783	WP_005686865.1/L. rhamnosus HN001
BWR10_11450	Hypothetical protein	69		
BWR10_11455	Hypothetical protein	60	ND	
BWR10_11460	Hypothetical protein	66		
BWR10_11465	Hypothetical protein	81	KY355782	
BWR10_11470	Bacteriocin immunity protein	99	KY355781	WP_031546828.1/L. rhamnosus HN001
BWR10_11475	Bacteriocin leader domain-containing protein	52		
BWR10_11480	Hypothetical protein	75		
BWR10_11485	Bacteriocin leader domain-containing protein	61	ND	
BWR10_11490	Hypothetical protein	61		
BWR10_11495	Hypothetical protein	61		
BWR10_11500	CAAX protease family protein	268	KY355779	WP_005686845.1/L. rhamnosus HN001
BWR10_11505	Rrf2 family transcriptional regulator	146	KY355778	WP_005686843.1/L. rhamnosus HN001
BWR10_11510	MFS transporter	475	KY355777	WP_005686841.1/L. rhamnosus HN001
BWR10_11515	Hypothetical protein	110	KY355776	
BWR10_11520	Bacteriocin immunity protein*	113	KY355775	WP_005686837.1/L. rhamnosus GG
BWR10_11525	Aldo/keto reductase	317	KY355774	
BWR10_11530	Alpha-galactosidase	81	ND	
BWR10_11535	Alpha-galactosidase	57		
BWR10_11540	Oxidoreductase	244	KY355772	
BWR10_11545	NADH-dependent flavin oxidoreductase	381	KY355771	
BWR10_11550	Hypothetical protein	105	KY355770	
BWR10_11555	Class II fumarate hydratase	459	KY355769	

ND: sequences were not deposited. *Detected in RAST, PGAAP and BAGEL3.

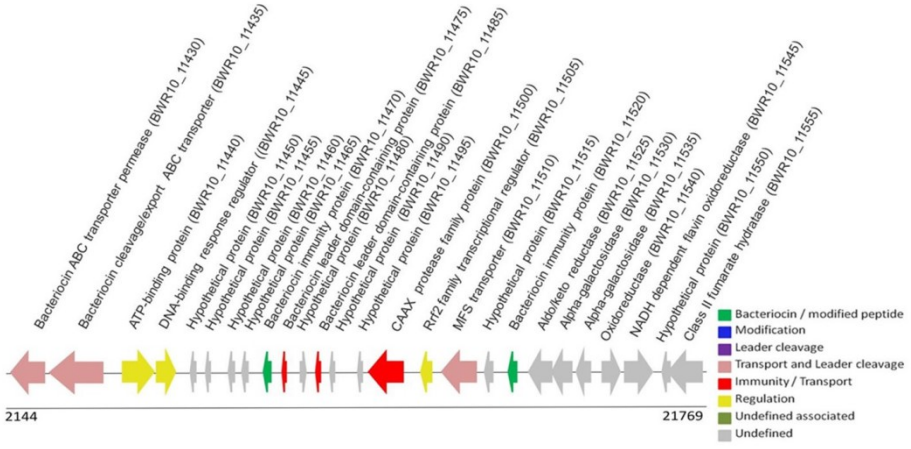

FIGURE 2 | BAGEL3 graphical output for putative prebacteriocin (orf010 in green—locus tag: BWR10_11520). The manual annotation of the predicted locus tag in PGAAP required for bacteriocin synthesis is identified according to the legend.

are highly conserved. The synthesis of class II bacteriocins is dependent on the expression of at least four genes that are organized in one or two operons, with relevant genes including: the prebacteriocin structural gene, an immunity protein-associated gene, a gene encoding an ABC-transporter that exports the bacteriocin simultaneous with the processing of

TABLE 2 | Antibacterial activity spectrum of *Lactobacillus rhamnosus* LL156.4 in the deferred agar-spot and spot-on-the lawn assays.

Indicator strain	Source	Deferred agar-spot assay[f]	Spot-on-the lawn assay[g]
Escherichia coli	ATCC[a] 5723	+[d]	–
Enteropathogenic E. coli (EPEC)	CDC[b] O111ab	+	–
Enterohemorrhagic E. coli (EHEC) Enteroinvasive E. coli (EIEC)	ATCC 43895 ATCC 43893	+ +	– –
Enterotoxigenic E. coli (ETEC)	H10407	+	–
Enteroaggregative E. coli (EAEC)	042	+	–
Klebsiella pneumoniae	ATCC 13822	+	–
Salmonella enterica serovar Typhimurium	ATCC 13311	+	–
Shigella sonnei	ATCC 11060	+	–
Listeria monocytogenes	ATCC 15313	+	+
Listeria monocytogenes	ATCC 19115	+	+
Listeria monocytogenes	ATCC 6477	+	+
Listeria monocytogenes	Scott A	+	+
Corynebacterium fimi	NCTC[c] 7547	+	+
Micrococcus luteus	ATCC 49732	+	+
Staphylococcus aureus	ATCC 29213	+	+
Bacillus cereus	ATCC 11778	+	+
Enterococcus faecalis	ATCC 19433	+	+
Lactobacillus acidophilus	ATCC 4356	–[e]	–
Lactobacillus brevis	ATCC 367	–	–
Lactobacillus rhamnosus	ATCC 7469	–	–
Lactobacillus delbrueckii subsp. lactis	ATCC 7830	+	+
Lactobacillus fermentum	ATCC 9338	+	+
Lactobacillus plantarum	ATCC 8014	+	+

[a]ATCC, American Type Culture Collection, Rockville, MD, USA.
[b]CDC, Center for Diseases Control, Atlanta, GA, USA.
[c]NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK
[d]Presence of inhibition zone (+)
[e]Absence of inhibition zone (–)
[g]Assay conducted with cells of L156.4.
[h]Assay conducted with the cell-free supernatant (CFS) at pH 7.

the leader sequence, and a gene encoding an accessory protein whose function remains unknown (Drider et al., 2006).

We annotated the whole genome using PGAAP, which uses a combination of gene prediction methods through a Hidden Markov Model (HMM) with an approach based on sequence similarity (Angiuoli et al., 2008). Moreover, we used the web-based software BAGEL3 for in silico prospection of class I, II and III bacteriocins through a knowledge-based bacteriocin database and motif databases (van Heel et al., 2013). Using this tool, we provided evidence of a putative bacteriocin produced by L. rhamnosus L156.4 isolated from the feces of NIH mice, which was further confirmed by in vitro assays. Reports on bacteriocins produced by L. rhamnosus remain scarce. Strains of this species have been isolated from the intestinal tract of humans and animals (Heilig et al., 2002) and to the best of our knowledge, bacteriocinogenic L. rhamnosus fecal strains have been detected only in human feces (Gorbach, 1996; Gill et al., 2000; Cukrowska et al., 2009; Dimitrijević et al., 2009; Aguilar-Uscanga et al., 2013). Thus, this is the first report of a putative bacteriocin produced by a L. rhamnosus strain (L156.4) that was obtained from the feces of an NIH mouse.

Using BAGEL3, we found that the ORF of the prebacteriocin in L. rhamnosus L156.4 showed 100% identity with that of L. rhamnosus GG (ATCC 53103), a probiotic strain (Szajewska and Kołodziej, 2015) commercialized by Chr. Hansen (Hørsholm, Denmark). Previous investigations with L. rhamnosus GG described an 8.7-Kb putative type IIb bacteriocin operon, which includes an exporter protein, an ABC/C39-type peptidase, a two-component signal transduction system, an immunity protein and the bacteriocin gene (Kankainen et al., 2009). PGAP, RAST, and BLASTp analyses allowed the detection of other ORFs that encoded proteins such as prebacteriocin (98 amino acids), an immunity protein, ABC transporter proteins, and regulatory proteins sharing 100% similarity with ORFs of L. rhamnosus HN001, a probiotic strain (Gill et al., 2000) commercialized by Danisco (DuPont, Las Vegas, NV, USA). Both predicted putative bacteriocins contain the Enterocin A domain (pfam08951), but the regions are different, and one of them is incomplete (orf010). Due to the similarities of our putative bacteriocin to that of L. rhamnosus GG (ATCC 53103), we conducted a comparative analysis of the whole genomes of these strains. In spite of the great synteny between the genes related to the putative bacteriocin, the cluster of the

TABLE 3 | Effect of temperature, pH, H_2O_2 and proteolytic enzymes on the antagonistic activity against *L. fermentum* ATCC 9338.

Treatments	Inhibitory activity of CFS[a]
pH	
3.0	+[a]
4.0	++[b]
5.0	++
6.0	++
7.0	++
8.0	+
9.0	+
ENZYMES	
Catalase	++
α-Chymotrypsin	–[c]
Proteinase K	–
Trypsin	–
Pepsin	–
CONTROLS	
MRS broth	–
CFS pH 3.0	+
CFS pH 4.2	++
CFS pH 7.5	++
Potassium phosphate 50 mM; pH 7.0	–
Tris-HCl 100 mM; pH 7.5	–
Glycine added at 20 mM $CaCl_2$, (50 mM; pH 3.0)	–
TEMPERATURE	
Control (25˚C)	++
60˚C, 30 min	++
80˚C, 30 min	++
100˚C, 30 min	++
121˚C, 15 min	++

[a]Presence of inhibition zone with growth of sparse colonies (+).
[b]Presence of clear inhibition zone (++).
[c]Absence of inhibition zone (–).

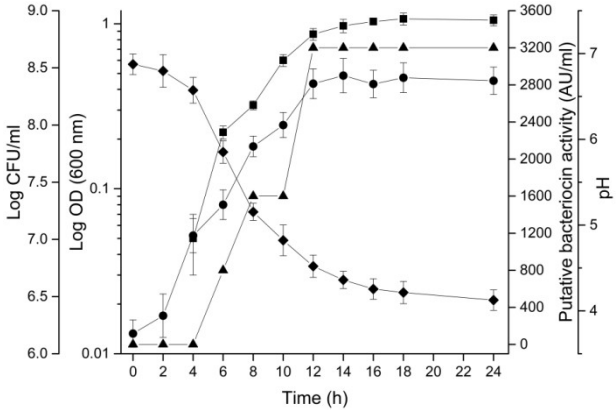

FIGURE 3 | Evaluation of production of putative bacteriocin in culture medium. Growth curve of L. rhamnosus LL156.4 in LAPTg broth at 37˚C in anaerobic conditions. Symbols: optical density (OD) 600 nm (•), Log CFU/ml ( ), pH ( ), putative bacteriocin activity (AU/ml) (N).

predicted bacteriocin in L. rhamnosus L156.4 is not identical to the one identified in L. rhamnosus GG (ATCC 53103). Moreover, the phylogenetic tree showed that L. rhamnosus L156.4 is more closely related to L. rhamnosus JCM1136 and NBRC3425 strains.

A search for the YGNGVXC motif, a characteristic of class IIa bacteriocins, was conducted in the L. rhamnosus L156.4 genome, but this pediocin-like sequence was not found. However, the CAAX amino terminal protease of self-immunity, which is indicative of class IIb bacteriocins, was detected (Pei and Grishin, 2001). Nevertheless, it is important to mention that our data were obtained from the analysis of a draft genome, and thus, it is possible that some information related to the bacteriocin is missing. More studies and analyses using a complete genome are being planned.

The antibacterial activity spectrum was evaluated using the deferred agar-spot and spot-on-the lawn assays (**Table 2**). Among gram-positive target strains, the inhibition of Enterococcus faecalis, L. fermentum, Lactobacillus delbrueckii subsp. lactis, Lactobacillus plantarum, and L. monocytogenes can be attributed to the putative bacteriocin, considering that the mentioned antibacterial spectrum and antilisterial activity are among the main features of class II bacteriocins produced by LAB (Klaenhammer, 1993; Drider et al., 2006). Our results present some overlapping features with other reports of antimicrobial activity of bacteriocins produced by L. rhamnosus strains against E. faecalis (Todorov and Dicks, 2005; Aguilar-Uscanga et al., 2013), Micrococcus luteus (Srinivasan et al., 2013), S. aureus (Sarika et al., 2010; Srinivasan et al., 2013; Jeong and Moon, 2015), L. monocytogenes (Aguilar-Uscanga et al., 2013; Srinivasan et al., 2013; Jeong and Moon, 2015) and E. coli (Todorov and Dicks, 2005). Class II bacteriocins kill bacteria by pore formation or by interfering with the integrity of the target cell membrane, inducing permeabilization and leakage of the intracellular content (Drider et al., 2006).

Bacteriocins can be effective against gram-negative bacteria, but this effect is limited due to the protective effect of the outer membrane (Helander et al., 1997; Cotter et al., 2005). Here, antagonism against the gram-negative enteropathogenic bacteria E. coli, Salmonella Typhimurium, Shigella sonnei, and Klebsiella pneumoniae can be the production of organic acids by L. rhamnosus L156.4, which is a facultatively heterofermentative species. The neutralization of the pH of the supernatant confirmed an effect of organic acids on pH decrease. The antimicrobial activities of the bacteriocin, organic acids, and the acidic pH are complementary and might be synergistic (Helander et al., 1997). The antimicrobial mechanism of these acids is mostly associated with their ability to cross the cytoplasmic membrane in its un-disassociated form, resulting in reduced intracellular pH and the disruption of the transmembrane proton motive force, particularly in gram-negative bacteria (Alakomi et al., 2000). Moreover, it has been demonstrated that membrane permeabilization by lactic acid can potentiate the effect of antimicrobial peptides, suggesting

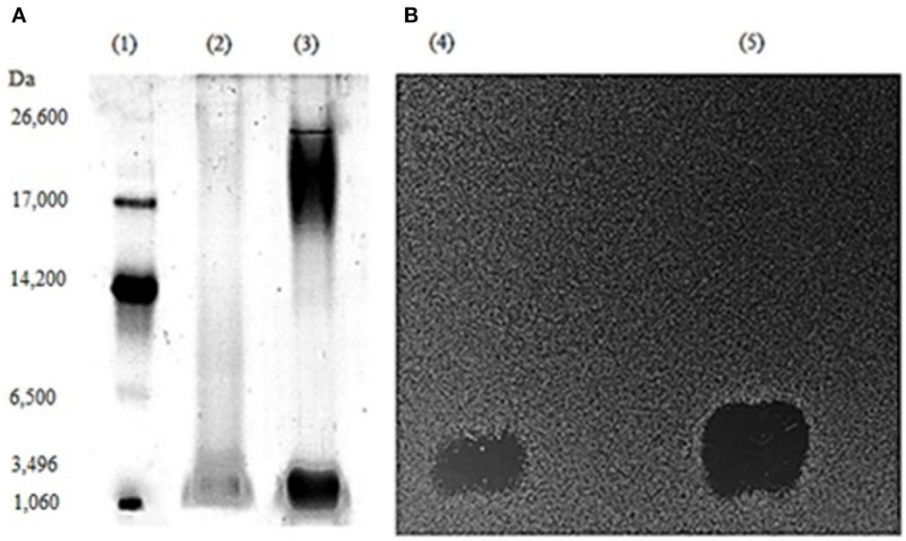

FIGURE 4 | Molecular mass evaluation by simultaneous visual detection of bacteriocin activity by Tricine SDS-PAGE. (A) Gel stained with Comassie Brilliant Blue R250: lanes 1, 2, and 3 represent molecular weight standards, cell free supernatant (CFS), and desalted ammonium sulfate precipitated supernatant (DCFS), respectively. (B) Gel overlaid with MRS soft agar inoculated with L. fermentum ATCC 9338: lanes 4 and 5 represent CFS and DCFS, respectively.

a synergic behavior of these compounds (Niku-Paavola et al., 1999).

Physicochemical characterization assays were performed with the CFS of L. rhamnosus L156.4 using L. fermentum ATCC 9338 as the indicator species (**Table 3**). The putative antimicrobial compound present in the CSF was heat-resistant and remained active at pH-values ranging from 3 to 9, and it remained stable at all tested temperatures. In addition, the CSF lost its activity after treatment with proteases, confirming its proteinaceous nature, indicating that L. rhamnosus L156.4 is a bacteriocin-producer strain. The possibility of the inhibitory effect observed against the indicator strain being caused by hydrogen peroxide was discarded, given that the producer strain was cultured anaerobically and that the antibacterial effect was not altered after treatment with catalase.

The physicochemical characteristics described in this study for the inhibitory product of L. rhamnosus L156.4 had also been observed for other bacteriocins. Rhamnosin A is a small non lanthionine-containing bacteriocin produced by L. rhamnosus strain 68, which also retained its biological activity after thermal treatment (95˚C, 30 min) and was sensitive to the proteolytic activity of pepsin and trypsin (Dimitrijević et al., 2009). Similarly, the bacteriocin produced by L. rhamnosus GP1 was stable at pH-values ranging from 2.5 to 8.5, and after autoclaving at 121˚C for 20 min (Sarika et al., 2010).

Previous reports have demonstrated the influence of the culture medium composition on bacterial growth and production of antimicrobial compounds. Although MRS medium is generally used for antagonism assays and physicochemical characterization, LAPTg broth was chosen for evaluation of bacteriocin production and partial purification because it contains lower amounts of potentially interfering proteins or peptides than does MRS. The same medium was used in other studies for bacteriocin purification (Ocaña et al., 1999; Tomás et al., 2002). Tomás et al. (2002) used a complete factorial design to compare the production of bacteriocin by L. salivarius subsp. salivarius CRL 1328 in LAPTg, and in an initial pH of 6.5, its maximum bacteriocin activity (1,280 AU/ml) was detected after a 6 h incubation at 37˚C. In similar conditions, we observed the production of 3,200 AU/ml after a 12 h incubation at 37˚C, and confirmed a positive correlation between the bacterial growth and the putative bacteriocin production. This pattern was already described for other lactic acid bacteria (Ocaña et al., 1999; Tomás et al., 2002). Nevertheless, Todorov and Dicks (2005) detected a high level of bacteriocin production (12,800 AU/ml) by L. rhamnosus strains ST461BZ and ST462BZ culture in MRS medium after a 15 h of incubation at 30˚C.

The protein profile examined on a SDS-PAGE-Tricine gel showed a diffuse band of equal mobility pattern (1–3 kDa) in both samples CSF and DCFS samples, which presented an antagonistic activity in situ against the indicator strain (**Figure 4**). As estimated by the same method, other bacteriocins produced by L. rhamnosus strains showed molecular sizes, ranging from 2.8 to 8.0 kDa (Li et al., 2005; Todorov and Dicks, 2005; Aguilar-Uscanga et al., 2013; Srinivasan et al., 2013). Molecular masses of 6433.8 and 6,502 Da were obtained by mass spectrometry analyses of rhamnosin A (Dimitrijević et al., 2009) and a bacteriocin described by Yue et al. (2013), respectively.

CONCLUSIONS

A putative bacteriocin produced by L. rhamnosus L156.4 was predicted in silico and inhibited the growth of several bacteria in vitro, including gram-positive human and foodborne bacterial pathogens. Its antilisterial activity supports further studies in

order to explore it for food preservation and for use as a probiotic.

AUTHOR CONTRIBUTIONS

RN and JN designed the study and drafted the manuscript. AS performed in vitro experiments. VA, SS, LO, and AM performed next generation sequencing and in silico analyses. RN, MD, AM, and Vd analyzed the results and wrote the manuscript. RN and MD reviewed the final version of the manuscript. All authors read and approved the manuscript after contributing with suggestions.

ACKNOWLEDGMENTS

We are thankful to Jamil Silvano Oliveira for the technical support during this research. This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Universidade CEUMA and Pró-reitoria de Pesquisa da UFMG (PRPq). MD is a fellow from FAPEMIG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00876/full#supplementary-material

Supplementary Figure S1 | Gene synteny between *L. rhamnosus* GG (ATCC 53103) and *L. rhamnosus* L156.4. Lactobacillus rhamnosus GG (ATCC 53103) (top) was used as a reference for the comparison analysis. The genomes are represented according to the nucleotide conservation and synteny. Low similarity regions are represented as white regions inside the blocks, highlighted by a black (*). Regions of deletions are represented as blank spaces between the blocks and by the letter (A), and an inversion region are represented by letter (B). To perform the genome synteny analysis, we used the software Mauve, which compares the genomes by identifying and clustering homologous genes between the genomes into large collinear blocks of genes.

Supplementary Figure S2 | ACT comparison between *L. rhamnosus* GG (ATCC 53103) and *L. rhamnosus* L156.4. On top, the complete genome of L. rhamnosus GG (ATCC 53103); on bottom, the contig of L. rhamnosus L156.4 that contains the predicted bacteriocin. Regions of similarity between the two genomes are marked in red; the bacteriocin is highlighted in yellow and is present in both genomes (green rectangle). In addition, regions of deletions and insertions are represented by blank spaces.

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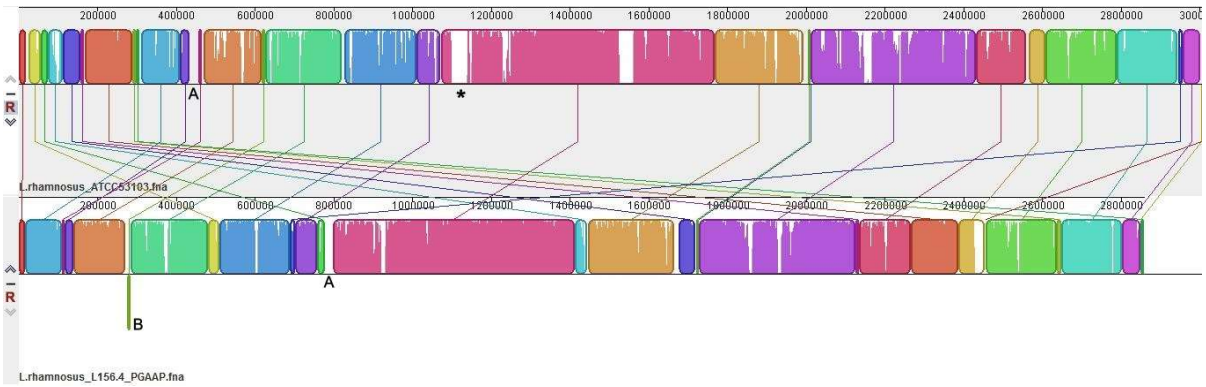
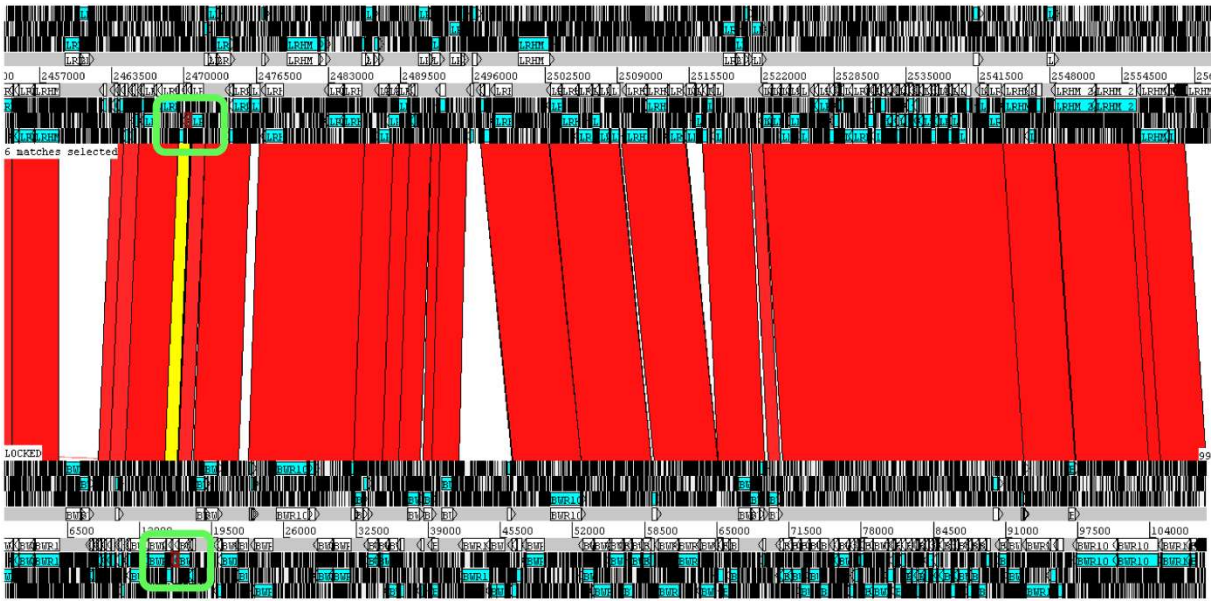
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

VI.1.1 Supporting information



VI.1.1.1 Supplementary Figure S1. Gene synteny between *L. rhamnosus* GG (ATCC 53103) and *L. rhamnosus* L156.4. *Lactobacillus rhamnosus* GG (ATCC 53103) (top) was used as a reference for the comparison analysis. The genomes are represented according to the nucleotide conservation and synteny. Low similarity regions are represented as white regions inside the blocks, highlighted by a black (*). Regions of deletions are represented as blank spaces between the blocks and by the letter **(A)**, and an inversion region are represented by letter **(B)**. To perform the genome synteny analysis, we used the software Mauve, which compares the genomes by identifying and clustering homologous genes between the genomes into large collinear blocks of genes.



VI.1.1.2 Supplementary Figure S2. ACT comparison between *L. rhamnosus* GG (ATCC 53103) and *L. rhamnosus* L156.4. On top, the complete genome of *L. rhamnosus* GG (ATCC 53103); on bottom, the contig of *L. rhamnosus* L156.4 that contains the predicted bacteriocin. Regions of similarity between the two genomes are marked in red; the bacteriocin is highlighted in yellow and is present in both genomes (green rectangle). In addition, regions of deletions and insertions are represented by blank spaces.

VI.1.2 DISCUSSÃO

O isolamento e a identificação de *L. rhamnosus* L156.4 possibilitou trazer ao público o primeiro relato de uma bactéria, produtora de bacteriocinas, isolada de fezes de camundongos NIH. As análises realizadas *in vitro* permitiram, além de confirmar a atividade de exclusão predita pelas bacteriocinas *in silico*, demonstrar o ampla espectro antagonista e caracterização físico-química das bacteriocinas desta bactéria. Das 23 linhagens patogênicas testadas, *L. rhamnosus* L156.4 não inibiu apenas 3 espécies de *Lactobacillus* pelo método *Deferred Agar-spot.*

Sendo a atividade antagonista/exclusão, uma das características de um organismo probiótico, os resultados obtidos nos leva a aprofundar as análises relacionadas à probiose da linhagem *L. rhamnosus* L156.4, como por exemplo o fato das proteínas relacionadas à bacteriocina serem 100% similares a linhagem probiótica *L. rhamnosus* HN001 (Gill et al., 2000).

VI.2 - Capítulo II

Analyses of the probiotic property and stress resistance-related genes of *Lactococcus lactis* subsp. *lactis* NCDO 2118 through comparative genomics and *in vitro* assays

Letícia C. Oliveira, Tessália D. L. Saraiva, Wanderson M. Silva, Ulisses P. Pereira, Bruno C. Campos, Leandro J. Benevides, Flávia S. Rocha, Henrique C. P. Figueiredo, Vasco Azevedo, Siomar C. Soares

O artigo acima foi publicado na revista "Plos One". Nele, nós utilizamos tanto uma abordagem *in silico* quanto *in vitro* no intuito de identificar possíveis genes relacionados com a capacidade probiótica da linhagem de *Lactococcus lactis* subsp. *lactis* NCDO 2118. Para tanto, realizamos análises comparativas da linhagem supracitada com mais 15 genomas completos da espécie *Lactococcus*, observando a filogenia, sintenia gênica e as ilhas genômicas identificadas. Além disso, buscando aprofundar as análises nas características probióticas da linhagem em estudo, identificamos regiões de fago, bacteriocinas, genes de resistência à bile e ao estresse ácido, além de genes relacionados à adesão e à resistência a antibióticos. A proteômica também foi uma abordagem utilizada neste estudo com o objetivo de identificar possíveis genes que codificam proteínas potenciais, que fossem secretadas e expressas em *L. lactis* NCDO 2118, importantes na caracterização da atividade anti-inflamatória e imunomodulatória desta linhagem.

PLOS ONE

RESEARCH ARTICLE

Analyses of the probiotic property and stress resistance-related genes of *Lactococcus lactis* subsp. *lactis* NCDO 2118 through comparative genomics and *in vitro* assays

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OPEN ACCESS

Citation: Oliveira LC, Saraiva TDL, Silva WM, Pereira UP, Campos BC, Benevides LJ, et al. (2017) Analyses of the probiotic property and stress resistance-related genes of *Lactococcus lactis* subsp. *lactis* NCDO 2118 through comparative genomics and *in vitro* assays. PLoS ONE 12(4): e0175116. https://doi.org/10.1371/journal.pone.0175116

Editor: Luca Cocolin, University of Torino, ITALY

Received: November 14, 2016

Accepted: March 21, 2017

Published: April 6, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Support was provided by: National Counsel of Technological and Scientific Development [http://cnpq.br/]; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior [https://www.capes.gov.br/]; Fundação de Amparo à Pesquisa de Minas Gerais [http://www.fapemig.br/en/].

Abstract

Lactococcus lactis subsp. *lactis* NCDO 2118 was recently reported to alleviate colitis symptoms via its anti-inflammatory and immunomodulatory activities, which are exerted by exported proteins that are not produced by *L. lactis* subsp. *lactis* IL1403. Here, we used *in vitro* and *in silico* approaches to characterize the genomic structure, the safety aspects, and the immunomodulatory activity of this strain. Through comparative genomics, we identified genomic islands, phage regions, bile salt and acid stress resistance genes, bacteriocins, adhesion-related and antibiotic resistance genes, and genes encoding proteins that are putatively secreted, expressed *in vitro* and absent from IL1403. The high degree of similarity between all *Lactococcus* suggests that the Symbiotic Islands commonly shared by both NCDO 2118 and KF147 may be responsible for their close relationship and their adaptation to plants. The predicted bacteriocins may play an important role against the invasion of competing strains. The genes related to the acid and bile salt stresses may play important roles in gastrointestinal tract survival, whereas the adhesion proteins are important for persistence in the gut, culminating in the competitive exclusion of other bacteria. Finally, the five secreted and expressed proteins may be important targets for studies of new anti-inflammatory and immunomodulatory proteins. Altogether, the analyses performed here highlight the potential use of this strain as a target for the future development of probiotic foods.

Competing interests: The authors have declared that no competing interests exist.

Introduction

The genus *Lactococcus* is part of the lactic acid bacteria (LAB), one of the most biotechnologically important groups of bacteria, which is composed of *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Weissella* and others [1]. LAB species share in common the ability to convert sugar (mainly glucose) into lactic acid through specific metabolic pathways. Additionally, these species are facultative anaerobic, catalase negative and non-motile. Moreover, there is a close phylogenetic relationship between the bacteria of this group [2].

Many LAB species are biotechnologically important due to their safety aspects, achieved because they have been used for years in the preservation and maintenance of food [3]. Previous studies highlight the importance of genome sequencing in the discovery of new features related to LAB: genes coding for proteolytic enzymes (which participate in cheese maturation) in *Lactobacillus helveticus* [4], identification of citrate catabolic pathways in *Lactobacillus casei* [5], and genes responsible for decarboxylation of alpha-keto acid branched chain in *Lactococcus lactis* [6; 7].

Genome sequencing studies have also helped in the elucidation of probiotic effects exerted by LAB. For instance, in *Lactobacillus reuteri*, genome analyses have focused on the capacity to adapt to nutrient availability and environmental conditions of the GI tract, the adhesion mechanisms, the production of antimicrobial compounds, and the mechanisms of immunomodulation, such as the synthesis of pro-inflammatory extracellular polymeric substances (EPS compounds) [8]. Moreover, *Lactobacillus rhamnosus* and *L. casei* strains isolated from marketed probiotic products were compared with the well-studied *L. rhamnosus* GG and *L. casei* BL23, mainly focusing on pilus gene clusters and metabolic pathways analyses [9]. Interestingly, a new adhesion-associated protein, *cwaA*, was identified through genome sequencing and comparative genomics analyses of *Lactobacillus plantarum* NL42. The expression of cwaA in *L. lactis* has significantly increased its autoaggregation, hydrophobicity and exclusion ability, where the mutant strain was able to inhibit the adhesion of *Staphylococcus aureus* and *Escherichia coli* to HT-29 cells [10]. Another study illustrated the mechanisms by which *Lactobacillus* species from the intestinal niche have adapted to the gastrointestinal tract (GIT) by acquiring traits, such as stress tolerance, carbohydrate absorption, adhesion to epithelial cells and mucus [11].

Additionally, many species of this group are important for their probiotic effects, such as the genus *Lactobacillus*, which is used in the production of the fermented milk Yakult [12], and *Bifidobacteria*, widely known for their beneficial effects on the host intestinal tract [13]. However, although several works highlight the probiotic effects of LAB, most focus on *Lactobacillus* and *Bifidobacterium* species [14], whereas few studies report the beneficial effects of *L. lactis* strains. For instance, *Lactococcus lactis* subsp. *cremoris* FC has an important anti-inflammatory activity [15]. The probiotic properties of *L. lactis* subsp. *cremoris* IBB477 have attracted attention due to their adhesion mechanisms and survival in the intestinal environment [16; 17]. Additionally, it was recently demonstrated, through the evaluation of three *L. lactis* strains *in vitro*, that *Lactococcus lactis* subsp. *lactis* NCDO 2118 has anti-inflammatory and immunomodulatory activity that can alleviate colitis symptoms [18]. This strain was described as a gamma-aminobutyric acid (GABA) producer [19]. It has been extensively used for heterologous expression [20], and its probiotic effect is associated with exported proteins [18].

Here, we use comparative genomics and *in silico* analyses to provide insights into the probiotic nature of *L. lactis* NCDO 2118. The criteria for screening LAB strains before their use as probiotics include assessing functional features, such as the ability to resist environmental conditions found in the digestive tract (low gastric pH and bile salts) and the ability to antagonize or competitively exclude pathogens, which is achieved by secreting antimicrobial substances

or competing for nutrients and epithelial adhesion sites. LAB produce different antimicrobial components, such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, bacteriocins and adhesion inhibitors. The adhesiveness of LAB may involve passive forces, electrostatic interactions, hydrophobic steric forces, lipoteichoic acids, and lectins [21]. The hydrophobic nature of the outermost surface of microorganisms facilitates the adhesion of bacteria to the host epithelium, thereby conferring competitive advantages during the colonization of the GIT [22]. The antimicrobial susceptibility of intestinal microorganisms is an important criterion for the selection of probiotic strains, mainly due to the potential transfer of those genes to pathogenic or commensal bacteria that inhabit the GIT [23]. In the following sections, we present comparative genomic analyses of *L. lactis* NCDO 2118 and other *Lactococcus* species and predict genes that putatively code for acid stress resistance proteins, bacteriocins, adhesins and exported proteins.

Results

General features, phylogenomics and synteny analyses

The general genomic features of all genomes used in this work are summarized in Table 1.

Briefly, *Lactococcus garvieae* strains have the highest G+C content, ~38.80%, whereas the lowest G+C contents, ~34.86%, were from *L. lactis* NCDO 2118 and *L. lactis* KF147, both isolated from vegetables. Additionally, the genome sizes of the *Lactococcus* species range from ~1.95 Mb to ~2.60 Mb, and the two *L. garvieae* strains have the smallest genomes.

In this work, the only species harboring plasmids were *L. lactis* NCDO 2118, *L. lactis* KF147, *Lactococcus lactis* subsp. *lactis* KLDS 40325 and *Lactococcus lactis* subsp. *lactis* CV56 strains, *Lactococcus lactis* subsp. *cremoris* A76, *Lactococcus lactis* subsp. *cremoris* SK11 and *Lac- tococcus lactis* subsp. *cremoris* UC5099 (*L. cremoris* UC5099) strains, where the latter harbored the greatest number of plasmids (Table 1).

From the heatmap created with Gegenees (Fig 1), it is possible to visualize a high similarity between the subspecies of *Lactococcus*, with nucleotide similarities ranging from 40% to 100%. Additionally, the species and subspecies clustered separately, creating 3 green blocks of strains at the chart, represented by *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, with similarities ranging from 91% to 100%, and *L. garvieae*, in which the two strains of this species were 100% similar to each other.

On the phylogenetic tree created using 16S, the species and subspecies also clustered together, forming two main clades corresponding to the best similarity among *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (Fig 1). Additionally, *L. garvieae* strains appeared in an outside node compared to *L. lactis* species and are the two most distinct and distant species of *Lactococcus* on the heatmap and phylogenetic tree. Briefly, on the heatmap, the degree of intraspecies similarity varies from 91% to 100%, whereas interspecies similarity varies from 40% to 65%.

From the genome synteny analysis (S1 Fig), all strains from *L. lactis* subsp. *lactis* presented a high degree of synteny, where the most conserved genome compared to *L. lactis* NCDO 2118 (chosen as reference genome) was *L. lactis* KF147. Additionally, we performed a comparison with the plasmids of *L. lactis* NCDO 2118 and *L. lactis* KF147 strains. However, we verified a high degree of similarity from the beginning to the end of each plasmid sequence, meaning that they possibly harbored the same plasmid (data not shown).

Metabolic pathways prediction

To identify conserved or non-conserved metabolic pathways, we used three different datasets, consisting of (1) the closely related *L. lactis* NCDO 2118, *L. lactis* KF147 and *L. lactis* IL1403,

Table 1. Complete genomes and genomic features of *Lactococcus* species and *Streptococcus thermophilus* used in genomic comparisons.

Strain	Size (bp)	GC%	Genes	Proteins	Source	Accession Number	Plasmids	Pseudogenes	Reference
***Lactococcus lactis* subsp. *lactis* NCDO 2118**	2,554,693	34,86	2,471	2,386	Frozen peas	CP009054	1	52	[24]
***Lactococcus lactis* subsp. *lactis* IL1403**	2,365,589	35,30	2,406	2,277	Dairy starter	AE005176	-	45	[3]
***Lactococcus lactis* subsp. *lactis* KF147**	2,598,144	34,86	2,662	2,473	Mung Bean	CP001834	1	93	[25]
***Lactococcus lactis* subsp. *lactis* KLDS 40325**	2,589,250	35,39	2,732	2,593	Fermented milk	CP006766	1	56	[26]
***Lactococcus lactis* subsp. *lactis* IO-1**	2,421,471	35,10	2,318	2,224	Water (drain pit of a kitchen sink)	AP012281	-	-	[27]
***Lactococcus lactis* subsp. *lactis* CV56**	2,399,458	35,09	2,549	2,408	Healthy woman's vagina	CP002365	5	51	[28]
***Lactococcus lactis* subsp. *lactis* S0**	2,488,699	35,20	2,482	2,311	Fresh raw milk	CP010050	-	88	Unpublished
***Lactococcus lactis* subsp. *lactis* AI06**	2,398,091	35,04	2,320	2,178	Açaí palm	CP009472	-	61	[29]
***Lactococcus lactis* subsp. *cremoris* A76**	2,452,616	35,88	2,845	2,769	Dairy starter	CP003132	4	-	[30]
***Lactococcus lactis* subsp. *cremoris* KW2**	2,427,048	35,70	2,353	2,268	Fermented corn	CP004884	-	1	[31]
***Lactococcus lactis* subsp. *cremoris* MG1363**	2,529,478	35,70	2,597	2,434	Dairy starter	AM406671	-	82	[32]
***Lactococcus lactis* subsp. *cremoris* NZ9000**	2,530,294	35,70	2,594	2,510	Dairy starter	CP002094	-	-	[33]
***Lactococcus lactis* subsp. *cremoris* SK11**	2,438,589	35,82	2,739	2,501	Dairy starter	CP000425	5	144	[34]
***Lactococcus lactis* subsp. *cremoris* UC5099**	2,250,427	35,76	2,401	2,109	Dairy starter	CP003157	8	188	[35]
Lactococcus garvieae* ATCC49156	1,950,135	38,80	2,024	1,947	Fish (*Alosa fallax*)	AP009332	-	0	[36]
Lactococcus garvieae* Lg2	1,963,964	38,80	2,045	1,968	Fish (*Alosa fallax*)	AP009333	-	0	[36]
Streptococcus thermophilus LMD-9 **	1,856,368	39.08	1960	1743	Dairy starter	CP000419	2	132	[37]

* *Lactococcus garvieae* are fish pathogens

** *Streptococcus thermophilus* was used as a closely related outgroup in the analyses

https://doi.org/10.1371/journal.pone.0175116.t001

(2) all strains from *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (non-pathogenic dataset), and (3) all strains from this study (including *L. garviae*). The number of metabolic pathways harbored by each genome varies from 148 to 206, with a general mean of ~183 pathways. Both *L. garvieae* strains contained 148 metabolic pathways, *L. lactis* subsp. *lactis* showed an average of ~192 metabolic pathways, and *L. lactis* subsp. *cremoris* showed ~186 pathways.

The main differences were that the strain *L. lactis* NCDO 2118 contains more peptidoglycan biosynthesis pathways than *L. lactis* KF147 and *L. lactis* IL1403 strains. Other exclusive metabolic features of *L. lactis* NCDO 2118 in this context were complete anaerobic respiration pathways, fermentation of pyruvate to acetate, fermentation of fumarate, complete heterolactic fermentation, valine degradation, L-serine degradation, ammonia assimilation to glutamate, complete superpathway of acetate utilization and formation, protein degradation, initial pathway of sucrose degradation I, valine degradation, lysine degradation I and acyl-ACP thioesterase pathway (S1 Table).

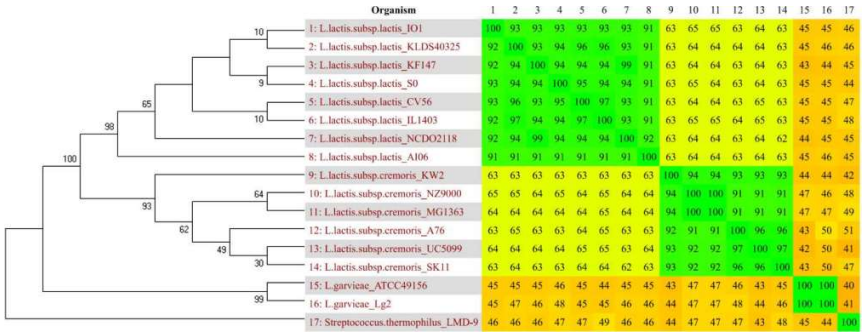

Fig 1. 16S phylogenetic tree and genomic heatmap of *Lactococcus* genus. The *Streptococcus thermophilus* LMD-9 (position 17) was added to root the tree. The species in comparison are distributed from 1 to 17 in the same order, both vertically and horizontally. The numbers in the heatmap show the percentage of similarity between the species, varying from yellow (low similarity) to green (high similarity), or from 40% to 100%, respectively. The heatmap and the phylogenetic tree were created with the software Gegenees and Mega (Neighbor-Joining method with 1000 bootstraps replicates), respectively.

https://doi.org/10.1371/journal.pone.0175116.g001

Genome plasticity

We identified 5 prophages in *L. lactis* NCDO 2118, of which 2 were incomplete, and 3 were considered intact (Table 2). The three intact phages harbored important genes such as *rusA*, *arsC1*, *arsC3*, *amtB*, *rpmE2*, *carA*, *pyrB*, *pyrP* and *pepT*.

Additionally, we used BRIG to visualize the plasticity events from phage sequences (Fig 2). According to the BRIG analyses, phage 1 was incomplete in all species, except for the reference genome *L. lactis* NCDO 2118 and *L. lactis* KF147. Both phages 2 and 3, predicted as intact in the reference, were also present in *L. lactis* KF147, *L. lactis* IL1403, *Lactococcus lactis* subsp. *cremoris* NZ9000 and *L. cremoris* MG1363, whereas the former phage was also found in *Lactococcus lactis* subsp. *cremoris* KW2. Phage 4, also intact in the reference genome, was present in all other species. Phage 5, predicted as incomplete in the reference genome, was absent in *L. lactis* IO-1, *L. cremoris* KW2, *L. cremoris* UC5099 and partially present in both *L. garvieae* strains.

Table 2. Intact and incomplete phages predicted in *L. lactis* subsp. *lactis* NCDO 2118.

Phages	Genes	Proteins
Region 1 –*Intact phage*	*rusA e arsC1*	Integrase, Prophage, Phage antirepressor, Transcriptional regulator, Recombinase, Endodeoxyribonuclease, Aminotransferase, Phage terminase small subunit, Peptidase, Bacteriophage lysine, Arsenate reductase
Region 2 –*Intact phage*	*amtB*, *kinA*, *llra*, *rpmE2*, *arsC3*, *carA*, *pyrB*, *pyrP*	Ammonium transporter, Sensor protein kinase, Two-component system regulator, 50S ribosomal protein L31 type B, Universal stress protein, Arsenate reductase, Bacteriophage lysine, Phage tail protein, Head-tail joining protein, Capsid protein, Phage ATP-dependent endopeptidase, Phage terminase small subunit, Endonuclease, Terminase, Replisome organizer, BRO-like protein, DNA binding protein, Phage integrase, Carbamoyl-phosphate synthase small chain, Aspartate carbamoyltransferase, Uracil transporter
Region 3 –*Intact phage*	*pepT*, *ppaC*, *pflA*, *ysiA*, *ysiB*	Amino Acid permease, Peptidase T, Manganese-dependent inorganic pyrophosphatase, Pyruvate-formate lyase activating enzyme, Permease, Phage protein, Integrase
Region 4 – *Incomplete phage*	*ardA*, *ecfA1*, *ecfA2*, *ecfT*, *dapH*, *yciA*	Peptidoglycan hydrolase, Antirestriction protein, Integrase, ATPase, Energy-coupling factor transporter, Thiol-disulfide isomerase, N-acetyldiaminopimelate deacetylase
Region 5 – *Incomplete phage*	*glnA*	Integrase, Bacteriocin, DNA primase, Glutamine synthetase

Phage locations were predicted using the software PHAST.

https://doi.org/10.1371/journal.pone.0175116.t002

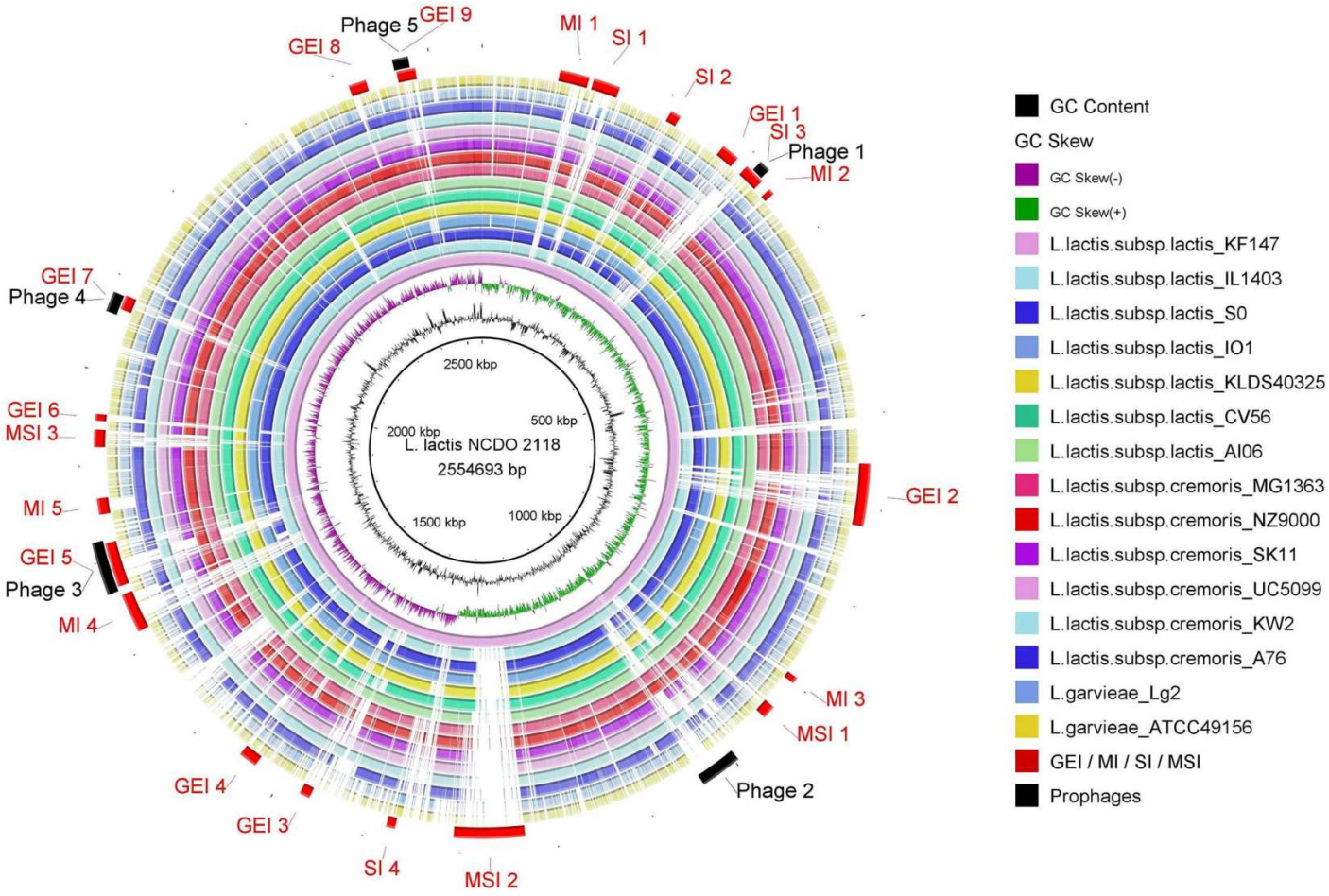

Fig 2. Circular comparison of the *Lactococcus* genus using *L. Lactis* NCDO 2118 as a reference. Each ring of the circle corresponds to a specific complete genome represented in the legend on the right. The similarity between species is represented by the intensity of the color. Darker colors represent higher similarities than bright ones. Deleted regions are represented by blank spaces inside the circles. (GEI = Genomic Island; MI = Metabolic Island; SI = Symbiotic Island; MSI = Miscellaneous Island, harboring both metabolic and symbiotic factors). Genomic islands and phage sequences were predicted with GIPSy and PHAST, respectively. The circular genomic comparisons were created with BRIG.

https://doi.org/10.1371/journal.pone.0175116.g002

In the GIPSy predictions, we identified 9 Genomic Islands (GEIs), 5 Metabolic Islands (MIs), 4 Symbiotic Islands (SIs) and 3 Miscellaneous Islands (MSIs), which were predicted as harboring both metabolic and symbiotic related factors. The GEIs are listed in S2 Table.

All SIs were only partially present in the other strains, except for SI4, which was absent from all *L. garviae* strains, *L. lactis* subsp. *cremoris* strains and *L. lactis* IL 1403 (Fig 2). Addi- tionally, all MIs presented regions of deletions in the pathogenic species *L. garviae*. The most prominent GEIs were MI3, which was only present in the two strains isolated from plants (*L. lactis* NCDO 2118 and *L. lactis* KF147), and MSI 2, which presented the biggest region of deletion in all *Lactococcus*, except for *L. lactis* NCDO 2118 and *L. lactis* KF147.

Antibiotic resistance

LAB that are widely used as probiotics or in starter cultures have the potential to host antibiotic resistance genes, thereby presenting a risk of transferring such genes to many lactic acid bacteria and other pathogenic bacteria [23]. In the antibiogram assay, *L. lactis* NCDO 2118 was

Table 3. Antibiotic susceptibility of *L. lactis* NCDO 2118.

Antibiotic susceptibility assay			
Antibiotic	**Concentration**	**Inhibition zone diameter (mm)**	**Susceptibility***
Ceftriaxone	30 μg	31	S
Erythromycin	10 μg	31	S
Tetracycline	30 μg	25	S
Ampicillin	30 μg	35	S
Vancomycin	10 U	0	R
Penicillin	30 μg	35	S
Amikacin	30 μg	15	R
Chloramphenicol	30 μg	28	S
Oxacillin	1 μg	14	R

* R = resistant, S = susceptible.

https://doi.org/10.1371/journal.pone.0175116.t003

susceptible to ceftriaxone, erythromycin, tetracycline, ampicillin, penicillin and chloramphenicol and resistant to vancomycin, oxacillin and amikacin antibiotics (Table 3). Additionally, we tried to correlate the antibiogram profile with the genome content of *L. lactis* NCDO 2118, which presented 22 antibiotic resistance-related genes putatively coding for a VanZ family protein (NCDO2218_1094), penicillin-binding proteins (NCDO2118_0402, NCDO2118_0445, NCDO2118_0526, NCDO2118_0880 and NCDO2118_2216), and multidrug efflux pump proteins (Table 4). Additionally, no antibiotic resistance related gene presented deviation in its genomic signature.

Table 4. Genes putatively coding for antibiotic resistance-related proteins.

Query ID	Product	Gene	G+C Content	Codon Usage
NCDO2118_0089	Multidrug resistance protein	*sugE*	NORMAL	NORMAL
NCDO2118_0090	Multidrug efflux transporter	*blt*	NORMAL	NORMAL
NCDO2118_0108	Multidrug resistance efflux pump	*pmrB*	NORMAL	NORMAL
NCDO2118_0144	MFS transporter	*ybfD*	NORMAL	NORMAL
NCDO2118_0258	Multidrug resistance ABC transporter	-	NORMAL	NORMAL
NCDO2118_0259	Multidrug ABC transporter ATP-binding protein	-	NORMAL	NORMAL
NCDO2118_0363	MFS transporter	*napC*	NORMAL	NORMAL
NCDO2118_0369	Multidrug ABC transporter ATP-binding protein	*lmrC*	NORMAL	NORMAL
NCDO2118_0370	Multidrug transporter	*lmrD*	NORMAL	NORMAL
NCDO2118_0402	Penicillin-binding protein 2B	*pbp2B*	NORMAL	NORMAL
NCDO2118_0445	Penicillin-binding protein 1B	*pbp1B*	NORMAL	NORMAL
NCDO2118_0526	Penicillin-binding protein 1A	*ponA*	NORMAL	NORMAL
NCDO2118_0593	Multidrug transporter	-	NORMAL	NORMAL
NCDO2118_0645	Multi-drug resistance efflux pump	*pmrA*	NORMAL	NORMAL
NCDO2118_0726	Multidrug resistance ABC transporter ATP-binding and permease protein	*lmrA*	NORMAL	NORMAL
NCDO2118_0880	Penicillin-binding protein 2X	*pbpX*	NORMAL	NORMAL
NCDO2118_0930	Multidrug resistance protein B	-	NORMAL	NORMAL
NCDO2118_1094	VanZ family protein	-	NORMAL	NORMAL
NCDO2118_1401	Multidrug MFS transporter	-	NORMAL	NORMAL
NCDO2118_1736	Multidrug transporter	*yqiA*	NORMAL	NORMAL
NCDO2118_1995	MFS transporter permease	*yteD*	NORMAL	NORMAL
NCDO2118_2216	Penicillin-binding protein 2a	*pbp2A*	NORMAL	NORMAL

G+C content and codon usage information were retrieved from GIPSy analyses.

https://doi.org/10.1371/journal.pone.0175116.t004

Identification of genes involved in acid stress and bile salt resistance

We searched the genome sequence of *L. lactis* NCDO 2118 for genes previously shown to be differentially expressed on cells cultivated under low and optimum pH (5.1 and 6.5, respectively) in *L. cremoris* MG1363 [38] (Table 5). Additionally, we also searched for genes differentially regulated by bile exposure in *Bifidobacterium animalis* and *Bifidobacterium longum* NCIMB 8809 [39; 40] and/or identified on the total proteome and surfome of *Lactobacillus rhamnosus* GG using proteomics analyses (Table 5). Here, we identified some genes in *L. lactis* NCDO 2118 that were previously reported to be involved in the acid stress response, including

Table 5. Genes coding for proteins involved in acid stress and bile salt resistance.

Locus_tag	EC Number	Gene	Product	Stress response
NCDO2118_1870	-	*atpC*	ATP synthase epsilon chain	Acid stress
NCDO2118_1871	3.6.3.14	*atpD*	ATP synthase subunit beta	Acid stress
NCDO2118_1872	-	*atpG*	ATP synthase gamma chain	Acid stress
NCDO2118_1873	3.6.3.14	*atpA*	ATP synthase subunit alpha	Acid stress
NCDO2118_1874	-	*atpH*	ATP synthase subunit delta	Acid stress
NCDO2118_1875	-	*atpF*	ATP synthase subunit b	Acid stress
NCDO2118_1876	-	*atpB*	ATP synthase subunit a	Acid stress
NCDO2118_1877	-	*atpE*	ATP synthase subunit C	Acid stress
NCDO2118_1384	1.1.1.27	*ldh*	L-lactate dehydrogenase	Acid stress
NCDO2118_0475	-	*ptcC*	PTS system, cellobiose-specific IIC component	Acid stress
NCDO2118_0542	1.2.1.12	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase	Acid stress
NCDO2118_0399	5.4.2.11	*gpmA*	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	Acid stress/bile resistance
NCDO2118_2272	5.3.1.9	*pgi*	Glucose-6-phosphate isomerase	Acid stress
NCDO2118_0096	2.7.1.40	*pyk1*	Pyruvate kinase	Acid stress
NCDO2118_1385	2.7.1.40	*pyk2*	Pyruvate kinase	Acid stress
NCDO2118_0240	2.7.2.3	*pgk*	Phosphoglycerate kinase	Acid stress/bile resistance
NCDO2118_0417	-	*recA1*	Protein RecA	Acid stress
NCDO2118_1251	-	*recA2*	Protein RecA	Acid stress
NCDO2118_0540	-	*clpE*	ATP-dependent Clp protease ATP-binding subunit	Acid stress
NCDO2118_0453	-	*groL*	60 kDa chaperonin	Acid stress
NCDO2118_1545	-	*clpB*	Chaperone protein	Acid stress
NCDO2118_0467	1.15.1.1	*sodA*	Superoxide dismutase	Acid stress
NCDO2118_0073	2.7.6.5	*relA*	GTP pyrophosphokinase	Acid stress
NCDO2118_0637	4.2.1.11	*eno*	Enolase	Acid stress/bile resistance
NCDO2118_1019	-	*dnaK*	Chaperone protein	Acid stress/bile resistance
NCDO2118_1594	3.5.99.6	*nagB*	Glucosamine-6-phosphate deaminase/isomerase	Bile resistance
NCDO2118_1909	3.4.24.-	*pepO*	Endopeptidase O	Bile resistance
NCDO2118_0941	5.4.99.9	*glf*	UDP-galactopyranose mutase	Bile resistance
NCDO2118_0500	6.3.4.2	*pyrG*	CTP synthase	Bile resistance
NCDO2118_0035	1.8.1.4	*pdhd*	Pyruvate dehydrogenase	Bile resistance
NCDO2118_2145	6.1.1.19	*argS*	Arginyl-tRNA synthetase	Bile resistance
NCDO2118_1958	-	*oppA*	Oligopeptide-binding protein	Bile resistance
NCDO2118_2203	-	*rpsC*	30S ribosomal protein S3	Bile resistance
NCDO2118_2191	-	*rpsE*	30S ribosomal protein S5	Bile resistance
NCDO2118_2208	-	*rplD*	50S ribosomal protein L4	Bile resistance
NCDO2118_2197	-	*rplE*	50S ribosomal protein L5	Bile resistance
NCDO2118_2193	-	*rplF*	50S ribosomal protein L6	Bile resistance

https://doi.org/10.1371/journal.pone.0175116.t005

genes coding for chaperones (*dnaK*) and stringent response. Additionally, DnaK and Enolase are plasminogen receptors involved in bile modulation during intestinal colonization.

Additionally, we assayed *L. lactis* NCDO 2118 to see how it responds to the challenges of acid pH and bile salt secretion in the gastrointestinal tract. When in contact with artificial gastric juice, 48% of the *L. lactis* NCDO 2118 was not inhibited and was able to grow after acid pH challenge, whereas the contact with bile salts inhibited 95% of the bacteria growth, showing a high sensibility, as a result of three independent experiments (S2 Fig).

Identification of genes coding for adhesins and adhesion-related proteins

Based on literature data, we predicted proteins involved in the adhesion mechanisms of *L. lactis* NCDO 2118, shown in Table 6. *L. lactis* NCDO 2118 harbors 19 genes putatively coding for adhesion-related proteins, such as the gene *chiA* (NCDO2118_2053) and the genes coding for the *Chitin binding protein* (CBP–NCDO2118_2054) and the laminin-binding protein (NCDO2118_1446).

To determine whether *L. lactis* NCDO 2118 exhibited adhesive ability, corroborating the *in silico* data, we performed microbial adhesion to solvents (MATS) experiments, which demonstrated a moderate cell surface hydrophobicity, as suggested by Nader-Macías *et al*., (2008) [41], with 52% association with xylene.

Bacteriocins and other competitive exclusion mechanisms

To predict putative bacteriocins, we used the software BAGEL [42]. In addition to identification, BAGEL also classifies the bacteriocins into three classes: (i) lanthionine-containing bacteriocins/lantibiotics, (ii) non-lanthionine-containing bacteriocins and (iii) bacteriolysins/non-bacteriocin lytic proteins [43].

In *L. lactis* NCDO 2118, BAGEL predicted one bacteriocin for each of the three classes (Fig 3): a lanthipeptide (class I), NCDO2118_1768 (putative Bacteriocin-lactococcin-A—class II)

Table 6. Proteins potentially involved in the adhesion mechanisms of *L. lactis*.

Locus_tag	Gene	Product
NCDO2118_0315		Hypothetical protein
NCDO2118_0552		Hypothetical protein
NCDO2118_0647	*pycA*	Pyruvate carboxylase
NCDO2118_0684		ChW repeat-/cell adhesion domain-containing transglutaminase-like protease
NCDO2118_0727		Hypothetical protein
NCDO2118_0774		Flagellar hook-length control protein FliK
NCDO2118_0776		Hypothetical protein
NCDO2118_0806	*exoA*	Exodeoxyribonuclease
NCDO2118_0857		Hypothetical protein
NCDO2118_1205		Hypothetical protein
NCDO2118_1365		Hypothetical protein
NCDO2118_1446	*bmpA*	Basic membrane protein A (laminin-binding protein)
NCDO2118_1515	*ypdD*	Sugar hydrolase
NCDO2118_1627		Hypothetical protein
NCDO2118_2053	*chiA*	Chitinase
NCDO2118_2054		Chitin binding protein
NCDO2118_2211		Hypothetical protein
NCDO2118_2278		Fibronectin-binding protein
NCDO2118_2284		Hypothetical protein

https://doi.org/10.1371/journal.pone.0175116.t006

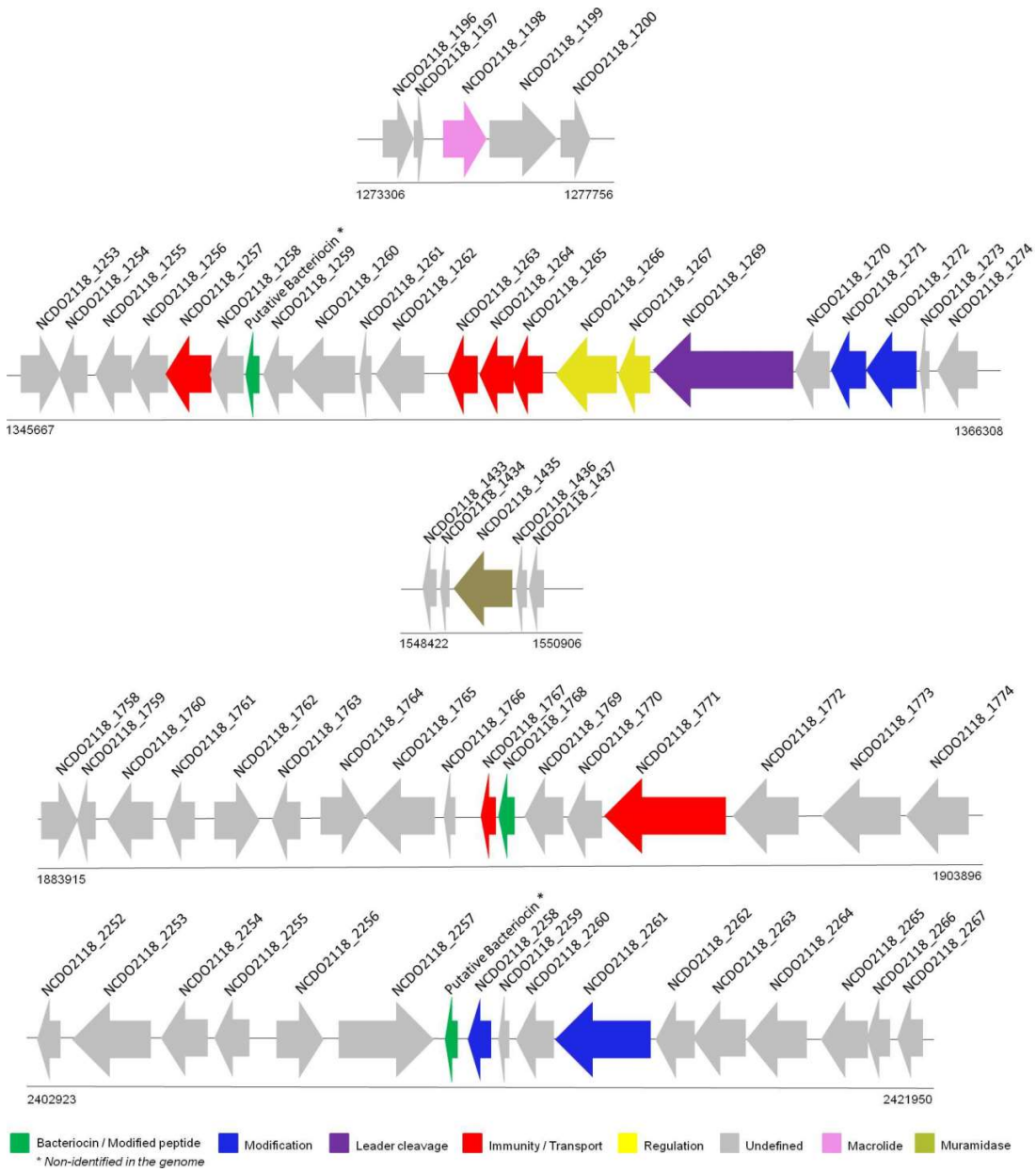

Fig 3. Regions of bacteriocins predicted with BAGEL in *L. lactis* NCDO 2118. BAGEL predicted three putative bacteriocins, one of each class. (A) Putative bacteriocin/Class I predicted on orf010 (pseudogene) and *nisZ* was found with manual curation. (B) Putative bacteriocin/Class II predicted on orf027 (pseudogene). (C) Putative bacteriocin/Sactipeptidase predicted on orf011 (this region was not previously characterized in the *L. lactis* subsp. *lactis* NCDO 2118 genome). All putative bacteriocins were also identified in Bactibase.

https://doi.org/10.1371/journal.pone.0175116.g003

and a putative bacteriocin (class III), located between NCDO2118_2257 and NCDO2118_2258. The class III putative bacteriocin was not described in the *L. lactis* NCDO 2118 genome, possibly because the gene-finding methodology failed to predict it. The bacteriocin of class I is a lantibiotic Nisin coded by the *nisZ* gene (NCDO2118_1272), a natural variant of *nisA* [44]. Briefly, Nisin is commonly produced by strains of *L. lactis*, and the cluster of genes coding for the nisin biosynthesis proteins consists of 11 genes: *nisABTCIP* (biosynthesis and immunity), nis*FEG*

(immunity) and the two-component regulatory system *nisRK* [45]. *L. lactis* NCDO 2118 harbors a *nisBCIP* operon (where *nisP* is a pseudogene), a *nisRK* two-component system and a *nisFEG* operon. Additionally, BAGEL has predicted the presence of another putative bacteriocin between NCDO2118_1258 and NCDO2118_1259 that is located close to the class I cluster of genes. However, the amino acid sequence predicted from this region only presents similarity to a hypothetical protein. Lactococcin A is a class IId, non-pediocin-like, single-peptide bacteriocin normally produced by strains of *L. lactis*. Four genes are responsible for the biosynthesis of lactococcin: the lactococcin-A coding gene, one immunity gene and the dedicated ABC transporter system along with its accessory protein. *L. lactis* NCDO 2118 harbors an immunity protein (NCDO2118_1767) and lactococcin-A (NCDO2118_1768). As for the class III prediction, the predicted putative bacteriocin is located upstream of two hypothetical proteins (NCDO2118_2258 and NCDO2118_2259); however, little is known about the organization of the gene cluster of class III bacteriocins [45], and the putative bacteriocin predicted by BAGEL only presents similarity to hypothetical proteins in GENBANK.

Moreover, an additional bacteriocin-coding gene was harbored by GEI 9 (S2 Table), which was not predicted by BAGEL. Through blast analyses, we found a significant amino acid similarity, with identities varying from 76% to 98%, between this gene and a bacteriocin-coding gene from other *L. lactis* in the UNIPROT and NCBI BLAST databases. However, many of the genes were also described as hypothetical proteins. In addition, we also searched for other genes that could possibly play a role in the competitive exclusion of other bacteria. A *Lyzozyme M1* and a Macrolide biosynthetic protein encoding genes were also included in S3 Table after manual curation in the *L. lactis* NCDO 2118 genome.

In the present study, a deferred agar spot assay was used for the initial determination of antagonistic activity via diffusible compound(s) produced by *L. lactis* NCDO 2118. To assay whether *L. lactis* NCDO 2118 could affect the growth of pathogenic bacteria, we used an approach to measure its antagonistic activity against the strains *Salmonella enterica* ATCC 14028, *Escherichia coli* ATCC 25723, *Staphylococcus aureus* 29213, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 15313, *Enterococcus faecalis* ATCC 19433 and *Pseudomonas aeruginosa* ATCC 5853. *L. lactis* NCDO 2118 showed no effect on the growth of the above-mentioned pathogenic strains.

In silico identification of putatively secreted proteins

Here, we strove to predict genes encoding secreted proteins from *L. lactis* NCDO 2118 that are absent from the genomes of the strains *L. lactis* IL1403 and *L. cremoris* MG1363, as the secreted proteins of *L. lactis* NCDO 2118 are possibly responsible for the immunomodulatory effects of this transient bacterium inside the host [18].

To predict the secreted proteins, we used the software SurfG+, which classifies the proteins using an identification approach based on the presence/absence of signal peptides, signal retention and transmembrane helix [46], which are correlated with the cell wall thickness of the bacteria. To determine the cell wall thickness, we made photomicrographs of *L. lactis* NCDO 2118 (Fig 4); the cell wall was measured more than 270 times, showing an average size of ~20 nm, and this value was used to determine the motifs. If none of the motifs were found in the protein sequence, SurfG+ characterized the protein as cytoplasmic (CYT) [47]. Using SurfG+, we predicted 94 secreted proteins in *L. lactis* NCDO 2118.

From this data, the secreted proteins of *L. lactis* NCDO 2118 were compared to the proteins identified in *L. lactis* IL1403 using OrthoMCL [48]. In this comparison, 26 of the secreted proteins were exclusive from *L. lactis* NCDO 2118. Because the probiotic effect was searched using secreted proteins previously expressed *in vitro*, we searched for proteins that were expressed in

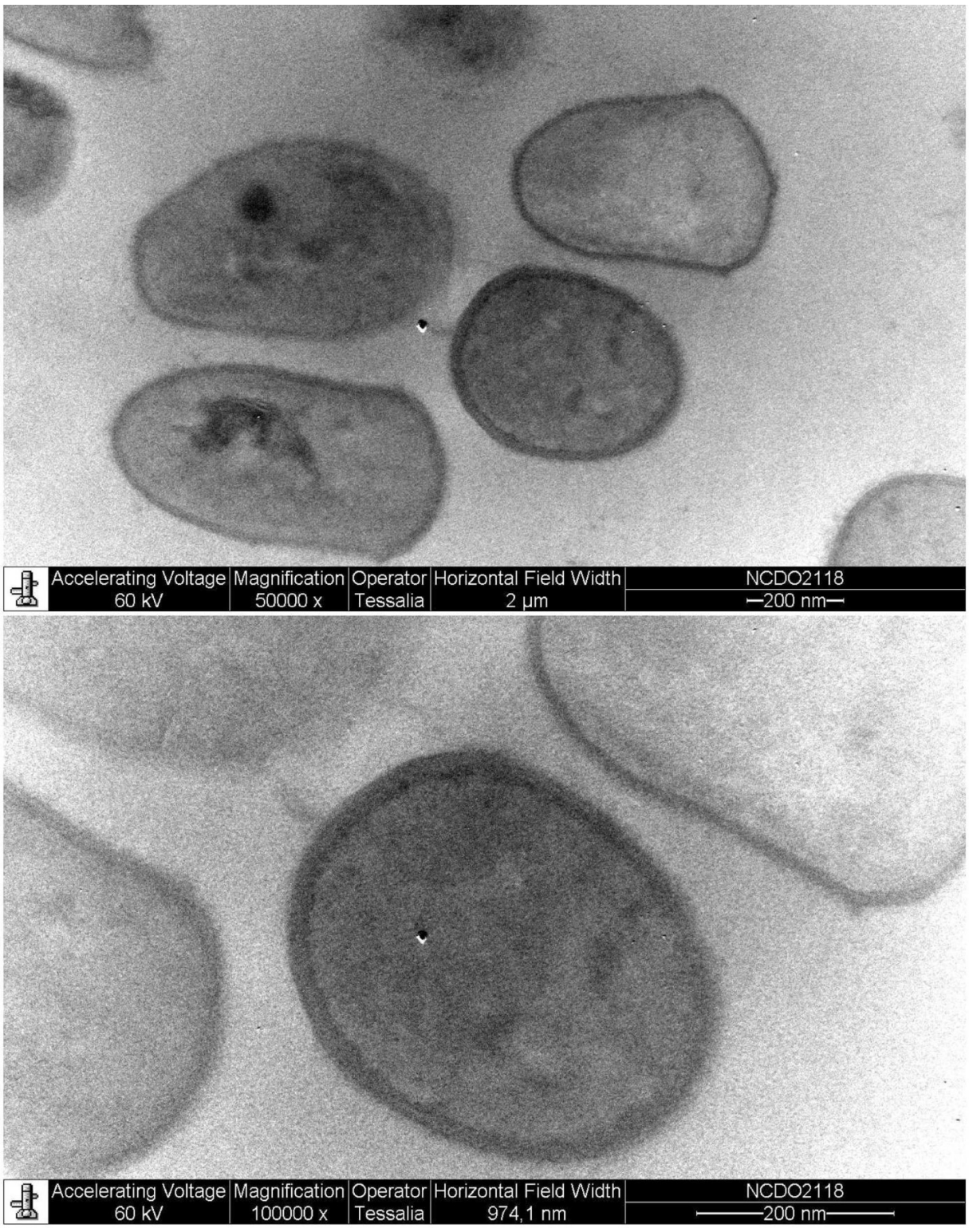

Fig 4. Photomicrograph of *L. lactis* NCDO 2118. The measurements of the membrane wall were performed with ImageJ software using images generated with electron microscopy with EM10A equipment (Zeiss). Top: magnification of 50,000 times; bottom: magnification of 100,000 times.

https://doi.org/10.1371/journal.pone.0175116.g004

Table 7. Prediction of exclusive secreted proteins of *L. lactis* NCDO 2118.

Locus tag	Gene	Start	Stop	Product	Orthology/Subcellular Location/Proteome
NCDO2118_0052	NCDO2118_0052	57803	58270	Hypothetical protein	Exclusive/ Secreted
NCDO2118_0128	*epsX*	133945	134712	Polysaccharide biosynthesis protein	Exclusive/ Secreted
NCDO2118_0139	*epsK*	144750	145652	Polysaccharide biosynthesis protein	Exclusive/ Secreted/ Expressed
NCDO2118_0140	*epsL*	145677	146600	Transcriptional regulator	Exclusive/ Secreted/ Expressed
NCDO2118_0212	NCDO2118_0212	214606	215988	Hypothetical protein	Exclusive/ Secreted/ Expressed
NCDO2118_0256	NCDO2118_0256	255719	256297	Hypothetical protein	Exclusive/ Secreted
NCDO2118_0291	NCDO2118_0291	285998	287113	Endoglucanase	Exclusive/ Secreted
NCDO2118_0294	NCDO2118_0294	288612	289397	Hypothetical protein	Exclusive/ Secreted
NCDO2118_0483	NCDO2118_0483	478392	479351	Hypothetical protein	Exclusive/ Secreted
NCDO2118_0533	NCDO2118_0533	527774	527965	Hypothetical protein	Exclusive/ Secreted
NCDO2118_0683	NCDO2118_0683	697697	698158	Hypothetical protein	Exclusive/ Secreted/ Expressed
NCDO2118_0684	NCDO2118_0684	698177	701176	ChW repeat-/cell adhesion domain-containing transglutaminase-like protease	Exclusive/ Secreted
NCDO2118_0882	NCDO2118_0882	918428	918706	Hypothetical protein	Exclusive/ Secreted
NCDO2118_0904	NCDO2118_0904	939391	940704	Hypothetical protein	Exclusive/ Secreted
NCDO2118_0942	NCDO2118_0942	985414	986700	Hypothetical protein	Exclusive/ Secreted
NCDO2118_0991	NCDO2118_0991	1034860	1035321	Hypothetical protein	Exclusive/ Secreted
NCDO2118_1361	NCDO2118_1361	1468537	1469364	Hypothetical protein	Exclusive/ Secreted
NCDO2118_1363	NCDO2118_1363	1474372	1475115	Hypothetical protein	Exclusive/ Secreted
NCDO2118_1364	NCDO2118_1364	1475137	1475901	Hypothetical protein	Exclusive/ Secreted
NCDO2118_1420	NCDO2118_1420	1537567	1538400	Hypothetical protein	Exclusive/ Secreted/ Expressed
NCDO2118_1459	NCDO2118_1459	1569307	1569477	Hypothetical protein	Exclusive/ Secreted
NCDO2118_1795	NCDO2118_1795	1927992	1929140	Transcriptional regulator	Exclusive/ Secreted
NCDO2118_2077	NCDO2118_2077	2227730	2228593	Hypothetical protein	Exclusive/ Secreted
NCDO2118_2151	NCDO2118_2151	2304776	2305051	Hypothetical protein	Exclusive/ Secreted
NCDO2118_2232	NCDO2118_2232	2371307	2372062	Hypothetical protein	Exclusive/ Secreted
NCDO2118_2330	NCDO2118_2330	2482143	2482712	Hypothetical protein	Exclusive/ Secreted

Exclusive, secreted and expressed proteins were predicted using OrthoMCL, SurfG+ and proteomic analyses, respectively.

https://doi.org/10.1371/journal.pone.0175116.t007

L. lactis NCDO 2118 *in vitro* using proteomics analyses. Five proteins were both present in the 26 secreted proteins that were exclusive from *L. lactis* NCDO 2118 and in the 867 expressed proteins from proteomic analyses (Table 7). The complete lists of genes identified in proteomic analyses, in the prediction of subcellular location and the exclusive proteins of *L. lactis* NCDO 2118 are described in S4 Table.

Discussion

Genomic characterization of *L. lactis* NCDO 2118 and comparison with other species

The genomic lengths of the *Lactococcus* species analyzed here are highly variable (from ~1.95 Mb to ~2.60 Mb). However, the finding that *L. garvieae* strains have the smallest genomes compared to *L. lactis* strains is in agreement with the lifestyle of *L. garvieae*, isolated from diseased fish. Because pathogenic bacteria may scavenge compounds from the host for their own metabolism, they tend to lose genes involved in biosynthetic pathways, thus, presenting smaller genomes [49].

The high similarity at the subspecies level may be related with some specific characteristics already described in literature, such as the propensity of *L. lactis* subsp *lactis* to form longer chains. Besides, *L. lactis* subsp. *lactis* are able to produce GABA, ammonia from arginine, carbon dioxide and diacetyl formation from citrate as opposing to *L. lactis* subsp. *cremoris* subspecies. Additionally, analyses using southern hybridization, PFGE, 16 rRNA and housekeeping genes (*atpA*, *rpoA*, *pheS*, *pepN*, *bcaT*, *pepX*) showed two separate clusters formed by *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* with a low degree of similarity between them [50–52].

From the genome synteny analyses, we have found a high degree of synteny between *L. lactis* KF147 and *L. lactis* IL1403, which was already reported in a previous work [25]. However, there was no other genome sequence of any *Lactococcus* species correlated with plants available at the time the work was performed. Here, we found that the most conserved genome compared to *L. lactis* NCDO 2118 was *L. lactis* KF147. The material of fermented plant covers a highly variable niche according to some characteristics as: chemical composition and physical conditions. Thus, plant-related strains posses a great metabolic diversity that certainly extrapolates that from dairy strains [53].

Finally, although *L. lactis* NCDO 2118 shares several pathways in common with *L. lactis* KF147 and *L. lactis* IL1403, it presents several exclusive metabolic features that may be explored for future utilization in industry.

Evaluation of safety aspects in the use of *L. lactis* NCDO 2118 by genome plasticity and antibiotic resistance approaches

Plasmid-linked antibiotic resistance is not very common among LAB, but it does occur, and safety implications should be taken into consideration. Strains harboring resistance plasmids should not be used as human or animal probiotics. Checking the ability of a proposed probiotic strain to act as a donor for conjugative antibiotic resistance genes may be a sensible precaution in some instances [54].

To provide a better understanding of the putative plasticity of *L. lactis* NCDO 2118, we have predicted putative phage and genomic islands of this species. The presence of phage regions may contribute to the acquirement of antibiotic resistance, the ability to survive in a new environment, the improvement of adhesion ability, or even to turning the bacteria pathogenic [55]. Here, we found 5 phages; the 3 intact phages harbored important genes such as *rusA*, *arsC1*, *arsC3*, *amtB*, *rpmE2*, *carA*, *pyrB*, *pyrP* and *pepT*. The *rusA* gene is associated with the prophage sequences of several genera of bacteria, including *Bacillus*, *Streptococcus*, *Staphylococcus*, and *Enterococcus*, and it is also present in *Lactococcus lactis* phage r1t [56]. The *arsC1* gene is related to arsenate resistance in *Corynebacterium glutamicum* [57]. *arsC3* codes for a thioredoxin-dependent arsenate reductase of the *Mycobacterium* sp. A33 [58]. *amtB* is a gene of the ammonia transporter family, which is found in eubacteria, archaea, fungus, plants and animals, whereas in prokaryotes, its homologue is co-transcribed with a PII paralogue, GlnK, in response to nitrogen limitation [59]. The *rpmE2* gene codes for a L31 ribosomal protein. The genes *carA*, *pyrB* and *pyrP* are organized as an operon in *L. cremoris* MG1363, where *pyrP* encodes a membrane-bound protein with high affinity to uracil permease and pyrimidines, and *pyrB* and *carA* encode pyrimidine biosynthetic enzymes [60]. Finally, the gene *pepT* encodes for a tripeptidase.

Additionally, we predicted 9 GEIs, 5 MIs, 4 SIs and 3 MSIs in the genome sequence of *L. lactis* NCDO 2118. Interestingly, all MIs present deletions in the pathogenic species *L. garvieae*, which is a common feature of pathogenic bacteria that adapted to scavenge nutrients from the host [61]. Additionally, MI3 is only present in the *L. lactis* NCDO 2118 and *L. lactis* KF147 and may be important for the adaptation of those strains to plants.

We have also assayed *L. lactis* NCDO 2118, aiming to characterize its antibiotic resistance profile. *L. lactis* NCDO 2118 is susceptible to most of the antibiotics assayed here. Although *L. lactis* NCDO 2118 presented resistance to oxacillin and susceptibility to penicillin, it only harbored genes coding for a VanZ family protein, which may be related to the vancomycin resistance, penicillin-binding proteins, and multidrug efflux pump proteins.

The efflux pumps are membrane transporter proteins responsible for the extrusion of relevant antibiotics, which are found in both Gram-positive and Gram-negative bacteria [62; 63]. Penicillin-binding proteins are transpeptidases or caboxypeptidases that harbor specific motifs that limit the active site serine penicillin-recognizing enzyme family, including class A and C β-lactamases [64]. Vancomycin is a glycopeptide antibiotic used in severe infections. Some species used in the food industry or found naturally in raw food material present an intrinsic resistance to vancomycin, including *L. rhamnosus*, *L. casei*, *Lactobacillus plantarum*, and *Leuconostoc lactis* [65].

Finally, although *L. lactis* NCDO 2118 does present genes putatively coding for antibiotic resistance-related proteins, none of those genes present anomalous G+C or codon usage deviation, nor are they harbored by the putative horizontally acquired regions predicted by GIPSy or PHAST. More interestingly, no Resistance Island was identified in *L. lactis* NCDO 2118, corroborating its safety aspects [66].

In vitro and in silico analyses of survival, exclusion mechanisms and probiotic properties of *L. lactis* NCDO 2118

Susceptibility of *L. lactis* NCDO 2118 to acid stress and bile salts. Concerning the acid stress, lowering the intracellular pH reduces the transmembrane pH difference and the activity of acid-sensitive enzymes and damages proteins and DNA [67].The first mechanism used by *L. lactis* species to cope with acid stress is to maintain a low intracellular pH (pHi) by using membrane ATPase FoF1 [68; 69] and the generation of alkaline substances through the catabolism of amino acids (deamination, for example) [70; 71]. Bile salts, on the other hand, are surface-active, amphipathic molecules with a potent antimicrobial activity, and they act as detergents that disrupt biological membranes [67]. The percentage of resistance to bile salts also tends to vary among LAB and even between strains of the same species [72].

Here, we have identified 25 and 16 genes previously shown to be involved in acid stress and bile resistance in other species, respectively. In an *in vitro* assay, however, only 48% of *L. lactis* NCDO 2118 was able to grow after pH challenge, and 95% of bacteria was inhibited by bile salts. Other authors have already found that bacteria with an intestinal origin tend to be more resistant to stomach acids [73]. Therefore, this finding corroborates our results because *L. lactis* NCDO 2118 was isolated from frozen peas. Most of the genes found in *L. cremoris* MG1363 were also identified in *L. lactis* NCDO 2118. Additionally, a work using proteomics analyses identified some genes related to acid response and they are present in *L. lactis* NCDO 2118 genome (*clpEP*, *ahpC*, *tig*, *hpr and luxS*) [74] showing that other approaches may better elucidate the mechanism of survival to acid stress on this strain.

The high susceptibility of *L. lactis* NCDO 2118 to bile salts, on the other hand, must be further explored *in vitro* and *in vivo* using transcriptomics analyses to determine the expression rates of the described genes.

Competitive exclusion mechanisms of *L. lactis* NCDO 2118. There are several mechanisms used by bacteria to competitively exclude other species, such as bacteriocin production, space competition through the use of adhesins or receptors that bind to specific surface features, predation and even rapid growth [75].

Adhesins are responsible for the recognition and colonization of host tissues through specific binding. This process may activate the innate host cells or the expression of new genes.

Adhesins may be characterized as hair-like attachments named pili or fimbriae or in other cases, named non-pilus adhesin, related to the microbial cell surface [76].

In *L. lactis* NCDO 2118, we have identified the gene *chiA* (NCDO2118_2053) and the genes coding for the *Chitin binding protein* (CBP–NCDO2118_2054) and the laminin-binding protein (NCDO2118_1446), which are normally related to adhesion in other bacteria. Chitin is degraded by chitinases that belong to members of the glycoside hydrolase of family 18 [77]. One example of bacteria that produces chitinase is *Serratia marcescens*, one of the most efficient organisms in chitin degradation [78]. When *E. coli* was cloned with a chitin-binding protein of *Serratia marcescens*, there was a significant increase in its ability to adhere to human colon cells [77].

Chitin-binding encoding genes are broadly distributed in many microorganisms. The *L. lactis* IL1403 genome, for example, harbors chitinolytic machinery represented by one family 33 CBP (*yucG;* referred as *Ll*CBP33A), one family 18 chitinase (*chiA*, referred as *Ll*Chi18A) and one family 20 *N*-acetylhexosaminidase [3; 79]. Another example of bacteria that present a high adhesion degree is *Borrelia burgdorferi*, which is able to bind to mammalian laminin, an important extracellular matrix (ECM) component [80]. A laminin-binding protein has also been identified in *L. lactis* NCDO 2118.

Additionally, we have found using MATS experiments that *L. lactis* NCDO 2118 presents a 52% of association to xylene, which supports the presence of genes coding for adhesion-related proteins in this strain. The hydrophobicity is directly related to the capacity of strains to adhere to surfaces. This capacity is determined by hydrophobic components present in the outer membrane of microorganisms, and it is known that hydrophobic interactions have an important role in the adhesion of bacteria to the epithelium. The application of MATS experiments facilitates a qualitative assessment of the polarity or non-polarity of the bacterial surface, which is important because it indicates the potential for probiotic adhesion to apolar surfaces in the intestinal and vaginal epithelia. However, this test is only a primary indicator of the adherence of microorganisms [81; 82].

The other bacterial competitive exclusion mechanism assayed here was the production of exclusion antimicrobial peptides, named bacteriocins. Bacteriocins produced by a bacterium may be activated against others, even ones from the same species, while the producer is immune to its own peptides [43]. This exclusion mechanism is very important for probioses, as it renders probiotic organisms able to compete with and kill pathogenic ones, promoting a health benefit to the host [2]. We have predicted one bacteriocin for each of the three classes in *L. lactis* NCDO 2118 (class I-III), which may be important for exclusion mechanisms of this bacteria. However, the lack of *nisT* and the pseudogenization of *nisP* on the class I gene cluster, the lack of ABC-transporters in the class II cluster and, also, the lack of information regarding the product of the putative bacteriocin in the class III cluster have to be further studied using *in vitro* analyses to elucidate whether those bacteriocins are produced and present antimicrobial activity or not.

We have also performed a deferred agar spot assay for the initial determination of antagonistic activity produced by *L. lactis* NCDO 2118. This test indicates the activity against various Gram-positive and -negative bacteria. This inhibitory effect may be due to H_2O_2, lactic acid, bacteriocins, antibiotic-like substances, or a combination of these compounds [83]. However, *L. lactis* NCDO 2118 showed no effect on the growth of the pathogenic strains assayed here.

Secreted proteins and immunomodulatory effects. According to Luerce *et al*., (2014), the secreted proteins of *L. lactis* NCDO 2118 are possibly responsible for the immunomodulatory effects of this transient bacterium inside the host. In a comparison of the anti-inflammatory effects between *L. lactis* NCDO 2118, *L. lactis* IL1403 and *L. cremoris* MG1363 strains, only the *L. lactis* NCDO 2118 supernatant was able to decrease the IL-8 production (45%), showing its immunomodulatory ability against inflammation [18].

Here, we predicted 5 proteins that are present in the 26 secreted proteins exclusive from *L. lactis* NCDO 2118 and in the 867 expressed proteins from proteomic analyses and may thus be related to the probiotic effect of this strain (Table 7). From those 5 exclusive, secreted and expressed genes of *L. lactis* NCDO 2118, *epsK* and *epsL* are part of the operon *epsABCDEFGHIJKLX*, whereas there is an *epsR* gene located in another genomic region.

The EPSs are a type of biopolymer able to facilitate intense interactions of biofilm cells through adhesion, aggregation of bacterial cells, cohesion of biofilms, protective barriers, and cell component export [84]. Through microarray and electron microscopy analyses, Denou *et al*., 2008 found an *eps* cluster of genes exclusive from a probiotic *Lactobacillus* strain compared to a type strain and they have shown that deletion of this cluster from the probiotic strain results in lack of the fuzzy layer on the outside of the cell wall [85].

Altogether, the lack of further knowledge of the *eps* cluster of genes and the presence of three other genes coding hypothetical exclusive/secreted/expressed proteins highlight the need for additional studies to better elucidate the underlying mechanisms involved in the anti-inflammatory and immunomodulatory activities of this strain.

Materials and methods

Genome sequences

The genome sequences of *L. lactis* NCDO 2118 [24] and 15 other strains of *Lactococcus* were retrieved from the GENBANK dataset of NCBI (Table 1). Briefly, the dataset is composed of 8 strains of *Lactococcus lactis* subsp. *lactis*, 2 of which were isolated from legumes (*L. lactis* NCDO 2118 and *Lactococcus lactis* subsp. *lactis* KF147), 6 *Lactococcus lactis* subsp. *cremoris* isolated from dairy or other fermented foods, and 2 *Lactococcus garvieae* isolated from diseased fish. *L. garvieae* was added to the analyses because it is a closely related pathogenic species. *S. thermophilus* LMD-9 was used as an outgroup to root the phylogenetic tree. Only complete genomes were used to avoid bias.

In silico analyses

Heatmap of genome similarities and 16S phylogenetic tree. The heatmap analyses of the 17 strains were performed with Gegenees [86]. The input files consisted of complete genomes in.fna format. *Streptococcus thermophilus* LMD-9, a closely related species, was used as an outgroup to root the tree. The analyses were performed with default parameters for comparative analyses using the alignment method BLASTn. Gegenees performs an all-versus-all alignment process of the fragments generated from the 17 genomes. The result was exported from Gegenees as a heatplot image. Additionally, a phylogenetic tree was made using the 16S sequences from all genomes as identified by RNAmmer [87]. After that, they were aligned in MUSCLE [88], and the phylogenetic tree was inferred using the Neighbor-Joining method with 1000 bootstrap replicates.

Genome synteny. The genome synteny analyses were performed using Mauve, with the "progressiveMauve" option and all genome sequences in the.fna format. Mauve predicts gene synteny by merging locally collinear blocks of conserved genome orthologous regions and ordering them according to a reference genome [89].

Genome plasticity. The genome plasticity analyses were performed by searching for horizontally acquired regions such as genomic islands and phage sequences. The genomic islands were searched using the software GIPSy: Genomic Island Prediction Software [90], which updates the methodology of the software PIPS: Pathogenicity Island Prediction Software. Briefly, GIPSy performs the prediction of four different classes of genomic islands: Pathogenicity Islands, Resistance Islands, Metabolic Islands and Symbiotic Islands. In this work, we

searched for metabolic and symbiotic islands in the genome of *L. lactis* NCDO 2118 using *Lactococcus lactis* subsp. *cremoris* MG1363 and *Lactococcus garviae* Lg2 genomes as subjects. After, we consolidated and manually curated the results. The choice of metabolic and symbiotic islands was made based on the lifestyle of *L. lactis* NCDO 2118, a strain isolated from vegetables, and its metabolic importance.

All the analyses were performed using GENBANK files and default parameters. The results were exported in tabulated format and used in BRIG (Blast Ring Image Generator) to generate circular genome comparative views [91]. Finally, the prophage prediction was performed using the GENBANK file and the software Phast [92], and the results were exported in table format and used as input in BRIG.

Bacteriocin prediction. The bacteriocin prediction was performed in BAGEL software using the.fna file from *L. lactis* NCDO 2118. Briefly, the software works with a curated dataset of bacteriocins, in which the input data are evaluated based on a Hidden Markov Model. The genetic information is analyzed based on combinations of PFAM domains [42]. For the putative bacteriocin predicted on *L. lactis* NCDO 2118 (NCDO2118_1768), we used the Transporter Classification Database (TCDB) [93] with an e-value of e-07.

Circular comparison map of genomic sequences. To create circular genome comparisons, we used the software BRIG and all genome sequences in the.fna format; we created the figure with *L. lactis* NCDO 2118 as reference strain. Additionally, we added the coordinates of the genomic islands and phage regions to the figure to visualize genome plasticity events. Finally, all genomes underwent BLAST analyses against the reference strain to create the circular comparison map.

Metabolic pathway prediction. A genome sequence in.fasta and a genome annotation in the.gbk format were used for reconstructing the *Lactococcus* species metabolic pathways. Posteriorly, the Pathway/Genome Databases (PGDB) for each of the 16 strains were computationally predicted using Pathway Tools software version 16.5 [94], developed by SRI International. The MetaCyc, a highly curated and non-redundant reference database of small-molecule metabolism, was used as a reference database for the PathoLogic component of the Pathway Tools software [95]. The metabolic pathways of *L. lactis* NCDO 2118 were used as a reference for the comparative analysis using the following comparisons: i) *L. lactis* NCDO 2118, *L. lactis* KF147 and *Lactococcus lactis* subsp. *lactis* IL1403, ii) non-pathogenic strains of *L. lactis* (*L. lactis* subsp. *lactis* and *cremoris*), and iii) all strains in this study.

Identification of the secretome. The prediction of the putative subcellular localizations of *L. lactis* NCDO 2118 proteins was performed *in silico* using SurfG+. This software contains such tools as *SignalP*, *LipoP* and *TMHMM* for the identification of motifs [46]. Interestingly, SurfG+ uses the size of the membrane wall to better differentiate the membrane (MEM) and potentially surface exposed (PSE) proteins. Here, the measurements of the membrane wall were performed with electron microscopy with EM10A equipment (Zeiss), as previously described [96].

L. lactis NCDO 2118 was grown at 30˚C for 18 h in M17 medium (Difco) containing 0.5% glucose [18] and then centrifuged. The resulting precipitate (~500 mL) was placed in an Eppendorf tube, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 6 h at 8˚C and washed three times with 0.1 M sodium cacodylate buffer (pH 7.2). After washing, the sample was post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) + 1.5% potassium ferrocyanide for 90 minutes, washed with 0.1 M with sodium cacodylate buffer (pH 7.2), dehydrated in a graduated ethanol series (50% EtOH, 70% EtOH, 95% EtOH, and 100% EtOH), and incorporated in Eponate–Araldite resin. Ultrathin sections were obtained using uranyl acetate and lead citrate and then verified by Zeiss-EM-10A [97]. The micrograph was obtained by one CCD Mega View camera. The thickness of the *L. lactis*

NCDO 2118 wall was determined from the image analysis micrograph in ImageJ software (available at imagej.nih.gov/ij/).

To measure the wall, we used at least five micrographs of *L. lactis* NCDO 2118 with magnifications of 50,000 and 100,000 times. We calculated the mean size of the cell walls, and the average number of amino acids for the obtained wall thickness was ~55 amino acids. This value was added to the SurfG+ software together with the.fasta sequence of amino acids (.faa) exported from the strain of interest.

After this process, we used OrthoMCL tool to predict the orthologous and paralogous genes between *L. lactis* NCDO 2118 and *L. lactis* IL1403.

In vitro analyses

Bacterial strains and growth conditions. For in vitro analyses, we used the probiotic strain *L. lactis* NCDO 2118 [18] and the pathogenic strains *Salmonella enterica* serovar *Typhimurium* ATCC 14028, *Escherichia coli* ATCC 25723, *Staphylococcus aureus* ATCC 29213, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 15313, *Enterococcus faecalis* ATCC 19433, and *Pseudomonas aeruginosa* ATCC 25853.

L. lactis NCDO 2118 was grown at 37˚C in MRS medium (Difco) without agitation for 18 hours. *L. monocytogenes* was cultured in TSB-YE for 24 hours at 28–30˚C. The pathogenic strains were grown in BHI medium (BD) for 24 hours at 37˚C. To prepare the solid and semi-solid culture media, we added 1.5% and 0.2–0.75% of agar, respectively.

***L. lactis* gastric juice susceptibility.** *L. lactis* NCDO 2118 stationary phase cells were suspended in either 0.9% saline solution (pH 7) or simulated gastric juice (NaCl 2 g/L, pepsin 3.2 g/L, adjusted to pH 2.5 with concentrated HCl) and incubated at 37˚C for 3 h. Solutions were centrifuged, the supernatant was discarded, and the pellets were suspended in MRS broth. Bacterial growth was evaluated by inoculating MRS broth with 2% v/v of control cells in saline and artificial gastric juice-treated cells onto microplates in triplicate, before incubating them in a Microplate Spectrophotometer System SpectraMax 340 (Molecular Devices Inc., Sunny- vale, CA, USA) at 37˚C for 18 h. The OD_{620nm} (optic density) was recorded at 30 min intervals. The percentage of growth inhibition was calculated as (1 –areaAGJ/areaCT) x 100, where area- AGJ and areaCT are the areas under the growth curve for the simulated gastric juice and con- trol, respectively. The total area under the curve was calculated by definite integration using the OriginPro 8.5 program (OriginLab Corporation, Northampton, MA, USA). The results were based on the average of three independent assays.

Susceptibility to bile salts. The susceptibility of *L. lactis* NCDO 2118 to bile salts was evaluated according to the method of Silva *et al*., (2013) [98]. For this, the *L. lactis* NCDO 2118 strain was grown in MRS medium at optical density of 0.6 and transferred (2% v/v) to MRS medium supplemented or not with 0.3% of Oxgall (Oxoid Ltd., Basingstoke, UK). The OD_{620nm} was recorded at 30 min intervals while incubating at 37˚C for 18 h in a microplate reader. The percentage of growth inhibition was calculated as (1 –areaBS/areaCT) x 100, where areaBS and areaCT are the areas under the growth curve for bile salt and control cells, respectively. The percentage of bacterial viability was determined in a Microplate Spectrophotometer System SpectraMax 340 (Molecular Devices Inc., Sunnyvale, CA, USA) in the same manner as described above. The results were based on an average of three independent assays.

Cell surface hydrophobicity. MATS was measured to evaluate the bacterial cell surface hydrophobicity [99]. Measurement of the cell surface hydrophobicity of *L. lactis* NCDO 2118 was performed with xylene using the MATS method. Bacterial stationary phase cultures were centrifuged, washed twice and adjusted to an OD_{600nm} of 0.6 with 0.1 M KNO_3, pH 6.2 (A_0). Then, xylene was added in suspension 16% (v/v) and maintained for 10 minutes at room

temperature. The tube was agitated vigorously, and after 30 minutes, the aqueous phase was collected for optical density OD_{600nm} measurement. The reduction percentage of optical density was calculated. The results were based on the average of three independent assays.

Antagonistic activity. Bacterial isolates were cultured in MRS broth for 24 h at 37˚C within an anaerobic chamber. A 5 μL aliquot of the culture was then spotted onto MRS agar. After incubation at 37˚C for 48 h under anaerobic conditions, the cells were killed by exposure to chloroform for 20 min. Residual chloroform was allowed to evaporate, and Petri dishes were overlaid with 3.5 mL of a soft agar containing brain heart infusion (Acumedia, Neogen Co., Lansing, MI, USA), tryptone soy broth (Difco) supplemented with 0.5% yeast extract (Acumedia), or Ellinghausen–McCullogh–Johnson–Harris with Leptospira enrichment EMJH (Difco) inoculated with 0.2 mL of a 24 h culture of *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 19433, *Pseudomonas aeruginosa* ATCC 25853, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25723, *Salmonella enterica* serovar *Typhimurium* ATCC 14028, *Leptospira interrogans* serovar *Icterohaemorrhagiae*, or *Listeria monocytogenes* ATCC 15313. After incubating at 37˚C for 24 h under aerobic or anaerobic conditions, depending on the indicator strain, the antagonistic activity was determined based on the presence of a growth inhibition zone, using the method of Tagg as modified by Branco *et al*., (2010) [100].

Antibiotic susceptibility. *L. lactis* NCDO 2118 antibiotic susceptibility was determined using antibiotic diffusion discs (Oxoid, England) on MRS plates. Bacteria were inoculated in MRS broth and incubated overnight at 37˚C. Solutions of 10^8 viable cells (McFarland scale) were prepared from the colonies in 3.5 mL of 0.9% buffered saline. The diluted culture (100 μL) was streaked onto MRS agar, and antibiotic discs were applied to the surface using an antibiotic disc dispenser. The discs included amikacin (30 μg), ampicillin (30 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), erythromycin (10 μg), oxacillin (1 μg), penicillin G (10 U), tetracycline (30 μg) and vancomycin (30 μg). The results were interpreted according to Charteris *et al*., (1998) [101].

Bacterial strain, growth conditions and preparation of proteins from culture filtrates for proteomic analysis. *L. lactis* NCDO 2118 and *L. lactis* IL1403 were pre-inoculated in M17 medium (Difco, New Jersey, USA) and incubated at 30˚C for 16 h. The precultures were then inoculated (1:100) in fresh M17 medium supplemented with 0.5% (w/v) glucose (M17Glc) at 30˚C until reaching an OD_{600} = 0.8 (three independent experiments). The cultures were then centrifuged for 20 min at 2,700 x g. The supernatants were filtered using 0.22-μm filters, 30% (w/v) ammonium sulfate was added to the samples, and the pH of the mixtures was adjusted to 4.0. Next, 20 mL of N-butanol was added to each sample. The samples were centrifuged for 10 min at 1,350 x g and 4˚C. The interfacial precipitate was collected and resuspended in 1 mL of 20 mM Tris-HCl pH 7.2 [102]. To perform label-free proteomic analysis, the protein extract was concentrated using a spin column with a 10 kDa threshold (Millipore, Billerica, MA, USA). The protein was denatured (0.1% *Rapi*GEST SF at 60˚C for 15 min) (Waters, Milford, CA, USA), reduced (10 mM DTT), alkylated (10 mM iodoacetamide) and enzymatically digested with trypsin (Promega, Sequencing Grade Modified Trypsin, Madison, WI, USA).

Proteomic analysis. Qualitative and quantitative nanoUPLC tandem nanoESI-HDMS^E (Nano Electrospray High Definition Mass Spectrometry) experiments were performed using both a 1 h reversed phase gradient from 7% to 40% (v/v) acetonitrile (0.1% v/v formic acid) at 500 nL min^{-1} and a nanoACQUITY UPLC 2D RPxRP Technology system [103]. A nanoACQUITY UPLC HSS T3 1.8 μm, 75 μm × 15 cm column (pH 3) was used with an RP XBridge BEH130 C18 5 μm 300 μm x 50 mm nanoflow column (pH 10). Typical on-column sample loads were 250 ng of the total protein digests for each of the 5 fractions (250 ng/fraction/load). All analyses were performed using nano-electrospray ionization in the positive ion mode nanoESI (+) and a NanoLockSpray (Waters, Manchester, UK) ionization source. The mass

spectrometer was calibrated using a MS/MS spectrum of [Glu^{1}]-Fibrinopeptide B human (Glu-Fib) solution (100 fmol.μL^{-1}) delivered through the NanoLockSpray source reference sprayer. The multiplexed data-independent (DIA) scanning with additional specificity and selectivity for non-linear 'T-wave' ion mobility (HDMS^{E}) experiments were performed using a Synapt G2-S HDMS mass spectrometer (Waters, Manchester, UK).

Following the identification of proteins, the quantitative data were packaged using dedicated algorithms [104; 105] and searching against a database with default parameters to account for ions [106]. The databases used were reversed "on-the fly" during the database queries and appended to the original database to assess the false positive rate during identification. For proper spectra processing and database searching conditions, the ProteinLynxGlobalServer v.2.5.2 (PLGS) with Identity^{E} and Expression^{E} informatics v.2.5.2 (Waters) was used. UniProtKB with manually reviewed annotations was used, and the search conditions were based on taxonomy (*L. lactis*). The maximum allowed missed cleavages by trypsin were up to one, variable modifications by carbamidomethyl (C), acetyl N-terminal, phosphoryl (STY) and oxidation (M) were allowed, and a peptide mass tolerance value of 10 ppm was used [107]. The collected proteins were organized by the PLGS Expression^{E} tool algorithm into a statistically significant list that corresponded to higher or lower regulation ratios among the different groups. For protein quantification, the PLGS v2.5.2 software was used with the Identity^{E} algorithm using the Hi3 methodology. The search threshold to accept each spectrum was the default value in the program with a false positive value of 4%. The quantitative values were averaged over all samples, and the standard deviations at $p < 0.05$ were determined using the Expression software [107].

Conclusions

Although *L. lactis* NCDO 2118 presented a high similarity to the other *L. lactis* strains, it presents an SI that is commonly shared with *L. lactis* KF147, along with high genomic synteny conservation with this strain. Additionally, the antibiotic resistance of this strain to vancomycin, amikacin and oxacillin could be an obstacle for its use as a probiotic. However, the absence of resistance-related genes in regions acquired by HGT and the absence of RIs in the genome sequence corroborates its safety aspects and supports its use as a probiotic strain. Moreover, the high susceptibility of *L. lactis* NCDO 2118 to acid and bile salts stresses have to be further evaluated in a complete digestion simulation, using transcriptomics and proteomics analyses, to elucidate whether the identified genes are differentially expressed in those environmental conditions.

Interestingly, the adhesion of *L. lactis* NCDO 2118 to xylene and the putative production of three classes of bacteriocins are important indicators of the exclusion mechanisms used by this strain. However, the *in vitro* analyses have not shown any sign of an antagonistic effect against the assayed pathogenic bacteria. Future works could also take advantage of combined transcriptomics and proteomics analyses of *L. lactis* NCDO 2118 *in vitro* before and after intestinal passage to evaluate the expression of the identified genes. Additionally, the identification of the EPS cluster of genes putatively associated with the probiotic effect of *L. lactis* NCDO 2118 could be further explored in 16S metagenomics analyses of gut microbiota, after expression, purification and administration of EPS proteins. Finally, through the analyses of the safety, survival and probiotic aspects of *L. lactis* NCDO 2118, we highlight here the potential use of this strain as a target for the future development of probiotic foods.

Supporting information

S1 Fig. Gene synteny between *Lactococcus lactis* subsp. *lactis* strains. *L. lactis* subsp. *lactis* NCDO 2118 (top) was used as a reference for the comparison analyses. The genomes are

represented according to the nucleotide conservation and synteny. Low similarity regions are represented as white regions inside the blocks, highlighted by a red ( ). Regions of deletions are represented as blank spaces between the blocks, letter (A). Insertion regions are highlighted with the letter (B), and inversion regions are represented by the letter (C). To perform the genome synteny analysis, we used the software Mauve, which compares the genomes by identifying and clustering homologous genes between the genomes into large collinear blocks of genes [89]. The most conserved genome compared to *L. lactis* NCDO 2118 was *L. lactis* KF147. Between these two strains, it is possible to see some regions of: deletion; insertion; inversion and specific areas with low or no similarity with the reference genome. The comparison of those features with other strains shows: a deletion on the genome position 1,200,000 of *Lactococcus lactis* subsp. *lactis* IO-1; a big inversion region in *Lactococcus lactis* subsp. *lactis* AI06 in the range from 800,000 to 1,600,000; a small insertion near the genome position 200,000 of *L. lactis* KLDS 40325 (in green); and a block on *Lactococcus lactis* subsp. *lactis* S0 (2,000,000 position) with low similarity to the reference genome.
(TIF)

S2 Fig. Growth curves of *L. lactis* NCDO 2118 under acid and bile salt stresses. (A) *L. lactis* subsp. *lactis* NCDO 2118 growth under acid stress conditions. Blue: (LL) *L. lactis* without acid contact. Red: (LLAT) *L. lactis* under acid treatment. (B) *L. lactis* growth under intestinal conditions. Blue: (LL) *L. lactis* without salt contact salt. Red: (LLOG) *L. lactis* growth with 0.3% ox gall.
(TIF)

S1 Table. Metabolic pathways exclusive of *Lactococcus lactis* subsp. *lactis* NCDO 2118. The metabolic pathways were predicted using the software Pathway Tools.
(XLS)

S2 Table. Putative genomic islands of *L. lactis* subsp. *lactis* NCDO 2118.
(XLSX)

S3 Table. Genes coding for bacteriocins, muramidases and macrolides. Bacteriocin regions were predicted using BAGEL.
(XLS)

S4 Table. Exclusive, expressed and secreted proteins of *L. lactis* NCDO 2118. The exclusive, secreted and expressed proteins were predicted using the software OrthoMCL, SurfG+ and proteomics analyses, respectively.
(XLS)

Acknowledgments

We thank the Institute of Biological Sciences of the Federal University of Pará and the Official Laboratory of Fisheries Ministry for the sequencing and assembly of *L. lactis* NCDO 2118. We also thank the Laboratory of Cellular and Molecular Genetics for the manual curation of the genome annotation.

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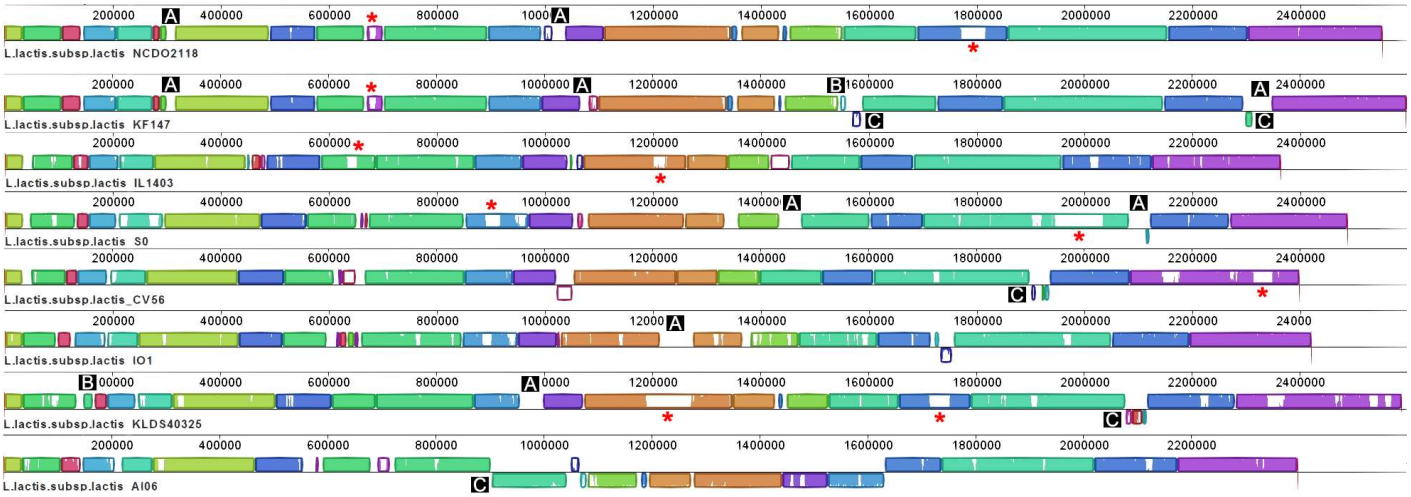
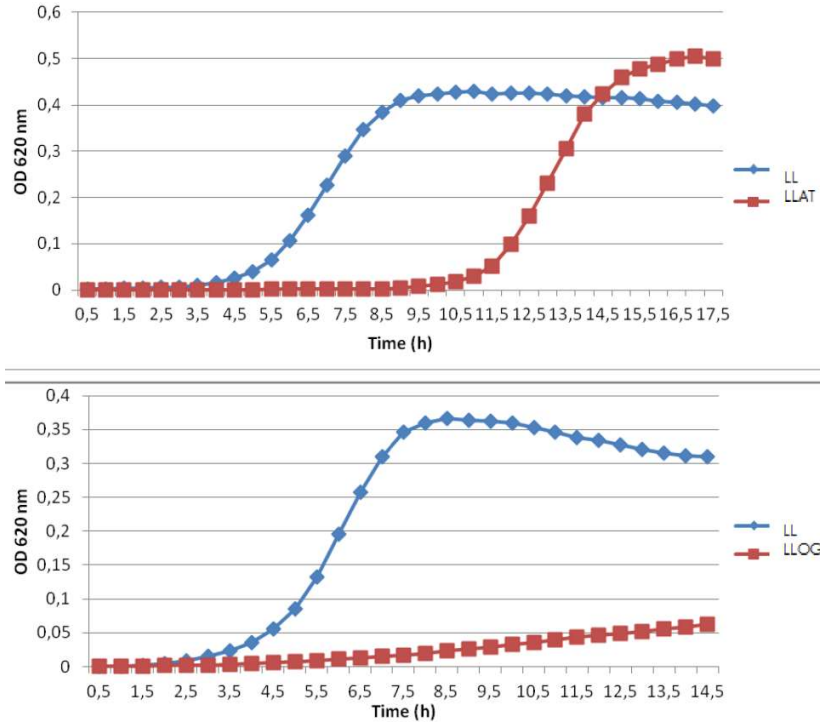
VI.2.1 Supporting information



VI.2.1.1 Fig S1. Gene synteny between *Lactococcus lactis* subsp. *lactis* strains.

L. lactis subsp. *lactis* NCDO 2118 (top) was used as a reference for the comparison analyses. The genomes are represented according to the nucleotide conservation and synteny. Low similarity regions are represented as white regions inside the blocks, highlighted by a red (*). Regions of deletions are represented as blank spaces between the blocks, letter (A). Insertion regions are highlighted with the letter (B), and inversion regions are represented by the letter (C). To perform the genome synteny analysis, we used the software Mauve, which compares the genomes by identifying and clustering homologous genes between the genomes into large collinear blocks of genes [89]. The most conserved genome compared to *L. lactis* NCDO 2118 was *L. lactis* KF147. Between these two strains, it is possible to see some regions of: deletion; insertion; inversion and specific areas with low or no similarity with the reference genome. The comparison of those features with other strains shows: a deletion on the genome position 1,200,000 of *Lactococcus lactis* subsp. *lactis* IO-1; a big inversion region in *Lactococcus lactis*subsp. *lactis* AI06 in the range from 800,000 to 1,600,000; a small insertion near the genome position 200,000 of *L. lactis* KLDS 40325 (in green); and a block on *Lactococcus lactis* subsp. *lactis* S0 (2,000,000 position) with low similarity to the reference genome.
https://doi.org/10.1371/journal.pone.0175116.s001



VI.2.1.2 Fig S2. Growth curves of *L. lactis* NCDO 2118 under acid and bile salt stresses.

(A) *L. lactis* subsp. *lactis* NCDO 2118 growth under acid stress conditions. Blue: (LL) *L. lactis*without acid contact. Red: (LLAT) *L. lactis* under acid treatment. (B) *L. lactis* growth under intestinal conditions. Blue: (LL) *L. lactis* without salt contact salt. Red: (LLOG) *L. lactis* growth with 0.3% ox gall.
https://doi.org/10.1371/journal.pone.0175116.s002

VI.2.1.3 Table S 2 Metabolic pathways exclusive of Lactococcus lactis subsp. lactis NCDO 2118. The metabolic pathways were predicted using the software Pathway Tools.

Locus tag	Gene	Enzyme	EC Number	Pathway
NCDO2118_1409	*axe*	cephalosporin-C deacetylase	EC:3.1.1.41	Biosynthesis of antibiotics
NCDO2118_0716	*apu*	Glucan 1,4-alpha-maltohydrolase	-	-
NCDO2118_0705	*rnmV*	ribonuclease M5	EC:3.1.26.8	-
NCDO2118_0711	-	4-alpha-glucanotransferase	EC:2.4.1.25	Starch and sucrose metabolism; Metabolic pathways
NCDO2118_0336	*yciA*	N-acetyldiaminopimelate deacetylase	EC:3.5.1.47	Lysine biosynthesis ; Metabolic pathways; Biosynthesis of secondary metabolites; Biosynthesis of amino acids
NCDO2118_0291	-	Endoglucanase	-	-
NCDO2118_2089	*lacA*	Galactoside O-acetyltransferase	-	-
NCDO2118_0606	-	DNA methylase	-	-
NCDO2118_1713	-	diamine N-acetyltransferase	EC:2.3.1.57	Arginine and proline metabolism; Metabolic pathways
NCDO2118_1878	*estA*	Carboxylic-ester hydrolases	EC:3.1.1.-	-
NCDO2118_1770	*ubiE*	demethylmenaquinone methyltransferase; 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase	EC:2.1.1.163; EC:2.1.1.201	Ubiquinone and other terpenoid-quinone biosynthesis; Metabolic pathways; Biosynthesis of secondary metabolites
NCDO2118_0503	-	Nucleoside 2-deoxyribosyltransferase	-	-
NCDO2118_0478	*ligA*	DNA ligase (NAD+)	EC:6.5.1.2	DNA replication; Base excision repair; Nucleotide excision repair; Mismatch repair
NCDO2118_0703	-	Endodeoxyribonucleases producing 5'-phosphomonoesters	EC:3.1.21.-	-
NCDO2118_0963	-	Recombinase	-	-
NCDO2118_1039	*nrdF*	ribonucleoside-diphosphate reductase	EC:1.17.4.1	Purine metabolism; Pyrimidine metabolism; Metabolic pathways
NCDO2118_0650	*icd*	isocitrate dehydrogenase (NADP+)	EC:1.1.1.42	Citrate cycle (TCA cycle); Glutathione metabolism; Metabolic pathways; Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics; Carbon metabolism; 2-Oxocarboxylic acid metabolism; Biosynthesis of amino acids
NCDO2118_1878	*estA*	Carboxylic-ester hydrolases	EC:3.1.1.-	-

NCDO2118_1386	*pfkA*	6-phosphofructokinase	EC:2.7.1.11	Glycolysis / Gluconeogenesis; Pentose phosphate pathway; Fructose and mannose metabolism; Galactose metabolism; Methane metabolism; Metabolic pathways; Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics; Carbon metabolism; Biosynthesis of amino acids; RNA degradation
NCDO2118_0345; NCDO2118_1716	-	Acting on ester bonds	EC:3.1.-.-	-
NCDO2118_0844	*mnmA*	tRNA-uridine 2-sulfurtransferase	EC:2.8.1.13	Sulfur relay system
NCDO2118_0816	*argF*	ornithine carbamoyltransferase	EC:2.1.3.3	Arginine biosynthesis; Metabolic pathways; Biosynthesis of secondary metabolites; Biosynthesis of antibiotics; Biosynthesis of amino acids
NCDO2118_2053	*chiA*	chitinase	EC:3.2.1.14	Amino sugar and nucleotide sugar metabolism; Metabolic pathways
NCDO2118_2088	*lacZ*	beta-galactosidase	EC:3.2.1.23	Galactose metabolism; Other glycan; Metabolic pathways
NCDO2118_0824	-	Haloacid dehalogenase	-	-
NCDO2118_0288	-	UDP-N-acetylglucosamine 2-epimerase (non-hydrolysing)	EC:5.1.3.14	Amino sugar and nucleotide sugar metabolism; Metabolic pathways
NCDO2118_1508	*lnbA*	beta-N-acetylhexosaminidase	EC:3.2.1.52	Other glycan degradation; Amino sugar and nucleotide sugar metabolism; Metabolic pathways
NCDO2118_0819	-	phosphatidylglycerol---membrane-oligosaccharide glycerophosphotransferase	EC:2.7.8.20	Glycerolipid metabolism; Metabolic pathways
NCDO2118_2175	*gmhA*	D-sedoheptulose 7-phosphate isomerase	EC:5.3.1.28	Lipopolysaccharide biosynthesis; Metabolic pathways
NCDO2118_2038	*metB*	cystathionine gamma-synthase	EC:2.5.1.48	Cysteine and methionine metabolism; Selenocompound metabolism; Sulfur metabolism; Metabolic pathways; Biosynthesis of secondary metabolites; Biosynthesis of antibiotics; Biosynthesis of amino acids;
NCDO2118_p0007	-	Transposase	-	-
NCDO2118_0479	-	diacylglycerol kinase (ATP)	EC:2.7.1.107	Glycerolipid metabolism; Glycerophospholipid metabolism; Metabolic pathways; Biosynthesis of secondary metabolites;
NCDO2118_1023	*fruC*	1-phosphofructokinase	EC:2.7.1.56	Fructose and mannose metabolism
NCDO2118_0751	-	3'(2'),5'-bisphosphate nucleotidase; oligonucleotidase	EC:3.1.3.7;EC:3.1.13.3	Sulfur metabolism; Metabolic pathways; Microbial metabolism in diverse environments
NCDO2118_1887; NCDO2118_2024	*birA; birA2*	biotin---[acetyl-CoA-carboxylase] ligase	EC:6.3.4.15	Biotin metabolism; Metabolic pathways
NCDO2118_0122	*glgB*	1,4-alpha-glucan branching enzyme	EC:2.4.1.18	Starch and sucrose metabolism; Metabolic pathways; Biosynthesis of secondary metabolites
NCDO2118_2132	*glk*	glucokinase	EC:2.7.1.2	Glycolysis / Gluconeogenesis; Galactose metabolism; Starch and sucrose metabolism; Amino sugar and nucleotide sugar metabolism; Streptomycin biosynthesis; Metabolic pathways;

				Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics; Carbon metabolism
NCDO2118_0584	*glyA*	glycine hydroxymethyltransferase	EC:2.1.2.1	Glycine, serine and threonine metabolism; Cyanoamino acid metabolism; Glyoxylate and dicarboxylate metabolism; One carbon pool by folate; Methane metabolism; Metabolic pathways; Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics; Carbon metabolism; Biosynthesis of amino acids
NCDO2118_1125	*frdC*	fumarate reductase (quinol)	EC:1.3.5.4	Citrate cycle (TCA cycle); Oxidative phosphorylation; Pyruvate metabolism; Butanoate metabolism; Metabolic pathways; Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics; Carbon metabolism; Two-component system
NCDO2118_1477	*rmaC*	NAD(P)H nitroreductase	-	-
NCDO2118_0400	*ahpC*	peroxiredoxin	EC:1.11.1.15	-
NCDO2118_0333	-	Thiol-disulfide isomerase	-	-
NCDO2118_2081	-	Protease	-	-
NCDO2118_0349	-	ribosomal-protein-alanine N-acetyltransferase	EC:2.3.1.128	-
NCDO2118_1853	*tyrA*	prephenate dehydrogenase	EC:1.3.1.12	Phenylalanine, tyrosine and tryptophan biosynthesis; Novobiocin biosynthesis; Metabolic pathways; Biosynthesis of secondary metabolites; Biosynthesis of antibiotics; Biosynthesis of amino acids
NCDO2118_1331	*aguA1*	alpha-glucuronidase	EC:3.2.1.139	-
NCDO2118_0714	*glgA*	starch synthase (glycosyl-transferring)	EC:2.4.1.21	Starch and sucrose metabolism; Metabolic pathways; Biosynthesis of secondary metabolites
NCDO2118_1592	*thiL*	acetyl-CoA C-acetyltransferase	EC:2.3.1.9	Fatty acid degradation; Synthesis and degradation of ketone bodies; Valine, leucine and isoleucine degradation; Benzoate degradation; Tryptophan metabolism; Pyruvate metabolism; Glyoxylate and dicarboxylate metabolism; Propanoate metabolism; Butanoate metabolism; Terpenoid backbone biosynthesis; Metabolic pathways; Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics; Carbon metabolism; Fatty acid metabolism; Two-component system
NCDO2118_1108	*cobQ*	Cobyric acid synthase	-	-
NCDO2118_1149	-	tRNA (cytidine34-2'-O)-methyltransferase	EC:2.1.1.207	-
NCDO2118_1089	*dgkA*	undecaprenol kinase	EC:2.7.1.66	Peptidoglycan biosynthesis
NCDO2118_1233	*ilvD*	dihydroxy-acid dehydratase	EC:4.2.1.9	Valine, leucine and isoleucine biosynthesis; Pantothenate and CoA biosynthesis; Metabolic pathways; Biosynthesis of secondary metabolites; Biosynthesis of antibiotics; 2-Oxocarboxylic acid metabolism; Biosynthesis of amino acids

NCDO2118_2174	-	D-glycero-alpha-D-manno-heptose 1,7-bisphosphate 7-phosphatase	EC:3.1.3.82; EC:3.1.3.83	Lipopolysaccharide biosynthesis; Metabolic pathways
NCDO2118_2070	-	Diadenosine polyphosphate hydrolase	-	-
NCDO2118_1274	*glpD*	glycerol-3-phosphate oxidase	EC:1.1.3.21	Glycerophospholipid metabolism; Biosynthesis of secondary metabolites
NCDO2118_1909	*pepO*	Metalloendopeptidases	EC:3.4.24.-	-
NCDO2118_0642	-	Dihydroorotate dehydrogenase	-	-
NCDO2118_0725	-	Lysophospholipase	-	-
NCDO2118_1506	-	Endo-beta-N-acetylglucosaminidase	-	-
NCDO2118_1295	-	UDP-N-acetylglucosamine 2-epimerase (non-hydrolysing)	EC:5.1.3.14	Amino sugar and nucleotide sugar metabolism; Metabolic pathways
NCDO2118_1779	*amyY*	alpha-amylase	EC:3.2.1.1	Starch and sucrose metabolism; Metabolic pathways
NCDO2118_0663	*scrB*	beta-fructofuranosidase	EC:3.2.1.26	Galactose metabolism; Starch and sucrose metabolism; Metabolic pathways
NCDO2118_2367	*gapB*	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	EC:1.2.1.12	Glycolysis / Gluconeogenesis; Metabolic pathways; Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics; Carbon metabolism; Biosynthesis of amino acids
NCDO2118_0168	*bglS*	6-phospho-beta-glucosidase	EC:3.2.1.86	Glycolysis / Gluconeogenesis; Starch and sucrose metabolism
NCDO2118_0446	*pepA*	glutamyl aminopeptidase	EC:3.4.11.7	-
NCDO2118_1096	*nucA*	5'-nucleotidase	-	-
NCDO2118_2064	-	tRNA-dihydrouridine synthase	-	-
NCDO2118_0544	-	In phosphorus-containing anhydrides	EC:3.6.1.-	RNA degradation
NCDO2118_1154	*folE*	GTP cyclohydrolase	-	-
NCDO2118_1568	*ypjA*	alcohol dehydrogenase	EC:1.1.1.1	Glycolysis / Gluconeogenesis; Fatty acid degradation; Tyrosine metabolism; Chloroalkane and chloroalkene degradation; Naphthalene degradation; Metabolic pathways; Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics; Degradation of aromatic compounds
NCDO2118_1980	-	Cysteine desulfurase	-	-

NCDO2118_1801	*ptcA2*	putrescine carbamoyltransferase	EC:2.1.3.6	-
NCDO2118_2136	*dinB*	DNA-directed DNA polymerase	EC:2.7.7.7	-
NCDO2118_0184	-	Dihydroxyacetone kinase	-	-
NCDO2118_0070	*prmA*	Methyltransferase	EC:2.1.1.-	-
NCDO2118_0499	-	Serine--pyruvate aminotransferase	-	-
NCDO2118_0849	*mutY*	Hydrolysing N-glycosyl compounds	EC:3.2.2.-	Base excision repair
NCDO2118_0183	*msrB*	eptide-methionine (R)-S-oxide reductase	EC:1.8.4.12	-
NCDO2118_1021	-	N-acyl-L-amino acid amidohydrolase	-	-
NCDO2118_1010; NCDO2118_0176	*preA*	heptaprenyl diphosphate synthase	EC:2.5.1.30	Terpenoid backbone biosynthesis; Biosynthesis of secondary metabolites
NCDO2118_1443; NCDO2118_1774	*kinB; kinF*	histidine kinase	EC:2.7.13.3	Two-component system; Quorum sensing
NCDO2118_2257	*adhE*	acetaldehyde dehydrogenase (acetylating)	EC:1.2.1.10; EC:1.1.1.1	Glycolysis / Gluconeogenesis; Fatty acid degradation; Tyrosine metabolism; Pyruvate metabolism; Chloroalkane and chloroalkene degradation; Naphthalene degradation; Butanoate metabolism; Metabolic pathways; Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics; Degradation of aromatic compounds
NCDO2118_0510; NCDO2118_0943	-	beta-N-acetylhexosaminidase	EC:3.2.1.52	Amino sugar and nucleotide sugar metabolism; Metabolic pathways; beta-Lactam resistance
NCDO2118_1142	-	Acyl-acyl-carrier-protein hydrolase	-	-
NCDO2118_1533	*purE*	5-(carboxyamino)imidazole ribonucleotide mutase	EC:5.4.99.18	Purine metabolism; Metabolic pathways; Biosynthesis of secondary metabolites; Biosynthesis of antibiotics
NCDO2118_0732; NCDO2118_0228	*qor*	Quinone oxidoreductase	-	-
NCDO2118_1155	*folP*	dihydropteroate synthase	EC:2.5.1.15	Folate biosynthesis; Metabolic pathways
NCDO2118_0413	*rheA*	RNA helicase	EC:3.6.4.13	RNA degradation
NCDO2118_1254	-	Adenylate cyclase	-	-
NCDO2118_0020	*tilS*	tRNAIle-lysidine synthase	EC:6.3.4.19	-

NCDO2118_0777; NCDO2118_0778	*lrgB; lrgA*	Murein hydrolase	-	-
NCDO2118_0736	*tadA*	tRNA(adenine34) deaminase	EC:3.5.4.33	-
NCDO2118_0812	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase	EC:1.2.1.38	Arginine biosynthesis; Metabolic pathways; Biosynthesis of secondary metabolites; Biosynthesis of antibiotics; 2-Oxocarboxylic acid metabolism; Biosynthesis of amino acids
NCDO2118_1158	*dukB*	Deoxyadenosine kinase	-	-
NCDO2118_0790; NCDO2118_1892	*fabG*	3-oxoacyl-[acyl-carrier-protein] reductase	EC:1.1.1.100	Fatty acid biosynthesis; Biotin metabolism; Biosynthesis of unsaturated fatty acids; Metabolic pathways; Fatty acid metabolism
NCDO2118_0079	-	1-acylglycerol-3-phosphate O-acyltransferase	EC:2.3.1.51	Glycerolipid metabolism; Glycerophospholipid metabolism; Metabolic pathways; Biosynthesis of secondary metabolites
NCDO2118_2383	*dacA*	serine-type D-Ala-D-Ala carboxypeptidase	EC:3.4.16.4	Peptidoglycan biosynthesis; Metabolic pathways
NCDO2118_0548; NCDO2118_0793	*fabZ1; fabZ2*	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	EC:4.2.1.59	Fatty acid biosynthesis; Biotin metabolism; Metabolic pathways; Fatty acid metabolism
NCDO2118_0435	*ldhB*	L-lactate dehydrogenase	EC:1.1.1.27	Glycolysis / Gluconeogenesis; Cysteine and methionine metabolism; Pyruvate metabolism; Propanoate metabolism; Metabolic pathways; Biosynthesis of secondary metabolites; Microbial metabolism in diverse environments; Biosynthesis of antibiotics
NCDO2118_0843; NCDO2118_2090	*galT*	UDP-glucose---hexose-1-phosphate uridylyltransferase	EC:2.7.7.12	Galactose metabolism; Amino sugar and nucleotide sugar metabolism; Metabolic pathways
NCDO2118_0609; NCDO2118_0762	*dnaC*	DNA helicase	EC:3.6.4.12	DNA replication
NCDO2118_1062; NCDO2118_2163	-	undecaprenyl-diphosphate phosphatase	EC:3.6.1.27	Peptidoglycan biosynthesis
NCDO2118_0115; NCDO2118_0527	*recU*	Acting on ester bonds	EC:3.1.-.-	-
NCDO2118_2002	*ytfB*	ADP-ribose diphosphatase	EC:3.6.1.13	Purine metabolism
NCDO2118_0272	*nrdG*	[formate-C-acetyltransferase]-activating enzyme	EC:1.97.1.4	-
NCDO2118_0271; NCDO2118_2032	*nrdD*	ribonucleoside-triphosphate reductase	EC:1.17.4.2	Purine metabolism; Pyrimidine metabolism; Metabolic pathways
NCDO2118_1394; NCDO2118_1395	*hepS*	heptaprenyl diphosphate synthase	EC:2.5.1.30	Terpenoid backbone biosynthesis; Biosynthesis of secondary metabolites

VI.2.1.4 Table S 3 Putative genomic islands of L. lactis subsp. lactis NCDO 2118.

MI1	*ctrA*	Amino acid permease
	NCDO2118_0079	1-Acyl-sn-glycerol-3-phosphate acyltransferase
	rmaD	Transcriptional regulator
	azoR1	FMN-dependent NADH-azoreductase 1
	secA	Protein translocase subunit SecA
	aroF	Phospho-2-dehydro-3-deoxyheptonate aldolase
	ptsH	Phosphocarrier protein HPr
	ptsI	Phosphoenolpyruvate-protein phosphotransferase
	NCDO2118_0086	Hypothetical protein
	NCDO2118_0087	Nucleoid-associated protein
	NCDO2118_0088	Alpha/beta hydrolase
	sugE	Multidrug resistance protein
	blt	Multidrug efflux transporter
	NCDO2118_0091	CAAX amino terminal protease
	NCDO2118_0092	ABC transporter
	NCDO2118_0093	ABC transporter
	NCDO2118_0094	ABC transporter
	NCDO2118_0095	Hypothetical protein
	pyk1	Pyruvate kinase
	NCDO2118_0097	Hypothetical protein
	ribA	GTP cyclohydrolase II
	NCDO2118_0099	Hypothetical protein
	tmk1	Thymidylate kinase
	pyrF1	Orotidine 5'-phosphate decarboxylase
	NCDO2118_0102	Hypothetical protein
	NCDO2118_0103	Hypothetical protein
	NCDO2118_0104	Transposase
	NCDO2118_0105	Phage integrase
	argG	Argininosuccinate synthase
	argH	Argininosuccinate lyase
	pmrB	Multidrug resistance efflux pump
	NCDO2118_0109	Transcriptional regulator
SI1	NCDO2118_0117	Transmembrane protein
	cbr	Carbonyl reductase
	NCDO2118_0119	Hypothetical protein
	dltE	Oxidoreductase
	NCDO2118_0121	Transcricional regulator
	glgB	1,4-alpha-glucan branching enzyme
	NCDO2118_0123	Hypothetical protein
	NCDO2118_0125	Integrase
	NCDO2118_0126	Hypothetical protein
	NCDO2118_0127	Hypothetical protein

	epsX	Polysaccharide biosynthesis protein
	epsA	Polysaccharide biosynthesis protein
	epsB	Polysaccharide biosynthesis protein
	epsC	Polysaccharide biosynthesis protein
	epsD	Polysaccharide biosynthesis protein
	epsE	Glycosyltransferase, group 1
	epsF	Polysaccharide biosynthesis protein
	epsG	Glycosyltransferase
	epsH	Glycosyltransferase, group 1
	epsI	Glycosyltransferase
	epsJ	Polysaccharide biosynthesis export protein
	epsK	Polysaccharide biosynthesis protein
	epsL	Transcriptional regulator
	NCDO2118_0141	Integrase
SI2	NCDO2118_0204	Epimerase
	NCDO2118_0205	Polysaccharide biosynthesis export protein
	NCDO2118_0206	Glycosyltransferase
	NCDO2118_0207	Hypothetical protein
	NCDO2118_0208	Glycosyltransferase
	NCDO2118_0209	Glycosyltransferase
	NCDO2118_0210	Glycerophosphodiester phosphodiesterase
	tagD1	Glycerol-3-phosphate cytidylyltransferase
	NCDO2118_0212	Hypothetical protein
	guaB	Inosine 5'-monophosphate dehydrogenase
GEI1	*enoA*	Enolase
	NCDO2118_0275	Hypothetical protein
	cspA1	Cold-shock protein
	cspC	Cold-shock protein
	cspB	Cold shock protein
	NCDO2118_0279	Hypothetical protein
	NCDO2118_0280	Hypothetical protein
	NCDO2118_0281	Hypothetical protein
	NCDO2118_0282	Stress response regulator
	corA	Mg2+ and Co2+ transporter
	ceo	N5-(Carboxyethyl)ornithine synthase
	NCDO2118_0285	Hypothetical protein
	NCDO2118_0286	Hypothetical protein
	epsR	Transcriptional regulator
	NCDO2118_0288	N-acetylglucosamine 2-epimerase
	NCDO2118_0289	Hypothetical protein
	NCDO2118_0290	Glycosyl transferase
	NCDO2118_0291	Endoglucanase
	NCDO2118_0292	Hypothetical protein
	NCDO2118_0293	Hypothetical protein

	NCDO2118_0294	Hypothetical protein
	NCDO2118_0295	Hypothetical protein
	NCDO2118_0296	Hypothetical protein
	NCDO2118_0297	ABC transporter, ATP-binding protein
	NCDO2118_0298	Transcriptional regulator
SI3	*enoA*	Enolase
	NCDO2118_0275	Hypothetical protein
	cspA1	Cold-shock protein
	cspC	Cold-shock protein
	cspB	Cold shock protein
	NCDO2118_0279	Hypothetical protein
	NCDO2118_0280	Hypothetical protein
	NCDO2118_0281	Hypothetical protein
	NCDO2118_0282	Stress response regulator
	corA	Mg2+ and Co2+ transporter
	ceo	N5-(Carboxyethyl)ornithine synthase
	NCDO2118_0285	Hypothetical protein
	NCDO2118_0286	Hypothetical protein
	epsR	Transcriptional regulator
	NCDO2118_0288	N-acetylglucosamine 2-epimerase
	NCDO2118_0289	Hypothetical protein
	NCDO2118_0290	Glycosyl transferase
	NCDO2118_0291	Endoglucanase
	NCDO2118_0292	Hypothetical protein
	NCDO2118_0293	Hypothetical protein
	NCDO2118_0294	Hypothetical protein
	NCDO2118_0295	Hypothetical protein
	NCDO2118_0296	Hypothetical protein
	NCDO2118_0297	ABC transporter, ATP-binding protein
	NCDO2118_0298	Transcriptional regulator
MI2	*phnD*	Phosphonate ABC transporter substrate-binding protein
	phnC	Phosphonates import ATP-binding protein
	phnB	Phosphonate ABC transporter permease
	phnE	Phosphonate ABC transporter permease
	NCDO2118_0359	2,3-cyclic-nucleotide 2-phosphodiesterase / 3-nucleotidase
	NCDO2118_0360	Hypothetical protein
	tpx	Thiol peroxidase
GEI2	*gltA*	Citrate synthase
	citB	Aconitate hydratase
	icd	Isocitrate dehydrogenase
	clpP	ATP-dependent Clp protease proteolytic subunit
	NCDO2118_0652	Hypothetical protein
	spxA	Arsenate reductase
	NCDO2118_0654	ABC transporter, permease protein

	pacL	Calcium-transporting ATPase
	brnQ	Branched-chain amino acid transport system carrier protein
	NCDO2118_0657	Acetyltransferase
	NCDO2118_0658	Hypothetical protein
	NCDO2118_0659	Cyclic-di-GMP-specific phosphodiesterase
	NCDO2118_0660	Hypothetical protein
	sacR	Sucrose operon repressor
	scrB	Sucrose-6-phosphate hydrolase
	scrA	PTS system, sucrose-specific IIABC component
	scrK	Fructokinase
	NCDO2118_0666	MFS transporter
	NCDO2118_0667	Transcriptional regulator
	rpiA1	Ribose-5-phosphate isomerase A
	NCDO2118_0669	Oxidoreductase
	NCDO2118_0671	Hypothetical protein
	NCDO2118_0672	Transcription antiterminator
	bglP1	PTS system, beta-glucoside-specific IIABC component
	NCDO2118_0674	6-phospho-beta-glucosidase
	NCDO2118_0675	Transcription antiterminator
	NCDO2118_0676	PTS system, beta-glucoside-specific IIABC component
	NCDO2118_0677	6-phospho-beta-glucosidase
	NCDO2118_0678	Transposase AB of ISLL6
	NCDO2118_0679	Hypothetical protein
	NCDO2118_0680	Hypothetical protein
	NCDO2118_0681	Hypothetical protein
	NCDO2118_0682	Peptidoglycan hydrolase
	NCDO2118_0683	Hypothetical protein
	NCDO2118_0684	ChW repeat/cell adhesion domain-containing transglutaminase-like protein
	NCDO2118_0685	Site-specific recombinase, DNA invertase Pin related protein
	NCDO2118_0686	Hypothetical protein
	NCDO2118_0687	Hypothetical protein
	NCDO2118_0689	Hypothetical protein
	NCDO2118_0690	Hypothetical protein
	NCDO2118_0691	Hypothetical protein
	NCDO2118_0692	Hypothetical protein
	NCDO2118_0693	Hypothetical protein
	NCDO2118_0694	Hypothetical protein
	NCDO2118_0695	ATP-binding protein
	NCDO2118_0696	Hypothetical protein
	NCDO2118_0697	hypothetical protein
	NCDO2118_0698	Hypothetical protein
	NCDO2118_0699	Transcriptional regulator
	NCDO2118_0700	Hypothetical protein

	tnpA1	Transposase A
	NCDO2118_0703	DNase
	ygiJ	DNA topology modulation protein FlaR
	rnmV	Ribonuclease M5
	rsmA	Ribosomal RNA small subunit methyltransferase A
MI3	NCDO2118_0857	Hipotetical protein
	NCDO2118_0858	Hypothetical protein
	NCDO2118_0859	Hypothetical protein
	NCDO2118_0860	Hypothetical protein
	NCDO2118_0861	Hypothetical protein
	NCDO2118_0862	Transposase
	NCDO2118_0863	Hypothetical protein
MSI1	NCDO2118_0898	ABC-type cobalamin
	NCDO2118_0899	Alpha/beta hydrolase family protein
	feuC	ABC-type Fe3+-siderophore transport system
	feuB	ABC-type Fe3+-siderophore transport system permease component
	feuA	Fe3+-siderophore binding protein
	NCDO2118_0903	NADP oxidoreductase
	NCDO2118_0904	Hypothetical protein
	NCDO2118_0905	Glyoxalase
	ftsW2	Cell division protein
	NCDO2118_0907	Transcriptional regulator
MSI2	NCDO2118_1183	Hypothetical protein
	NCDO2118_1184	Hypothetical protein
	npkK	Two-component system sensor histidine kinase
	npkR	Two-component system response regulator
	nrpA	Non-ribosomal peptide sythetase
	pksA	Acyl-CoA transferase
	pksB	Biosynthesis protein
	pksC	Biosynthesis protein
	NCDO2118_1191	ABC transporter
	NCDO2118_1192	ABC transporter
	NCDO2118_1193	Hypothetical protein
	npkS	Hybrid nonribosomal peptide
	nrpB	Thioesterase
	nrpC	4'-phosphopantetheinyl transferase
	NCDO2118_1197	Hypothetical protein
	NCDO2118_1198	Macrolide biosynthetic protein
	NCDO2118_1199	FAD dependent oxidoreductase
	NCDO2118_1200	Hypothetical protein
	NCDO2118_1201	Hypothetical protein
	NCDO2118_1202	Hypothetical protein

	NCDO2118_1204	Hypothetical protein
	NCDO2118_1205	Hypothetical protein
	NCDO2118_1206	Hypothetical protein
	kdpD	Osmosensitive K+ channel sensor histidine kinase
	kdpE	Osmosensitive K+ channel response regulator
	kdpA	Potassium-transporting ATPase A chain
	kdpB	Potassium-transporting ATPase B chain
	kdpC	Potassium-transporting ATPase C chain
	NCDO2118_1212	Transcriptional regulator
	NCDO2118_1213	Hypothetical protein
	rnr	Ribonuclease R
SI4	NCDO2118_1276	Hypothetical protein
	NCDO2118_1277	Hypothetical protein
	NCDO2118_1278	Na(+)/H(+) antiporter
	NCDO2118_1279	Hypothetical protein
	amyL	Alpha-amylase
	lctO	L-lactate oxidase
GEI3	*srtB*	Sortase
	NCDO2118_1360	Hypothetical protein
	NCDO2118_1361	Hypothetical protein
	NCDO2118_1362	Hypothetical protein
	NCDO2118_1363	Hypothetical protein
	NCDO2118_1364	Hypothetical protein
	NCDO2118_1365	Hypothetical protein
GEI4	*gpo*	Glutathione peroxidase
	acmC	N-acetyl-muramidase
	NCDO2118_1417	Calcium-transporting ATPase
	NCDO2118_1418	Hypothetical protein
	NCDO2118_1419	Hypothetical protein
	NCDO2118_1420	Hypothetical protein
	NCDO2118_1421	Hypothetical protein
	NCDO2118_1422	Hypothetical protein
	NCDO2118_1423	Hypothetical protein
	NCDO2118_1424	Universal stress protein
	NCDO2118_1425	Hypothetical protein
	arsC2	Arsenate reductase
	NCDO2118_1427	Hypothetical protein
	NCDO2118_1428	Hypothetical protein
	NCDO2118_1429	Phage protein
	NCDO2118_1430	Universal stress protein
	NCDO2118_1431	DNA-binding helix-turn-helix protein
	NCDO2118_1432	Hypothetical protein
	NCDO2118_1433	Hypothetical protein

	NCDO2118_1434	Hypothetical protein
	NCDO2118_1435	Lyzozyme M1 (1,4-beta-N-acetylmuramidase)
	NCDO2118_1436	Holin
	NCDO2118_1437	Hypothetical protein
MI4	*nagB*	Glucosamine-6-phosphate deaminase
	NCDO2118_1595	Hypothetical protein
	NCDO2118_1596	Transcriptional antiterminator
	fptA	Fructose-specific PTS system, component IIA
	fptB	Mannitol/fructose-specific PTS system, component IIB
	fptC	Mannitol/fructose-specific PTS system, component IIC
	amn	Alpha-mannosidase/fructosidase
	kdgA1	2-Keto-3-deoxy-6-phosphogluconate aldolase
	kdgK1	2-Dehydro-3-deoxygluconokinase
	NCDO2118_1603	Aldose-1-epimerase
	uxaA	Altronate dehydratase
	uxaB	Altronate oxidoreductase
	uxaC1	Uronate isomerase
	uxaT	Galacturonate transporter
	uxaR	Hexuronate operon repressor
	NCDO2118_1609	Hypothetical protein
	NCDO2118_1610	Hypothetical protein
	NCDO2118_1611	Hypothetical protein
	NCDO2118_1612	Hypothetical protein
	NCDO2118_1614	DNA segregation ATPase
	NCDO2118_1615	Hypothetical protein
	repB	Plasmid replication protein
	NCDO2118_1617	Excisionase
	queA	S-adenosylmethionine:tRNA ribosyltransferase-isomerase
	yqbH	Transcriptional regulator
	NCDO2118_1621	Hypothetical protein
	NCDO2118_1622	Hypothetical protein
	NCDO2118_1623	Hypothetical protein
	NCDO2118_1624	Hypothetical protein
	NCDO2118_1625	Hypothetical protein
	NCDO2118_1626	Hypothetical protein
	NCDO2118_1627	Hypothetical protein
	NCDO2118_1628	Hypothetical protein
	NCDO2118_1629	Hypothetical Protein
	obg	GTPase
GEI5	NCDO2118_1641	Hypothetical protein
	NCDO2118_1643	Hypothetical protein
	NCDO2118_1644	Universal stress protein
	NCDO2118_1645	Hypothetical protein
	arsC3	Arsenate reductase

	NCDO2118_1647	Bacteriophage lysin
	NCDO2118_1651	Hypothetical protein
	NCDO2118_1652	Phage tail protein
	NCDO2118_1653	Phage tail component
	NCDO2118_1654	Head-tail joining protein
	NCDO2118_1655	Hypothetical protein
	NCDO2118_1656	Capsid protein
	NCDO2118_1657	Phage ATP-dependent endopeptidase
	NCDO2118_1659	Head-tail joining protein
	NCDO2118_1661	Phage terminase small subunit
	NCDO2118_1662	Endonuclease
	NCDO2118_1663	Terminase
	NCDO2118_1664	Hypothetical protein
	NCDO2118_1665	Hypothetical protein
	NCDO2118_1666	Hypothetical protein
	NCDO2118_1667	Hypothetical protein
	NCDO2118_1669	Hypothetical protein
	NCDO2118_1670	Hypothetical protein
	NCDO2118_1671	Hypothetical protein
	NCDO2118_1672	Hypothetical protein
	NCDO2118_1675	Hypothetical protein
	NCDO2118_1678	Hypothetical protein
	NCDO2118_1679	Hypothetical protein
	NCDO2118_1680	Hypothetical protein
	NCDO2118_1681	Hypothetical protein
	NCDO2118_1682	Hypothetical protein
	NCDO2118_1683	Hypothetical protein
	NCDO2118_1684	Replisome organizer
	NCDO2118_1685	Hypothetical protein
	NCDO2118_1686	Hypothetical protein
	NCDO2118_1687	Hypothetical protein
	NCDO2118_1688	Hypothetical protein
	NCDO2118_1689	Hypothetical protein
	NCDO2118_1690	Hypothetical protein
	NCDO2118_1691	BRO-like protein
	NCDO2118_1692	DNA binding protein
	NCDO2118_1693	Hypothetical protein
	NCDO2118_1694	Hypothetical protein
	NCDO2118_1695	Hypothetical protein
	NCDO2118_1696	hypothetical protein
	NCDO2118_1697	Phage-related protein
	NCDO2118_1698	Phage integrase
MI5	*kdgA2*	2-dehydro-3-deoxyphosphogluconate aldolase / 4-hydroxy-2-oxoglutarate aldolase

	kdgK2	2-dehydro-3-deoxygluconokinase
	uxaC2	Uronate isomerase
	uxuT	Na-galactoside symporter
	uxuA	Mannonate dehydratase
	NCDO2118_1733	Hypothetical protein
	uxuB	Fructuronate reductase
	kdgR	Transcriptional regulator
	yqiA	Multidrug transporter
	rbsB	Ribose ABC transporter substrate binding protein
	rbsC	Ribose ABC transporter permease protein
	rbsA	Ribose import ATP-binding protein
	rbsD	D-ribose pyranase
	rbsK	Ribokinase
	rbsR	Ribose operon repressor
MSI3	NCDO2118_1786	Hypothetical protein
	NCDO2118_1787	Hypothetical Protein
	NCDO2118_1788	Hypothetical protein
	yrfA	Transcription regulator
	yrfB	NADH-dependent oxidoreductase
	NCDO2118_1793	Hypothetical protein
	NCDO2118_1794	Hypothetical protein
	NCDO2118_1795	Transcriptional regulator
	NCDO2118_1796	Hypothetical protein
	NCDO2118_1797	ABC transporter, permease protein
	arcC	Carbamate kinase
	aguA2	Agmatine deiminase
	yrfD	Amino acid antiporter
	ptcA2	Putrescine carbamoyltransferase
	llrH	Two-component system regulator
GEI6	NCDO2118_1786	Hypothetical protein
	NCDO2118_1787	Hypothetical Protein
	NCDO2118_1788	Hypothetical protein
	yrfA	Transcription regulator
	yrfB	NADH-dependent oxidoreductase
	NCDO2118_1793	Hypothetical protein
	NCDO2118_1794	Hypothetical protein
	NCDO2118_1795	Transcriptional regulator
	NCDO2118_1796	Hypothetical protein
	NCDO2118_1797	ABC transporter, permease protein
	arcC	Carbamate kinase
	aguA2	Agmatine deiminase
	yrfD	Amino acid antiporter
	ptcA2	Putrescine carbamoyltransferase
	llrH	Two-component system regulator

GEI7	*ysiB*	Permease
	NCDO2118_1923	Phage protein
	NCDO2118_1924	Phage associated protein
	NCDO2118_1925	Phage associated protein
	NCDO2118_1926	Hypothetical Protein
	NCDO2118_1927	Hypothetical Protein
	NCDO2118_1928	Hypothetical Protein
	NCDO2118_1929	Hypothetical protein
	NCDO2118_1930	Hypothetical Protein
	NCDO2118_1931	Hypothetical protein
	NCDO2118_1932	Hypothetical protein
	NCDO2118_1933	Hypothetical Protein
	NCDO2118_1934	Hypothetical protein
	NCDO2118_1935	Hypothetical Protein
	NCDO2118_1936	Hypothetical protein
	NCDO2118_1937	Integrase
	NCDO2118_1938	Hypothetical protein
	NCDO2118_1939	Hypothetical protein
GEI8	*ysiB*	Permease
	NCDO2118_1923	Phage protein
	NCDO2118_1924	Phage associated protein
	NCDO2118_1925	Phage associated protein
	NCDO2118_1926	Hypothetical Protein
	NCDO2118_1927	Hypothetical Protein
	NCDO2118_1928	Hypothetical Protein
	NCDO2118_1929	Hypothetical protein
	NCDO2118_1930	Hypothetical Protein
	NCDO2118_1931	Hypothetical protein
	NCDO2118_1932	Hypothetical protein
	NCDO2118_1933	Hypothetical Protein
	NCDO2118_1934	Hypothetical protein
	NCDO2118_1935	Hypothetical Protein
	NCDO2118_1936	Hypothetical protein
	NCDO2118_1937	Integrase
	NCDO2118_1938	Hypothetical protein
	NCDO2118_1939	Hypothetical protein
GEI9	NCDO2118_2304	Bacteriocin
	NCDO2118_2305	Hypothetical protein
	NCDO2118_2306	Hypothetical protein
	NCDO2118_2307	Hypothetical protein
	NCDO2118_2308	Hypothetical protein
	NCDO2118_2309	Hypothetical protein
	NCDO2118_2310	Hypothetical protein
	NCDO2118_2311	Hypothetical protein

	NCDO2118_2312	Hypothetical protein
	NCDO2118_2313	Hypothetical protein
	NCDO2118_2314	Hypothetical protein
	NCDO2118_2315	Hypothetical protein
	NCDO2118_2316	Hypothetical protein
	NCDO2118_2317	Hypothetical protein
	NCDO2118_2319	DNA primase
	NCDO2118_2320	Hypothetical protein
	NCDO2118_2321	Hypothetical protein
	NCDO2118_2322	Hypothetical protein
	NCDO2118_2323	Hypothetical protein
	NCDO2118_2324	Hypothetical protein
	glnA	Glutamine synthetase
	glnR	Glutamine synthetase repressor
	ptpL	Protein-tyrosine phosphatase
	NCDO2118_2328	ABC transporter, ATP-binding protein
	NCDO2118_2329	ABC transporter permease
	NCDO2118_2330	Hypothetical protein
	NCDO2118_2331	Transcriptional regulator

VI.2.1.5 Table S 4 Genes coding for bacteriocins, muramidases and macrolides.
Bacteriocin regions were predicted using BAGEL.

	Locus tag	Start	Stop	Gene	Product
Macrolide region prediction	**NCDO2118_1196**	1273306	1273974	*nrpC*	4'-phosphopantetheinyl transferase
	NCDO2118_1197	1273988	1274179	NCDO2118_1197	Hypothetical protein
	NCDO2118_1198	1274493	1275437	NCDO2118_1198	Macrolide biosynthetic protein
	NCDO2118_1199	1275501	1277006	NCDO2118_1199	FAD dependent oxidoreductase
	NCDO2118_1200	1277106	1277756	NCDO2118_1200	Hypothetical protein
Bacteriocin class I region prediction	**NCDO2118_1253**	1345667	1346536	*tnp*	Transposase B
	NCDO2118_1254	1346599	1347198	NCDO2118_1254	Adenylate cyclase
	NCDO2118_1255	1347239	1348024	NCDO2118_1255	ABC transporter
	NCDO2118_1256	1348027	1348845	NCDO2118_1256	ABC transporter
	NCDO2118_1257	1348838	1349836	NCDO2118_1257	ABC transporter
	NCDO2118_1258	1349837	1350556	NCDO2118_1258	Hypothetical protein
	NCDO2118_1259	1350754	1351383	NCDO2118_1259	Hypothetical protein
	NCDO2118_1260	1351399	1352835	NCDO2118_1260	AMP-dependent synthetase
	NCDO2118_1261	1352842	1353078	NCDO2118_1261	Acyl carrier protein
	NCDO2118_1262	1353109	1354176	NCDO2118_1262	Hypothetical protein
	NCDO2118_1263	1354816	1355460	*nisG*	Nisin transport protein
	NCDO2118_1264	1355447	1356175	*nisE*	Nisin transport protein

	NCDO2118_1265	1356177	1356854	*nisF*	Nisin transport protein
	NCDO2118_1266	1356953	1358296	*nisK*	Nisin biosynthesis sensor protein
	NCDO2118_1267	1358289	1358978	*nisR*	Nisin biosynthesis regulatory protein
	NCDO2118_1269	1361109	1361846	*nisI*	Nisin immunity protein
	NCDO2118_1270	1361843	1362604	*nisC*	Nisin biosynthesis protein
	NCDO2118_1271	1362673	1363779	*nisB*	Nisin biosynthesis protein
	NCDO2118_1272	1363903	1364055	*nisZ*	Lantibiotic nisin
	NCDO2118_1273	1365571	1366308	*glpF2*	Glycerol uptake facilitator
	NCDO2118_1274	1366361	1368190	*glpD*	Alpha-glycerophosphate oxidase
Muramidase region prediction	**NCDO2118_1433**	1548422	1548703	NCDO2118_1433	Hypothetical protein
	NCDO2118_1434	1548834	1549013	NCDO2118_1434	Hypothetical protein
	NCDO2118_1435	1549098	1550393	NCDO2118_1435	Lyzozyme M1 (1,4-beta-N-acetylmuramidase)
	NCDO2118_1436	1550390	1550614	NCDO2118_1436	Holin
	NCDO2118_1437	1550604	1550906	NCDO2118_1437	Hypothetical protein
Bacteriocin class II region prediction	**NCDO2118_1758**	1883915	1884700	NCDO2118_1758	Hydrolase
	NCDO2118_1759	1884746	1885138	NCDO2118_1759	Hypothetical protein
	NCDO2118_1760	1885273	1886253	*pta*	Phosphate acetyltransferase
	NCDO2118_1761	1886469	1887089	*udk*	Uridine kinase
	NCDO2118_1762	1887387	1888367	*yrbA*	Oxidoreductase
	NCDO2118_1763	1888634	1889233	*yrbB*	Transglycosylase
	NCDO2118_1764	1889891	1890850	NCDO2118_1764	Hypothetical protein
	NCDO2118_1765	1890875	1892413	NCDO2118_1765	Chloride channel protein
	NCDO2118_1766	1892582	1892815	NCDO2118_1766	Hypothetical protein
	NCDO2118_1767	1893188	1893502	NCDO2118_1767	Hypothetical protein
	NCDO2118_1768	1893520	1893849	NCDO2118_1768	Hypothetical protein
	NCDO2118_1769	1894024	1894857	*yrbI*	Transcriptional regulator
	NCDO2118_1770	1894893	1895651	*ubiE*	Demethylmenaquinone methyltransferase
	NCDO2118_1771	1895731	1898436	*pip*	Phage infection protein
	NCDO2118_1772	1898696	1900129	NCDO2118_1772	Phospho-beta-glucosidase
	NCDO2118_1773	1900636	1902378	*dxsB*	1-deoxyxylulose-5-phosphate synthase
	NCDO2118_1774	1902526	1903896	*kinF*	Histidine kinase
Bacteriocin class III region prediction	**NCDO2118_2252**	2402923	2403414	*comX*	Competence-specific transcriptional regulator
	NCDO2118_2253	2403671	2405401	*ezrA*	Septation ring formation regulator
	NCDO2118_2254	2405606	2406634	*tsf*	Elongation factor
	NCDO2118_2255	2406757	2407524	*rpsB*	30S ribosomal protein S2
	NCDO2118_2256	2407883	2408908	NCDO2118_2256	Hypothetical protein
	NCDO2118_2257	2409140	2411851	*adhE*	Alcohol dehydrogenase

	NCDO2118_2258	2412189	2412686	NCDO2118_2258	Hypothetical protein
	NCDO2118_2259	2412673	2412882	NCDO2118_2259	Hypothetical protein
	NCDO2118_2260	2412879	2413706	*ywdC*	Fe-S oxidoreductase
	NCDO2118_2261	2413699	2415822	*ywdD*	Fe-S oxidoreductase
	NCDO2118_2262	2415925	2416782	NCDO2118_2262	Transcriptional regulator
	NCDO2118_2263	2416893	2418032	NCDO2118_2263	hypothetical protein
	NCDO2118_2264	2418029	2419348	NCDO2118_2264	Radical SAM protein
	NCDO2118_2265	2419851	2420879	NCDO2118_2265	Hypothetical protein
	NCDO2118_2266	2420866	2421363	*coaD*	Phosphopantetheine adenylyltransferase
	NCDO2118_2267	2421411	2421950	NCDO2118_2267	Hypothetical protein

VI.2.1.6 Table S 5 Exclusive, expressed and secreted proteins of *L. lactis* NCDO 2118. The exclusive, secreted and expressed proteins were predicted using the software OrthoMCL, SurfG+ and proteomics analyses, respectively. F = forward, R = reverse.

Locus_tag	Gene	Start	Stop	Direction	Proteins
NCDO2118_0139	*epsK*	144762	145652	F	exopolysaccharide biosynthesis protein
NCDO2118_0140	*epsL*	145677	146600	R	LytR family transcriptional regulator
NCDO2118_0212	-	214606	215988	R	family 25 glycosyl hydrolase
NCDO2118_0683	-	697697	698158	F	hypothetical protein
NCDO2118_1420	-	1537567	1538422	R	hypothetical protein

VI.2.2 DISCUSSÃO

As análises comparativas destacaram a grande semelhança entre *L. lactis* NCDO 2118 e *L. lactis* KF147, além das ilhas compartilhadas entre elas. O fato destas duas linhagens serem isoladas de leguminosas nos leva a crer que estas ilhas possam estar relacionadas ao estilo de vida destas linhagens.

Mesmo tendo observado a resistência de NCDO 2118 *in vitro*, as análises *in silico* não predisseram nenhum gene relacionado à resistência e regiões adquiridas por transferência horizontal ou mesmo a presença de ilhas de resistência à antibióticos. Entretanto, na simulação do ambiente gástrico, apenas metade da porcentagem de *L. lactis* NCDO 2118 sobreviveu, enquanto que os sais biliares inibiram quase todo o crescimento da linhagem. A abordagem de proteômica veio acrescentar possíveis dados que possam estar ligados diretamente à capacidade anti-inflamatória e imunomodulatória de *L. lactis* NCDO 2118, entretanto, uma análise aprofundada se faz necessária para chegarmos a uma conclusão. Assim sendo, os resultados mostraram possibilidades que ainda precisam ser estudadas de forma integrada, caminhando para outras abordagens ômicas, como a transcriptômica, por exemplo.

VII DISCUSSÃO GERAL

Em ambos trabalhos (capítulos I e II) observamos o quanto é esclarecedor e importante a integração de técnicas no entendimento de um organismo probiótico. As abordagens *in silico* nos permite alcançar, de forma ágil, vários dados que possivelmente podem caracterizar este organismo, além de direcionar experimentos *in vitro*, nos quais estes podem confirmar os dados preditos.

Vimos no primeiro capítulo a utilização de experimentos *in vitro* confirmando uma característica antagonista predita em uma ferramenta online, o BAGEL. Sendo este genoma fechado futuramente, outras informações importantes poderão ser descobertas, sejam elas relacionadas ao seu potencial probiótico ou não. Assim, foram identificadas na linhagem *L. rhamnosus* L156.4, proteínas muito similares à bacteriocinas que já foram descritas em outras linhagens de *Lactobacillus,* idenficadas como probióticas: *L. rhamnosus* HN001 (Gill et al., 2000) e *L. rhamnosus* GG (ATCC 53103) (Kankainen et al., 2009). *L. rhamnosus* L156.4 demonstra ampla atividade antagonista e sendo esta atividade uma das características de um organismo probiótico, os resultados obtidos nos levam a aprofundar as análises relacionadas à probiose da linhagem *L. rhamnosus* L156.4.

No capítulo II, as análises comparativas entre as espécies de *Lactococcus* acabam destacando a grande semelhança entre as linhagens *L. lactis* NCDO 2118 e *L. lactis* KF147. O fato de possuírem um isolado semelhante, nos leva à hipótese de que seu estilo de vida pode refletir nos resultados encontrados. Focando na nossa linhagem de interesse, observamos que alguns dos dados *in silico* não foram comprovados nos experimentos *in vitro*. Apesar de *L. lactis* NCDO 2118 apresentar resistência a três dos 9 antibióticos testados, não foram observadas, *in silico,* alterações relacionadas ao seu conteúdo G+C e ao uso de códon. Ademais, a ausência de atividade antagonista dentre as bactérias patogênicas testadas, apresentou-se contradizendo as bacteriocinas preditas em *L. lactis* NCDO 2118. Esses resultados, além dos próprios experimentos mimetizando a ação do suco gástrico e sais biliares, precisariam ser realizados de forma mais aprofundada, testando a bactéria em condições diferentes, no sentido de esclarecer essas análises parciais. O mesmo se faz necessário com relação às proteínas secretadas, expressas e exclusivas de *L. lactis* NCDO 2118, das 5 preditas, 3 são proteínas hipotéticas e mesmo tendo encontrado dois genes *eps* já descritos na literatura como componentes de um *cluster eps* em uma linhagem probiótica de *Lactobacillus* (Denou et al.,

2008), novas análises e mesmo outras abordagens ômicas poderiam auxiliar na elucidação destes achados.

VIII CONCLUSÕES GERAIS

Por meio da genômica comparativa podemos observar características compartilhadas entre os genomas a serem analisados, que podem inclusive destacar regiões de alta ou baixa similaridade.

Nas análises com *L. rhamnosus* L156.4, apesar da linhagem apresentar várias proteínas relacionadas à bacteriocinas e bacteriocinas muito similares à outras linhagens de *L. rhamnosus,* conhecidas como probióticas, foi visto nas comparações, que a similaridade destas regiões aos outros genomas analisados é significativa, porém não são idênticas. Existem regiões de deleção e inserção, tanto nas comparações do Mauve, quanto do ACT.

Analisando *in vitro* a atividade antagonista de *L. rhamnosus* L156.4*,* observou-se um amplo espectro de exclusão com relação aos organismos utilizados, característica esta representativa pelas células e também pelo sobrenadante, que só não inibiu bactérias gram-negativas. Este mesmo sobrenadante passou por uma avaliação físico-química, a qual demonstrou que a atividade antagonista da linhagem em estudo se manteve significativa, demonstrando o potencial agressivo desta linhagem. Por fazer parte de um grupo de espécies já muito descritas e conhecidas como probióticas, um estudo aprofundado nessa área poderá elucidar novas características desta linhagem.

Nas análises com *L. lactis* NCDO 2118, os resultados comparativos acabaram por destacar as semelhanças desta linhagem com *L. lactis* KF147, isolados de ervilha congelada e feijão mungo, respectivamente. O fato de terem isolados semelhantes nos leva a correlacionar os resultados obtidos, principalmente em relação à ilha mista (MSI2) predita apenas para estas duas linhagens, representando genes relacionados a características metabólicas e simbióticas. A respeito das características probióticas, *L. lactis* NCDO 2118 foi capaz de crescer em meio ácido e apresentou adesão moderada, além de terem sido identificadas bacteriocinas de cada uma das três classes e proteínas relacionadas. A linhagem em estudo não mostrou crescimento no meio biliar e também não foi vista atividade antagonista *in vitro* contra as bactérias utilizadas. Por isso, faz-se necessário a utilização de novas abordagens para avaliação aos sais biliares e novos organismos para observação da atividade antagonista. O mesmo se aplica para as proteínas secretadas encontradas. Das cinco secretadas, expressas e exclusivas de *L. lactis* NCDO 2118, três são proteínas hipotéticas, das quais é necessário a busca por maiores informações e, as outras duas, fazem parte de um operon *eps* encontrado em uma linhagem probiótica de *Lactobacillus*.

IX PERSPECTIVAS

Para ***L. rhamnosus*** **L156.4:**

O genoma já depositado poderá ser analisado, comparando com outras espécies e linhagens de *Lactobacillus,* onde buscaríamos identificar possíveis genes e ou proteínas relacionadas às características probióticas. Destes resultados encontrados, os testes *in vitro* nos auxiliariam na confirmação e complementação destas análises.

Para *L. lactis* NCDO 2118:

Microbiota:

Utilizando um modelo de colite, buscaremos administrar a bactéria aos camundongos para posterior análise de metagenômica para análise da microbiota intestinal.

Metatranscriptoma:

Administrando a bactéria aos animais (camundongos *germ free*) induzidos à colite, buscaremos realizar o RNA-Seq do segmento intestinal, antes e após a passagem da bactéria probiótica pelo trato gastro-intestinal para posterior avaliação da expressão dos genes diferencialmente expressos.

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XI APÊNDICE

XI.1 *Curriculum vitae*

Dados pessoais

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Formação acadêmica

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Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Brasil
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Academia de Ciência e Tecnologia de São José do Rio Preto, AC&T, Brasil
Título: Comparação entre os diferentes fixadores e descalcificadores utilizados em biópsias de medula óssea.
Orientador: Renata Margarida Etchebehere

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Universidade de Uberaba, UNIUBE, Uberaba, Brasil
Título: Administrando um laboratóro de médio porte segundo a nova RDC (1986-2006)
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Áreas de atuação

1. Bioinformática
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Artigos completos publicados em periódicos

1. DE SAROM, ALISSA; KUMAR JAISWAL, ARUN; TIWARI, SANDEEP; **DE CASTRO OLIVEIRA, LETÍCIA**; BARH, DEBMALYA; AZEVEDO, VASCO; JOSE OLIVEIRA, CARLO; DE CASTRO SOARES, SIOMAR. Putative vaccine candidates and drug targets identified by reverse vaccinology and subtractive genomics approaches to control *Haemophilus ducreyi*, the causative agent of chancroid. Journal of the Royal Society Interface, v. 15, p. 20180032, **2018**.

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4. JUNIOR, A. F.; **Oliveira, L.C**.; ABURJAILE, F. F.; BENEVIDES, L. J. ; JAMAL, SYED BABAR; TIWARI, S.; SILVA, ARTUR; FIGUEIREDO, H. C. P.; GHOSH, P.; PORTELA, R. W.; WATTAM, A. R.; AZEVEDO, VASCO A. C. . Insight of Genus Corynebacterium: Ascertaining the Role of Pathogenic and Non-pathogenic Species. Frontiers in Microbiology, v. 8, p. 1937, **2017**.

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6. TIWARI, SANDEEP; JAMAL, SYED BABAR; **OLIVEIRA, LETICIA CASTRO**; CLERMONT, DOMINIQUE; BIZET, CHANTAL; MARIANO, DIEGO; DE CARVALHO, PAULO VINICIUS SANCHES DALTRO; SOUZA, FLAVIA; PEREIRA, FELIPE LUIZ; DE CASTRO SOARES, SIOMAR; GUIMARÃES, LUIS C.; DORELLA, FERNANDA; CARVALHO, ALEX; LEAL, CARLOS; BARH, DEBMALYA; FIGUEIREDO, HENRIQUE; HASSAN, SYED SHAH; AZEVEDO, VASCO; SILVA, ARTUR.
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8. DE CASTRO SOARES, SIOMAR; **DE CASTRO OLIVEIRA, LETÍCIA**; JAISWAL, A. K.; AZEVEDO, VASCO. Genomic Islands: an overview of current software tools and future improvements. Journal of Integrative Bioinformatics, v. 13, p. 301, **2016**.

9. **OLIVEIRA, L. C.**; SARAIVA, T. D. L.; SOARES, S. C.; RAMOS, R. T. J.; SA, P. H. C. G.; CARNEIRO, A. R.; MIRANDA, F.; FREIRE, M.; RENAN, W.; JUNIOR, A. F. O.; SANTOS, A. R.; PINTO, A. C.; SOUZA, B. M.; CASTRO, C. P.; DINIZ, C. A. A.; ROCHA, C. S.; MARIANO, D. C. B.; DE AGUIAR, E. L.; FOLADOR, E. L.; BARBOSA, E. G. V.; ABURJAILE, F. F.; GONCALVES, L. A.; GUIMARAES, L. C.; AZEVEDO, M.; AGRESTI, P. C. M. *et al.*

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10. PEREIRA, U.P.; SOARES, S.C.; BLOM, J.; LEAL, C.A.G.; RAMOS, R.T.J.; GUIMARÃES, L.C.; **Oliveira, L.C.**; ALMEIDA, S.S.; HASSAN, S.S.; SANTOS, A.R.; MIYOSHI, A.; SILVA, A.; TAUCH, A.; BARH, D.; AZEVEDO, V.; FIGUEIREDO, H.C.P.
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1. SILVA, W. M.; SOUZA, C. S. **L Castro-Oliveira** ; SOARES, S. C. SOUZA, G. F. M. H.; TAVARES, G. C.; FOLADOR, EDSON L.; PEREIRA, F. L.; FIGUEIREDO, H.C.P.; AZEVEDO, V. A. C. . Comparative proteomic analysis of four biotechnological strains Lactococcus lactis through label-free quantitative proteomics. Microbial Biotechnology (Online), **2018**.

Artigos submetidos e em revisão

1. **Journal:** Plos Computational Biology
   Article type: Review
   Title: Omics of probiotic bacteria: what features should I search for? Probiotic bacteria - omics approach
   Authors: Letícia de Castro Oliveira, Mariana Oliveira, Alissa de Sarom, Leandro de Jesus Benevides, Carlo José Freire Oliveira, Henrique Cesar Figueiredo; Vasco Ariston de Carvalho Azevedo and Siomar de Castro Soares

2. **Journal:** Heliyon
   Article type: Research article
   Title: The Pan-genome of the zoonotic neglected pathogen *Bartonella henselae* reveals two groups with different patterns of adaptation to hosts
   Authors: Leandro Gomes Alves, Arun Jaiswal, Sandeep tiwari, Leandro Benevides, Letícia Oliveira, Felipe Zen, Vasco Azevedo and Siomar Soares

3. **Journal:** Plos One
   Article type: Original Research
   Title: A Reverse Vaccinology and Subtractive Genomics Approach for the Common Therapeutics Identification against *Mycobacterium leprae* and *Mycobcaterium lepromatosis*
   Authors: Arun Kumar Jaiswal; Sandeep tiwari; Syed Babar Jamal; Letícia de Castro Oliveira; Helioswilton Sales Campos; Leonardo Eurípedes Andrade Silva; Carlo Jose Freire Oliveira; Vasco Azevedo; Siomar C. Soares; Virmondes Rodrigues Junior; Marcos Vinicius Silva

4. **Journal:** Nucleic Acids Research
   Article type: Original Research
   Title: In silico evaluation of safety aspects in the use of *Faecalibacterium* strains by genome plasticity and antibiotic resistance approaches.
   Authors: Benevides, Leandro; Oliveira, Letícia; Figueiredo, Henrique; Langella, Philippe; Azevedo, Vasco; Chatel, Jean-Marc; Soares, Siomar

Capítulos de livros publicados

1. SOARES, SC; RAMOS, R.T.J.; SILVA, W. M.; **Castro-Oliveira, L.**; AMORIM, LG; Hirata, R; Mattos-Guaraldi, A. L.; MIYOSHI, A; SILVA, A.; AZEVEDO, V
   Corynebacterium pathogenic species in next-generation genomic era: the use of EDGAR and PIPS software and the importance of pathogenicity islands identification in pan-genomic analyses of pathogenic species In: Microbial pathogens and strategies for combating them: science, technology and education..1ª ed.Badajoz: Formatex Research Center, 2013, v.3, p. 1584-1599. ISBN: 9788494213410