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Doutorado

ANÁLISE GENÔMICA E CARACTERIZAÇÃO FUNCIONAL DE *Limosilactobacillus fermentum* JAC 231 COMO CANDIDATO A PROBIÓTICO

CARMEM DUARTE LIMA CAMPOS

São Luís
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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Maranhão - UFMA, como requisito para obtenção do título de Doutora em Ciências da Saúde.

Orientador: Prof. Dr. Valério Monteiro-Neto

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BANCA EXAMINADORA

Prof. Dr. Valério Monteiro-Neto (Orientador)
UFMA

1º Prof. Dr. Afonso Gomes Abreu Júnior
UFMA

2º Profa. Dra. Cristina de Andrade Monteiro
IFMA

3º Prof. Dr. Rafael Cardoso Carvalho
UFMA

3º Profa. Dra. Mayara Cristina Pinto da Silva
UFMA

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“Quando acontecem as coisas mais importantes da nossa vida, muitas vezes, naquele momento, nem nos damos conta do que está acontecendo. Uma pessoa nem sempre está em condições de constatar: ‘Opa, estou amadurecendo!’ Muitas vezes é só quando olhamos para trás que nos damos conta do que aconteceu e, então, reconhecemos aquilo que as pessoas chamam de amadurecimento”

C. S. Lewis

Dedico

A Deus, a mim, a minha família, ao meu orientador, a todos que contribuíram e aqueles que serão beneficiados por esta pesquisa.

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“Nenhuma alta sabedoria pode ser atingida sem uma dose de sacrifício”.
- C. S. Lewis

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E, por fim, dedico este trabalho àqueles que serão positivamente impactados por esta pesquisa, na esperança de que os conhecimentos aqui produzidos contribuam para o progresso científico e para o bem-estar coletivo.

RESUMO

O gênero *Lactobacillus* é constituído por bactérias Gram-positivas catalase-negativas, tolerantes a ácidos, não formadoras de esporos e fermentadores de carboidratos. Uma recente reclassificação desse gênero levou a uma mudança de nomenclatura, gerando novos 23 gêneros. A espécie *Lactobacillus fermentum* foi renomeada de *Limosilactobacillus fermentum.* Linhagens de *L. fermentum* ocupam diversos nichos, e em humanos é considerada um indicador de uma microbiota normal e saudável. Muitas linhagens têm sido investigadas por atuarem na manutenção da função da barreira epitelial, exclusão competitiva, atividade imunomoduladora e síntese de substancias antimicrobianas (ácido lático, H_2O_2 e bacteriocinas) com atividade ampla contra microrganismos patogênicos, motivando seu uso como probiótico. A caracterização da atividade próbiotica e as tecnologias de sequenciamento do genoma têm sido ferramentas importantes para obter informações sobre os aspectos metabólicos e de segurança de cada linhagem direcionando pesquisas para seu o uso biotecnológico e terapêutico. Este estudo teve por objetivo sequenciar o genoma completo do isolado vaginal, *L. fermentum* JAC 231, analisando aspectos gerais e de segurança da linhagem, investigando as bases genotípicas e fenotípicas para seu potencial probiótico, como tolerância a condições gastrointestinais (sais biliares, adesão a mucina, adesão a células eucarióticas e pH), presença de genes de resistência e virulência. Aqui expandimos a caracterização da atividade antimicrobiana e anti-adesão dessa linhagem, avaliando o seu efeito sobre *Klebisiella pneumoneae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella enteritidis* ATCC 13076, *Enterococcus faecalis* ATCC 29212 e *Escherichia coli* enteropatogênica O127:H6 E2348/69-EPEC. A partir da análise de bioinformática identificamos que o genoma continha 2.064.918 pb, com conteúdo de GC de 51,4%, apresentando 1.993 sequências codificadoras de proteínas relacionadas, principalmente, ao metabolismo de proteínas, carboidratos e aminoácidos. A análise comparativa revelou, que *L. fermentum* JAC 231, compartilha 2.204 agrupamentos ortólogos com a outras cinco linhagens probióticas de *L. fermentum* (MCC 2760, AGR 1485, ATCC 23271, CECT 5716, e IFO 3956). A investigação do genoma demonstrou que *L. fermentum* JAC 231, possui genes que codificam para uma bacteriocina (Enterolisina A), genes de tolerância ao estresse, síntese de vitaminas e sobrevivência às condições gastrointestinais. Além disso, observamos a ausência de genes que codificam para fatores de virulência e a presença de genes de resistência intrínseca à vancomicina. Fenotipicamente, *L. fermentum* JAC 231 apresentou sensibilidade a maioria dos antibióticos testados, exceto vancomicina, como já visualizado na análise do genoma. Capacidade de tolerar o pH ácido (com crescimento de 107,7% no pH 2 e 75,29% no pH 4), assim como aos sais biliares nas concentrações de 0,5% (87,34) e 1% (84,76%). Demonstrou, ainda, capacidade de adesão a mucina e às células HeLa, apresentando crescimento logaritmo de 5.9 e 5.7, respectivamente. Adicionalmente apresentou atividade antagonista no teste de *overlay* inibindo o crescimento de todas as bactérias utilizadas com zonas de inibição que variaram de 16.6 ± 2.08 a 28.3 ± 2.88 mm, além do potencial anti-adesão observado, principalmente, nos ensaios de deslocamento e exclusão. Dessa forma, nosso estudo confirma as características e o perfil de segurança de *L. fermentum* JAC 231 como uma linhagem probiótica. A análise do genoma demonstrou ser eficaz para avaliar as propriedades benéficas dessa linhagem, mostrando correlação com os dados obtidos *in vitro*. Além disso, verificamos atividade antagonista e anti-adesão exibida por *L. fermentum* JAC 231. Estudos posteriores são necessários para elucidar os mecanismos pelos quais essa linhagem promissora exerce seus efeitos antimicrobianos de amplo espectro.

Palavras-chave: *Limosilactobacillus fermentum*; caracterização genômica; probióticos, antimicrobiano.

ABSTRACT

The genus *Lactobacillus* is composed of Gram-positive, catalase-negative, acid-tolerant, non-spore-forming, carbohydrate-fermenting bacteria. A recent reclassification of this genus led to a change in nomenclature, generating 23 new genera. The species *Lactobacillus fermentum* was renamed *Limosilactobacillus fermentum*. *L. fermentum* strains of occupy various niches, and in humans it is considered an indicator of a normal and healthy microbiota. Many strains have been investigated for their role in maintaining the epithelial barrier function, competitive exclusion, immunomodulatory activity and synthesis of antimicrobial substances (lactic acid, H_2O_2 and bacteriocins) with broad activity against pathogenic microorganisms, motivating their use as probiotics. In this sense, the characterization of probiotic activity and genome sequencing technologies have been important tools to obtain information about the metabolic and safety aspects of each strain, directing research towards its biotechnological and therapeutic use. This study aimed to sequence the complete genome of the vaginal isolate, *L. fermentum* JAC 231, analyzing general and safety aspects of the strain, investigating the genotypic and phenotypic bases for its probiotic potential, such as tolerance to diverse gastrointestinal conditions (bile salts, adhesion to mucin, adhesion to eukaryotic cells and pH), presence of resistance and virulence genes. Here we expand the characterization of the antimicrobial and anti-adhesion activity of this strain, evaluating its effect on *Klebisiella pneumoneae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella enteritidis* ATCC 13076, *Enterococcus faecalis* ATCC 29212 and enteropathogenic *Escherichia coli* O127:H6 E2348/69-EPEC. From the bioinformatics analysis, we identified that the genome contained 2.064.918 bp, with a GC content of 51.4%, presenting 1.993 protein-coding sequences related mainly to the metabolism of proteins, carbohydrates and amino acids. Comparative analysis revealed that *L. fermentum* JAC 231 shares 2.204 orthologous clusters with the other five probiotic strains of *L. fermentum* (MCC 2760, AGR 1485, ATCC 23271, CECT 5716, e IFO 3956). Genome investigation demonstrated that *L. fermentum* JAC 231 has genes encoding a bacteriocin (Enterolysin A), stress tolerance genes, vitamin synthesis, and survival under gastrointestinal conditions. Furthermore, we observed the absence of genes for virulence factors and the presence of genes for intrinsic resistance to vancomycin. Phenotypically, L. *fermentum* JAC 231, was susceptibility to most of the antibiotics tested, except vancomycin, as already visualized in the genome analysis. It was able to tolerate acid pH (with growth of 107.7% at pH 2 and 75.29% at pH 4), as well as bile salts at concentrations of 0.5% (87.34) and 1% (84.76%). It also demonstrated adhesion capacity to mucin and HeLa cells, presenting logarithmic growth of 5.9 and 5.7, respectively. Additionally, it presented antagonistic activity in the overlay test, inhibiting the growth of all bacteria used, with inhibition zones ranging from 16.6 ± 2.08 to 28.3 ± 2.88 mm, in addition to the anti-adhesion potential observed, mainly, in the displacement and exclusion tests. In this way, our study confirms the characteristics and safety profile of *L. fermentum* JAC 231 as a probiotic strain. Genome analysis proved to be effective in evaluating the beneficial properties of this strain, showing correlation with the data obtained *in vitro*. In addition, we verified the antagonistic and anti-adhesive activity displayed by *L. fermentum* JAC 231. Further studies are needed to elucidate the mechanisms by which this promising strain exerts its broad-spectrum antimicrobial effects.

Keywords: *Limosilactobacillus fermentum*; genomic characterization; probiotics, antimicrobial

LISTA DE SIGLAS E ABREVIATURAS

BAL - Bactérias do ácido lático

AMPs - *Antimicrobials Peptides*

GRAS – *General recognised as safe*

EFSA - *European Food Safety Authority*

RiPPs - *Ribosomally produced and posttranslationally modified peptides* -

FDA – *Food and Drug Administration*

MRS - De Man, Rogosa e Sharpe

BHI- *Brain Heart Infusion*

RAST - *Rapid Annotation using Subsystem Technology*

NCBI - *National Center for Biotechnology Information*

PBS - *Phosphate buffered saline*

HeLa - Células epiteliais cervicais humanas

DMEM - Meio de Eagle modificado por Dulbecco

MH - Müller Hinton

SUMÁRIO

7. MATERIAL SUPLEMENTAR

1. INTRODUÇÃO

O gênero *Lactobacillus* é constituído por bactérias Gram-positivas, catalase-negativas, tolerantes a ácidos, não formadoras de esporos e fermentadores de carboidratos (Zheng et al., 2020). A recente reclassificação do gênero *Lactobacillus* levou a mudança de nomenclatura, gerando 23 gêneros com as respectivas espécies. A espécie *Lactobacillus fermentum* foi designada de *Limosilactobacillus fermentum* (Zheng et al., 2020). Linhagens de *L. fermentum* ocupam diversos nichos e podem ser recuperadas de uma variedade de locais, tais como alimentos fermentados, plantas, tratos gastrointestinal e geniturinário humano, entre outros (Martín et al., 2005; Liu et al., 2014; Ouarabi et al., 2019; Park et al., 2023).

Além do uso já conhecido na indústria alimentícia, onde é utilizada na melhoria da qualidade de alimentos (Gu et al., 2023b; Zhang et al., 2022a), muitas linhagens de *L. fermentum* têm sido estudadas por apresentarem impactos positivos à saúde do hospedeiro, motivando seu uso como probiótico (Hill et al., 2014). O termo probiótico é definido como "microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefício à saúde do hospedeiro" (Hill et al., 2014). Alguns desses benefícios, já identificados em linhagens de *L. fermentum,* estão relacionados à manutenção da função da barreira epitelial, exclusão competitiva (Hill et al., 2014), atividade imunomoduladora (Kim et al., 2022) e síntese de substancias antimicrobianas (ácido lático, H_2O_2 e bacteriocinas) com atividade ampla contra microrganismos patogênicos(Sabia et al., 2014; Mann et al., 2021; Abramov et al., 2023a)

Espécies relacionadas ao gênero *Lactobacillus*, como *L. fermentum*, fazem parte da microbiota humana. Na vagina são considerados como bioindicadores de uma microbiota normal e saudável, através da inibição da proliferação de patógenos oportunistas (Hossain, 2022; Ozen; Piloquet; Schaubeck, 2023). Na microbiota vaginal, *L. fermentum* é considerado um bioindicador de equilíbrio e proteção, atuando na prevenção de infecções por meio da produção de ácido lático, que reduz o pH local e cria um ambiente hostil à proliferação de patógenos oportunistas como *Gardnerella vaginalis* e *Candida albicans* (Santos et al., 2018; Choi et al., 2022).

Já no intestino, essa espécie contribui para a integridade da barreira epitelial, competindo por nutrientes e espaço com microrganismos nocivos, além de secretar bacteriocinas e outros compostos antimicrobianos que inibem o crescimento de bactérias como *Escherichia coli* e

Clostridium difficile (Da Costa Lima et al., 2025; Monteiro et al., 2019; Riaz; Kashif Nawaz; Hasnain, 2010). Essa ação sinérgica nas duas regiões reforça o papel dos *Lactobacillus* como agentes probióticos fundamentais na defesa contra infecções e na promoção da homeostase microbiana.

Existe um consenso que as características probióticas são especificas de linhagens de uma determinada espécie (Hill et al., 2014). Além disso, a validação de um microrganismo como um probiótico requer análises mais profundas que vão além de suas características benéficas, e envolvem a avaliação da segurança para consumo humano (tais como a origem, serem isentos de vetores gênicos capazes de transferir resistência a antibióticos e de fatores de virulência) e tolerância a condições gastrointestinais (pH ácido, enzimas, sais biliares, etc.)(Hill et al., 2014; De Melo Pereira et al., 2018; Koutsoumanis et al., 2024).

O uso de métodos para a caracterização de atividade probiótica, as tecnologias de sequenciamento e analise do genoma têm sido ferramentas importantes para obtenção de informações acerca dos aspectos metabólicos e de segurança de cada linhagem bem como direcionar pesquisas para seu o uso biotecnológico e terapêutico (Peng, Ed-Dra e Yue, 2023; Santos, dos *et al.*, 2021; Zhang, L. *et al.*, 2022).

Em um estudo anterior, nosso grupo realizou a identificação de novos candidatos a probióticos de amostras isoladas da cavidade vaginal de mulheres sem sintomas de infecção no trato genital. Um desses microrganismos, *L. fermentum* JAC 231, apresentou atividade antimicrobiana e antivirulência sobre espécies de *Candida albicans*, interferindo na produção de enzimas hidrolíticas, formação de hifas, coagregação, adesão a células epiteliais, biofilme em formação e biofilme pré-formado (Campos et al., 2022 – Dados não publicados)

Neste estudo, investigamos os aspectos probióticos de *L. fermentum* JAC 231. Para isso, realizamos a análise do genoma de *L. fermentum* JAC 231, bem como a caracterização da segurança e capacidade de sobreviver as condições gastrointestinais. Adicionalmente, avaliamos o efeito de *L. fermentum* JAC 231 contra bactérias patogênicas avaliando sua atividade antimicrobiana e interferência na capacidade de adesão às células eucarióticas.

2. REFERENCIAL TEÓRICO

2.1. Probióticos

Etimologicamente, o termo "Probiótico" vem da palavra grega "*pro-bios*" que significa "para a vida" e é definido como "microrganismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro" (Hill et al., 2014). Existe um consenso que um dos principais benefícios dos probióticos é promover uma microbiota intestinal saudável. No entanto, atualmente, suas vantagens se estendem podendo ser utilizados na prevenção e tratamento de vulvovaginites (Vivekanandan et al., 2024; Yefet et al., 2024), infecções orais (Kamal et al., 2020), respiratórias (Maldonado et al., 2012), dermatológicas (Cukrowska et al., 2021), atividade imunoreguladoras (Huang; Chie; Wang, 2018), e, mais recentemente, psicobiótica (Zhu et al., 2023).

Vários microrganismos são considerados probióticos, sendo o grupo das bactérias do ácido lático (BAL), principalmente do gênero *Lactobacillus*, e *Bifidobacterium* os mais comuns. No entanto, outros microrganismos, tais como algumas espécies *Streptococcus*, *Leuconostoc, Saccharomyces* (levedura), *Enterococcus* e *Bacillus* também tem sido usados (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) et al., 2018; Mishra; Acharya, 2021). Apesar de muitas linhagens probióticas serem descritas como seguras e os relatos de efeitos adversos sejam raros, existe um consenso de que os benefícios são específicos de uma linhagem ou grupo (Hill et al., 2014). Por isso vários critérios de segurança e eficácia terapêutica devem ser preenchidos para que um microrganismo seja considerado um bom probiótico (Figura 1).

Figura 1. Critérios para seleção de probióticos (Mishra; Acharya, 2021; Koutsoumanis et al., 2024; Merenstein et al., 2023).

Os probióticos exercem seus efeitos benéficos por meio de diversos mecanismos de ação, entre os quais se destacam o fortalecimento da barreira epitelial, o aumento da adesão à mucosa intestinal, a inibição da adesão de microrganismos patogênicos, a exclusão competitiva desses patógenos, a produção de substâncias antimicrobianas e a modulação do sistema imunológico (De Melo Pereira et al., 2018; Merenstein et al., 2023). Além da aplicação desses microrganismos como probióticos tem sido amplamente explorado a capacidade dessas linhegns de sintetizar uma variedade de compostos bioativos, como ácidos orgânicos, ácidos graxos de cadeia curta, enzimas, bacteriocinas, biossurfactantes e exopolissacarídeos, que apresentam propriedades antimicrobianas contra microrganismos patogênicos ou atuam como moduladores da resposta imunológica (Sabia et al., 2014; Cortés-Zavaleta et al., 2014; Kumar et al., 2020; Jeyanathan et al., 2021; Bhawal et al., 2022; Abramov et al., 2023a; Bauer Estrada et al., 2025).

2.2. Bactérias do Ácido Lático – BAL

As BAL constituem um grupo de microrganismos Gram-positivas caracterizados pela capacidade de produzir ácido lático como única ou principal fonte de carbono apartir da fermentação de carboidratos. Geralmente são cocos ou bastonetes e possuem forte tolerância a pH ácido, o que lhes confere vantagens ecológicas em diferentes nichos (Liu et al., 2014; George et al., 2018).

Essas bactérias estão entre os microrganismos mais utilizados na fermentação de alimentos, bem como na melhoria do sabor e textura em produtos alimentícios fermentados. Suas propriedades probióticas têm sido exploradas e várias linhagens têm demonstrado capacidade de inibir o crescimento de microrganismos patogênicos, degradar micotoxinas, produzir substâncias antimicrobianas, estimular o sistema imune, entre outros efeitos (George et al., 2018; Kim et al., 2022; Sørensen et al., 2022; Koutsoumanis et al., 2024).

Esse extenso grupo é formado por vários gêneros bacterianos com grande potencial probiótico, sendo as mais conhecidas as seguintes: *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Limosilactobacillus*, *Levilactobacillus e Lactococcus*. Nesse contexto o gênero *Limosilactobacillus,* especialmente a espécie *L. fermentum* tem se destacado nos últimos anos (Maldonado et al., 2012; Gu et al., 2023a; Ozen; Piloquet; Schaubeck, 2023; Wang et al., 2023; Arroyo-Calatayud et al., 2025a).

2.3. Papel dos probióticos na defesa contra microrganismos patogênicos

O microbioma do corpo humano é composto por uma diversidade de microrganismos - bactérias, arqueas, fungos, protozoários e até mesmo vírus - bem como seus metabólitos e componentes estruturais, que interagem com as condições ambientais circundantes e influenciam diretamente os processos fisiológicos do hospedeiro (Berg et al., 2020; Agrawal et al., 2025). Estes microrganismos que habitam diferentes superfícies e cavidades do corpo humano, como pele, mucosas, trato gastrointestinal, geniturinário e respiratório, coexistem de maneira simbiótica com o organismo. A perturbação desse ecossistema microbiano, conhecido como disbiose, pode comprometer o equilíbrio natural da microbiota podendo criar um ambiente favorável a atividade patogênica de microrganismos autóctones e ao desenvolvimento de diversas patologias (Lloyd-Price; Abu-Ali; Huttenhower, 2016; Agrawal et al., 2025).

Entre os componentes benéficos da microbiota humana, especialmente no intestino, destacam-se as BAL, como os *Lactobacillus* spp. amplamente reconhecidas por suas propriedades probióticas (Liu et al., 2014). Essas bactérias competem com microrganismos patogênicos por nichos ecológicos e substratos disponíveis, atuando como barreiras biológicas naturais. Os mecanismos pelos quais as BAL exercem esse antagonismo incluem competição por nutrientes e sítios de adesão, bem como a produção de metabólitos antimicrobianos que contribuem para a inibição do crescimento de patógenos (Figura 2) (Zuo et al., 2019; Anjana; Tiwari, 2022; Kaur et al., 2023).

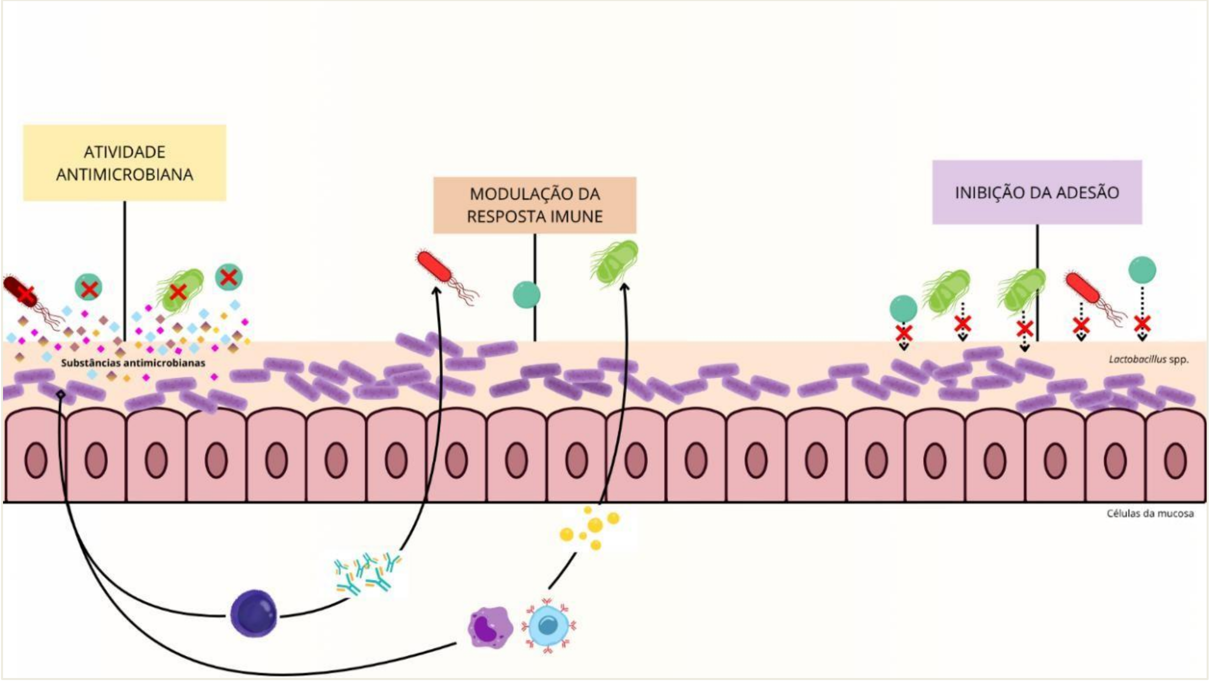

Figura 2. Atividade antimicrobiana dos probióticos (*Lactobacillus* spp.) na microbiota. De modo geral, os probióticos atuam na proteção do hospedeiro por meio de três principais mecanismos: secreção de substâncias antimicrobianas que inibem patógenos, modulação da resposta imune por interação com células imunológicas, e inibição da adesão de microrganismos patogênicos às células da mucosa.

A atividade antimicrobiana desses microrganismos benéficos também está relacionada a produção de diversas substancias (bacteriocinas, ácidos orgânicos - principalmente ácido lático e acético - e peróxido de hidrogênio) que reduzem o pH local e inibem o crescimento de diversos microrganismos, como *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes* e *Staphylococcus aureus* (Monteagudo-Mera et al., 2019; Phuengjayaem; Booncharoen; Tanasupawat, 2021; Anjana; Tiwari, 2022).

A modulação imunológica também representa um componente essencial da atividade antimicrobiana exercida por microrganismos na microbiota. Diversas espécies apresentam potencial imunomodulador, entre as quais tem se destacado *Limosilactobacillus fermentum*, que tem o potencial de estimular a produção de citocinas anti-inflamatórias, modular a atividade de células imunológicas e reduzir a secreção de mediadores inflamatórios (Maldonado et al., 2012; Rodríguez-Sojo et al., 2021; Kaur et al., 2022; Ozen; Piloquet;

Schaubeck, 2023). Esses efeitos fortalecem a barreira intestinal e diminuem a translocação bacteriana, contribuindo para a manutenção da homeostase imunológica e dificultando o estabelecimento de infecções oportunistas.

A capacidade antagonista das bactérias probióticas também se relaciona com a interferência em mecanismos de *quorum sensing*, sistema de comunicação bacteriana que regula a expressão de genes de virulência em patógenos. Ao interferirem nesses sinais, os probióticos reduzem a formação de biofilmes e a produção de fatores de virulência, ampliando seu efeito protetor (Wickström et al., 2013; Čitar et al., 2015; Gu et al., 2023a; Itapary Dos Santos et al., 2019).

A presença de organismos probióticos na microbiota não apenas favorece o equilíbrio ecológico intestinal, mas também atua de modo direto e indireto no controle de microrganismos patogênicos, representando uma estratégia biológica natural de manutenção da saúde e prevenção de doenças infecciosas (Plaza-Diaz et al., 2019; Liu; Wang; Wu, 2022).

2.4. *Limosilactobacillus fermentum*

Espécies do gênero *Limosilactobacillus*, como *L. fermentum*, são encontradas em diferentes nichos, como cereais e materiais vegetais fermentados espontaneamente, além de produtos lácteos, esterco, esgoto, e nos tratos gastrointestinal e genital de humanos, sendo um importante componente da microbiota humana (Ksiezarek et al., 2022). Além, de possuírem grande importância comercial na produção de alimentos fermentados (produtos lácteos, vegetais fermentados), produtos à base de carne e biotecnologia, suas aplicações terapêuticas como probióticos têm sido largamente explorados (Gil-Campos et al., 2012; Maldonado et al., 2012; Ozen; Piloquet; Schaubeck, 2023; Huang et al., 2023).

Várias linhagens de *L. fementum* demonstram a capacidade de inibir a colonização de outras espécies microbianas, sendo também usadas para prevenir a deterioração de alimentos (Zhang et al., 2022b). Seu efeito promotor da saúde tem sido investigado, e várias pesquisas tem demonstrado sua ação antimicrobiana contra patógenos orais, intestinais e vaginais (Wickström et al., 2013; Carmo et al., 2016; Ozen; Piloquet; Schaubeck, 2023; Park et al., 2023).

2.4.1. Propriedades probióticas de *L. fermentum*

O crescente interesse nas promissoras propriedades funcionais de *L. fermentum* tem impulsionado diversas investigações sobre sua aplicação na promoção da saúde humana. Reconhece-se que essa espécie é capaz de aderir às células epiteliais, sintetizar compostos antimicrobianos e ativar receptores que desencadeiam a expressão de genes associados ao sistema imunológico (Archer; Kurrey; Halami, 2018; Rodríguez-Sojo et al., 2021; Kaur et al., 2023, 2022). Esta espécie é geralmente reconhecida como segura, recebendo o selo GRAS (*General Recognised as Safe*) estando incluída nas listas oficiais das autoridades de segurança alimentar (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) et al., 2018; Koutsoumanis et al., 2024).

Uma das linhagens mais amplamente estudadas em relação às suas propriedades funcionais é a *L. fermentum* CECT 5716, que foi isolada do leite humano (Ozen; Piloquet; Schaubeck, 2023). Esta linhagem apresentou atividades imunomoduladoras, anti-inflamatórias e anti-infecciosas (contra *Staphylococcus aureus*); além de apresentar sensibilidade a todos os antibióticos propostos pelos padrões da Autoridade Europeia para a Segurança dos Alimentos (*European* Food Safety Authority- EFSA), e a análise do genoma revelou que não possui genes ou elementos transmissíveis de resistência a antibióticos, características que permitiram sua aplicação em produtos alimentícios (Gil-Campos et al., 2012; Cárdenas et al., 2015).

Para além disso, outras linhagens de *L. fermentum* têm sido investigadas por seus efeitos promotores na saúde, como listados na tabela 1.

Tabela 1. Aplicações probióticas de linhagens *L fermentum*

Propriedade	Linhagens	Referências
Antimicrobiana		
Antibacteriana	*L. fermentum* 3872 *L. fermentum* UCO-979C	(Paucar-Carrión et al., 2022; Abramov et al., 2023a)
Antifúngica	*L. fermentum* ATCC 23271 *L. fermentum* LF5 (I-789)	(Carmo et al., 2016; Dos Santos et al., 2021; Pane; Chisari, 2024)
Antiparasitária	*L. fermentum* AD1 = CCM7421	(Bucková et al., 2018)
Antiviral	*L. fermentum* CJL-112 *L. fermentum* UCO-979C	(Yeo et al., 2014; Valdebenito-Navarrete et al., 2023)
Antitumoral	*L. fermentum* YL-11	(Wei et al., 2019)
Antidiabéticas	*L. fermentum* MF423 *L. fermentum* L-18	(Ai et al., 2021; Kaur et al., 2023)

Imunomoduladora	*L. fermentum* NCDC 400 *L. fermentum* MN410703 e MN410702 *L. fermentum* SMFM2017-NK2	(Ann; Choi; Yoon, 2023; Nataraj et al., 2024; Prakash et al., 2023)
Antialérgica	*L. fermentum* CECT5716	(Amar et al., 2024)
Redução de colesterol	*L. fermentum* TY-S11 *L. fermentum* YDJ-6	(Deng et al., 2024; Chen et al., 2025)
Modulação do eixo intestino-cérebro	*L. fermentum* PS150 *L. fermentum* L18	(Cheng et al., 2025; Kaur et al., 2023)

Há um consenso que os benefícios à saúde associados aos candidatos probióticos são específicos de cada linhagem, com algumas apresentando mais de uma atividade funcional, conforme apontado na tabela 2 (Chandrasekaran; Weiskirchen; Weiskirchen, 2024; EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) et al., 2018; Hill et al., 2014; Merenstein et al., 2023; Mishra; Acharya, 2021; Plaza-Diaz et al., 2019). Destaca-se ainda, a capacidade de certas linhagens de *L. fermentum* de secretar metabolitos com a atividades biológicas, bem como sintetizar vitaminas, exopolissacarídeos (EPS), bacteriocinas e H_2O_2, que já foram descritos em *L. fermentum* e contribuem para a modulação da microbiota, a inibição de patógenos e o fortalecimento da barreira intestinal, ampliando o potencial terapêutico dos probióticos (Sabia et al., 2014; Kaur et al., 2022).

2.4.2. Moléculas com atividade antimicrobiana produzidas por *L. fermentum*

Diversas linhagens de *Limosilactobacillus fermentum* são reconhecidas pela capacidade de produzir compostos antimicrobianos que desempenham papel fundamental na inibição de microrganismos patogênicos e na manutenção da homeostase das mucosas humana. Entre esses compostos destacam-se o ácido lático, o peróxido de hidrogênio (H_2O_2) e diferentes classes de bacteriocinas. A seguir, apresentam-se os principais mecanismos associados a essas substâncias.

2.4.2.1. Ácido lático

O ácido lático, produzido por bactérias do gênero *Lactobacillus*, é um dos principais agentes antimicrobianos naturais da microbiota vaginal e intestinal, atuando na inibição direta de patógenos oportunistas (Liu et al., 2014; Peng; Ed-Dra; Yue, 2023).

O ácido lático é um metabólito chave gerado por bactérias ácido-láticas, especialmente por espécies como *L. fermentum*, durante a fermentação de carboidratos. Sua produção está diretamente associada à capacidade dessas bactérias de manter um ambiente hostil à colonização de microrganismos patogênicos. Na microbiota vaginal, o ácido lático é responsável por manter o pH ácido (entre 3,8 e 4,5), condição que dificulta a sobrevivência de agentes como *Gardnerella vaginalis*, *Escherichia coli* e *Candida albicans* (Carmo et al., 2016; Scillato et al., 2021; Gu et al., 2023a; Bauer Estrada et al., 2025; Da Costa Lima et al., 2025).

Além da acidificação do meio, o ácido lático produzido por linhagens de *L. fermentum* possui propriedades antimicrobianas diretas. Estudos demonstram que ele interfere na permeabilidade da membrana celular de microrganismos patogênicos, comprometendo o transporte ativo e a integridade estrutural dessas células (Sengupta et al., 2015; Scillato et al., 2021; Da Costa Lima et al., 2025)..

Na microbiota intestinal, o ácido lático também contribui para a manutenção da barreira epitelial, reduzindo inflamações e prevenindo a translocação bacteriana para a corrente sanguínea (Da Costa Lima et al., 2025). Essa função é essencial para evitar quadros de disbiose e doenças inflamatórias intestinais. Além disso, o ácido lático favorece a colonização de microrganismos benéficos, criando um ambiente competitivo que limita o crescimento de linhagens patogênicas(Anjana; Tiwari, 2022; Cortés-Zavaleta et al., 2014; Liu et al., 2014).

O ácido lático não é apenas um subproduto metabólico, mas um componente funcional da microbiota humana, com papel ativo na proteção contra infecções e na promoção da saúde mucosal. Sua presença é um indicativo de equilíbrio microbiano e de eficácia das bactérias probióticas na defesa do organismo (Cortés-Zavaleta et al., 2014; Liu et al., 2014). Essa ação é complementada pela produção de outros compostos antimicrobianos, como peróxido de hidrogênio e bacteriocinas, que atuam sinergicamente para reforçar a defesa da mucosa intestinal e vaginal

2.4.2.2. Peróxido de hidrogênio (H_2O_2)

Diversas espécies de *Lactobacillus*, incluindo *L. fermentum*, são reconhecidas por sua capacidade de sintetizar peróxido de hidrogênio como parte de seu metabolismo aeróbico. Esse composto desempenha um papel essencial na proteção das mucosas humanas, especialmente na vagina e no intestino, onde atua como barreira química contra microrganismos invasores (Hung et al., 2003). O H_2O_2 age por meio da geração de espécies reativas de oxigênio (ROS), que danificam estruturas celulares de patógenos, como proteínas, lipídios e ácidos nucleicos, levando à sua inativação ou morte (Tachedjian et al., 2017)

Na microbiota vaginal, a produção de peróxido de hidrogênio por *L. fermentum* está associada à manutenção de um ambiente ácido e oxidativo, que dificulta a colonização de agentes como *G.vaginalis*, *N. gonorrhoeae* e *C. albicans*. Essa atividade antimicrobiana é potencializada pela presença de ácido lático e bacteriocinas, formando um sistema de defesa sinérgico que protege contra infecções urogenitais (Hung et al., 2003; O'Hanlon; Moench; Cone, 2013; Petrariu et al., 2024)

No intestino, embora o ambiente seja menos propício à produção de H_2O_2 devido à menor disponibilidade de oxigênio, *L. fermentum* ainda pode contribuir para o controle de patógenos por meio de mecanismos semelhantes, especialmente em regiões mais oxigenadas ou durante estágios inflamatórios que alteram a composição microbiana local. Estudos recentes demonstram que linhagens de *L. fermentum* isoladas de ambientes humanos apresentam genes relacionados à produção de H_2O_2 e à resistência ao estresse oxidativo, o que reforça seu papel como probiótico funcional (Wang; Zhang; Zhang, 2021)

Além disso, a produção de peróxido de hidrogênio por *L. fermentum* tem sido explorada em aplicações biotecnológicas e terapêuticas, como em formulações probióticas voltadas à saúde feminina e à prevenção de infecções bacterianas recorrentes (Arroyo-Calatayud et al., 2025b; Shukla; Nair, 2025). A capacidade de modular o ambiente microbiano e de atuar diretamente contra patógenos torna essa espécie uma candidata promissora para estratégias de microbiota dirigida.

2.4.2.3. Bacteriocinas

(Christmann et al., 2023)De modo geral, as bacteriocinas – peptídeos antimicrobianos produzidos por bactérias - como *L. fermentum*, têm sido utilizadas no processamento e fermentação de alimentos atuando como bioconservantes naturais produzidas por BAL (Wayah; Philip, 2018; Heredia-Castro et al., 2021). Essas biomoléculas são definidas como peptídeos antimicrobianos produzidos por bactérias Gram-positivas e Gram-negativas que inibem espécies intimamente relacionadas (Darbandi et al., 2022; Ismael; Huang; Zhong, 2024) (Darbandi *et al.*, 2022; Riley e Wertz, 2002). Seu uso na inativação de patógenos, incluindo o seu espectro de atividade antimicrobiana, tolerância ao pH e ao calor e natureza não tóxica têm se destacado, além de não demostrarem efeito negativo sobre a microbiota humana devido a sua sensibilidade a proteases digestivas (Darbandi et al., 2022).

Existe um debate longo quanto à classificação das bacteriocinas. Originalmente, (Klaenhammer, 1993) propôs a divisão em quatro classes principais de bacteriocinas.

Atualmente, aceita-se a divisão em três classes principais: Classe I (peptídeos sintetizados ribossomalmente e modificados pós-traducionalmente - *ribosomally produced and posttranslationally modified peptides* - RiPPs), Classe II (bacteriocinas não modificadas) e Classe III (Estas são não modificadas e maiores que 30 kDa). Na tabela 2, descrevemos a classificação das bacteriocinas mais aceita (Klaenhammer, 1993; Alvarez-Sieiro et al., 2016).

Tabela 2. Classificação das bacteriocinas

Bacteriocinas de BAL			Exemplos
Termo estável (<10kDa)			
	Classe I	RiPPs - sofrem modificação enzimática durante a biossíntese, resultando em moléculas com aminoácidos incomuns.	
		• Classe Ia. ou lanthipeptídeos (tipos I, II, III e IV)	Nisina, bovicina HJ50
		• Classe Ib. ou peptídeos ciclizados da cabeça à cauda	Carnociclina A
		• Classe Ic. ou sacibióticos	Subtilosina A e Thuricin CD
		• Classe Id. ou peptídeos lineares contendo azol(in) (laps)	Estreptolisina S
		• Classe Ie. ou glicocinas	Glicocina F
		• Classe If. ou peptideos de lasso	Microcina J25
	Classe II	Bacteriocinas não modificadas, logo não necessitam de enzimas para sua maturação além de uma peptidase líder e/ou um transportador.	
		• Classe IIa. ou bacteriocinas semelhantes à pediocina	Pediocina PA-1
		• Classe IIb. ou bacteriocinas de dois peptídeos	Plantaricina J/K
		• Classe IIc. ou bacteriocinas sem líder	Enterocina L50
		• Classe IId. ou bacteriocinas de peptídeo único não semelhantes à pediocina.	Lactococina 972, lactococina A e enterocina B
Termo lábil (>10 kDa)			
	Classe III	Termolábeis, são compostos por bacteriolisinas e bacteriocinas não líticas.	Zoocina A, millericina B e caseicina

Atualmente, a nisina e pediocin PA-1 são as únicas bacteriocinas isoladas de BAL aprovadas pela FDA (Food and Drug Administration) para a conservação de alimentos laticínios e enlatados, porém seu uso é restrito devido ao seu estreito intervalo de pH, alto custo de produção e à sua baixa solubilidade em água (Christmann et al., 2023; Field et al., 2023). Contudo, vários estudos têm descrito a identificação de bacteriocinas de linhagens de *L. fermentum,* com atividade inibitória sob diversos microrganismos patogênicos (Riaz; Kashif Nawaz; Hasnain, 2010; Sabia et al., 2014; Heredia-Castro et al., 2021; Abramov et al., 2023a).

(Abramov et al., 2023a; Wayah; Philip, 2018)

A bacteriocina classe III (BLF3872) identificada por Abramov et al., (2023), produzida por *L. fermentum* LF3872 apresentou atividade sobre várias linhagens de *S. aureus* resistentes a antibióticos além de a linhagem formar agregados com as linhagens patogênica de *S. aureus* sugerindo uma ação direta da bacteriocina sobre os microrganismos. Em outro trabalho, Sabia et al., (2014) identificaram uma substância semelhante à bacteriocinas produzidas por uma linhagem de *L. fermentum* oriunda da cavidade vaginal, com atividade antagônica contra *Streptococcus agalactiae* e *C. albicans*.

2.5. Aplicações biotecnológicas e terapêuticas *L. fermentum*

L. fermentum tem emergido como uma espécie probiótica de amplo potencial biotecnológico e terapêutico, com aplicações que abrangem a formulação de alimentos funcionais até o desenvolvimento de estratégias para prevenção e tratamento de doenças (Gajendran; Rajamani, 2024; Kalhoro et al., 2023; Naghmouchi et al., 2020). Diversas linhagens específicas de *L. fermentum* tem demonstrado propriedades antioxidantes, antimicrobianas e imunomoduladoras, sendo capazes de produzir compostos bioativos como ácido lático, peróxido de hidrogênio e bacteriocinas, que atuam na inibição de patógenos intestinais e urogenitais (Lee et al., 2023; Wang et al., 2023; Wang; Zijlstra; Gänzle, 2020; Lee et al., 2025; Arroyo-Calatayud et al., 2025b).

Sua capacidade de sobreviver ao ambiente gastrointestinal e aderir à mucosa intestinal favorece sua utilização em suplementos probióticos voltados à saúde digestiva e imunológica (Raveschot et al., 2018). Recentemente, de *L. fermentum* tem sido incorporado a fermentação de alimentos, como iogurtes e bebidas vegetais, com benefícios sensoriais e nutricionais. Paralelamente, vários estudos explorado sua ação na redução de inflamações intestinais e na regulação da microbiota em condições como obesidade e síndrome metabólica (Wang et al., 2023; Dos Santos Nascimento et al., 2024; Shukla; Nair, 2025). Em contextos clínicos, linhagens de têm sido investigadas como adjuvantes em terapias antimicrobianas e como agentes preventivos contra infecções bacterianas recorrentes, reforçando sua versatilidade como ferramenta biotecnológica promissora (Chandla; Harjai; Shukla, 2022; Abramov et al., 2022, 2023b; Bauer Estrada et al., 2025).

Diante do crescente número de evidências sobre o potencial funcional de *L. fermentum*, torna-se imprescindível a realização de estudos *in vitro* que permitam avaliar, de forma controlada, os mecanismos de ação, a eficácia antimicrobiana e a interação com células hospedeiras. Esses ensaios fornecem evidências preliminares essenciais para a seleção de linhagens promissoras e para o delineamento de aplicações clínicas e tecnológicas seguras (De Melo Pereira et al., 2018; Plaza-Diaz et al., 2019).

Paralelamente, a caracterização genômica dessas linhagens representa uma ferramenta estratégica para a identificação de genes relacionados à produção de metabólitos bioativos, à tolerância a condições adversas, adesão à mucosa intestinal e aspectos ligados a resistência a antibióticos (De Melo Pereira et al., 2018; Naghmouchi et al., 2020; Rodríguez-Sojo et al., 2021). A integração entre abordagens fenotípicas e genotípicas é crucial para o avanço no desenvolvimento de probióticos de nova geração, com eficácia comprovada e aplicações direcionadas à promoção da saúde humana.

3. OBJETIVOS

3.1. Objetivo geral

Realizar a caracterização genômica e fenotípica acerca do potencial probiótico de *Limosilactobacillus fermentum* JAC 231 e avaliar sua atividade antimicrobiana e antiadesiva frente a microrganismos de interesse clinico.

3.2. Objetivos específicos

- Realizar a análise genômica global do isolado clinico vaginal *Limosilactobacillus fermentum* JAC 231 em relação aos diferentes processos biológicos da bactéria;
- Identificar os genes associados à colonização, produção de eventuais bacteriocinas e tolerância às condições adversas do trato gastrointestinal, evidenciando as características do seu potencial probiótico na comparação genômica com outras linhagens da mesma espécie;
- Investigar a presença de genes e o fenótipo do isolado bacteriano relacionados aos aspectos da segurança do isolado para uso como probiótico;
- Verificar a atividade inibitória do crescimento sobre diferentes microrganismos de interesse clínico;
- Investigar a atividade antiadesiva sobre diferentes microrganismos de interesse clínico;

4.

Capítulo 1: Comprehensive Genomic Characterization and Probiotic Evaluation of *Limosilactobacillus fermentum* JAC 231 Isolated from Vaginal Microbiota

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RESEARCH

Comprehensive Genomic Characterization and Probiotic Properties Evaluation of *Limosilactobacillus fermentum* JAC 231 Isolated from Vaginal Microbiota

Carmem Duarte Lima Campos[1] · Amanda Graziella Goncalves Mendes[1] · José Lima Pereira-Filho[1] · Viviane da Silva Sousa Almeida[1] · Ana Beatriz Santos Sousa[1] · Israel Viegas Moreira[1] · João Lucas do Carmo Lima[1] · Aleania Polassa Almeida Pereira[1] · Allysson Kayron Carvalho Silva[2] · Laís Araújo Souza Wolff[2] · Kátia Regina Assunção Borges[2] · Cristianne Roberta Rhoden[3] · Cinara Regina Aragão Vieira Monteiro[1] · Cristina de Andrade Monteiro[4] · Elizabeth Soares Fernandes[5] · Valério Monteiro-Neto[1]

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Abstract

Limosilactobacillus fermentum JAC 231, a strain isolated from the vaginal microbiota of an asymptomatic woman, was investigated for its genomic features, safety, probiotic potential, and antimicrobial properties in this study. Whole-genome sequencing revealed a 2.06 Mbp genome with 51.4% GC content and 1,993 predicted coding sequences (CDS). Functional annotation indicated that the genes were related to carbohydrate metabolism, stress tolerance, adhesion, and bacteriocin biosynthesis. Comparative analysis revealed that *L. fermentum* JAC 231 shared 1,587 orthologous groups with the other five probiotic strains of *L. fermentum*. No virulence factors or transferable antibiotic resistance genes were detected, and only an incomplete prophage region was identified, which supports the safety of this strain. Phenotypically, JAC 231 exhibited tolerance to acidic pH and bile salts and demonstrated high adhesion capacity to porcine gastric mucin (5.97 $\log_{10}$ CFU/mL) and HeLa epithelial cells (5.79 $\log_{10}$ CFU/mL). Antimicrobial susceptibility testing indicated resistance to vancomycin and moderate susceptibility to ciprofloxacin, features considered intrinsic and nontransferable. The strain exhibited broad-spectrum antimicrobial activity, with inhibition zones ranging from 16.60 ± 2.08 mm to 28.30 ± 2.88 mm against various Gram-positive and Gram-negative pathogens. Agar diffusion assays indicated that the antimicrobial activity of the cell-free supernatant was primarily due to the production of organic acids. Furthermore, JAC 231 significantly reduced pathogen adherence to epithelial cells in competitive, exclusion, and displacement assays. Collectively, these genomic and functional attributes support the safety and probiotic potential of *L. fermentum* JAC 231 and underscore its promise as a candidate for therapeutic applications targeting the urogenital and gastrointestinal microbiota.

Keywords *Limosilactobacillus fermentum* · Probiotic · Vaginal microbiota · Whole-genome sequencing · Antimicrobial activity · Mucosal adhesion · Safety assessment

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✉ Valério Monteiro-Neto
valerio.monteiro@ufma.br

Cristina de Andrade Monteiro
cristinamonteiro@ifma.edu.br

[1] Centro de Ciências Biológicas e da Saúde, Programa de Pós-Graduação em Ciências da Saúde – PPGCS, Universidade Federal do Maranhão, Avenida dos Portugueses, 1966, campus Bacanga, São Luís, MA 65080-040, Brazil

[2] Programa de Doutorado em Biotecnologia – Rede Nordeste de Biotecnologia (RENORBIO), Universidade Federal do Maranhão, Avenida dos Portugueses, 1966, campus Bacanga, São Luís, MA 65080-040, Brazil

[3] Laboratório Cedro, Avenida Silva Maia, 81, Centro, São Luís, MA 65020-570, Brazil

[4] Present address: Instituto Federal de Educação Ciência e Tecnologia do Maranhão, Avenida Getúlio Vargas, 4, Monte Castelo, São Luís, MA 65030-005, Brazil

[5] Department of Animal and Veterinary Sciences - ANIVET Ruminant Nutrition (RUN), Aarhus University, Viborg, Denmark

Introduction

In 2020, Zheng and colleagues performed a comprehensive taxonomic revision of the genus *Lactobacillus*, which resulted in the creation of several new genera and the reclassification of *Lactobacillus fermentum* as *Limosilactobacillus fermentum* [1]. This species is classified within the lactic acid bacteria (LAB) group, comprising Gram-positive, catalase-negative, acid-tolerant, nonsporulating, and strictly fermentative microorganisms [2]. Strains of *L. fermentum* are highly versatile and inhabit a wide variety of ecological niches, including fermented foods, plant materials, and the gastrointestinal and urogenital tracts of humans [3–7].

In addition to their well-established technological applications in the food industry, particularly in enhancing food quality and shelf life [8, 9], several *L. fermentum* strains have shown promising probiotic attributes. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [8]. Notably, *L. fermentum* strains exhibit various beneficial activities, including reinforcement of epithelial barrier function, modulation of host immune responses [4, 9], competitive exclusion of pathogens [10, 11], and production of antimicrobial substances such as organic acids, hydrogen peroxide, and bacteriocins with a broad spectrum of activity [12–14]. These properties confirm previous investigations into the therapeutic potential of *L. fermentum* strains against disorders affecting the gastrointestinal and vaginal tracts [15–17].

Recent comparative genomic analyses have also highlighted the probiotic potential of *L. fermentum* strains isolated from the human oral cavity, such as *L. fermentum* SD7, which displays aggregation-related genes, absence of virulence and resistance determinants, and strong phylogenetic coherence within distinct clades, underscoring its candidacy for food and healthcare applications [18].

The efficacy and safety of probiotics are strain-specific, necessitating the rigorous evaluation of individual strains rather than extrapolating data across species [8]. Hence, the thorough characterization of probiotic candidates involves not only the demonstration of beneficial physiological effects but also comprehensive safety assessments, such as confirming the absence of antibiotic resistance transferability and virulence factors, and testing the strain's tolerance to gastrointestinal stresses, such as acidic pH, digestive enzymes, and bile salts [8, 19, 20]. With advances in genome sequencing technologies, genomic analyses have emerged as essential tools for detailed characterization, providing insights into the genetic features related to the safety, metabolic capabilities, and probiotic potential of individual strains [21–24]. Some genomic studies on various probiotic *Lactobacillus* species have underscored the importance of genome-level characterization for reliably predicting probiotic functions and safety in humans [24–27].

In a previous study by our group, novel probiotic candidates were isolated from the vaginal microbiota of healthy women without symptoms of genital infection. Among these isolates, *L. fermentum* JAC 231 demonstrated significant antifungal and antivirulence effects against *Candida albicans*, including the reduction of hydrolytic enzyme secretion, inhibition of hyphal formation, impairment of coaggregation and adhesion to epithelial cells, and disruption of biofilm development [28]. Building on these promising results, the present study aimed to sequence and comprehensively analyze the genome of *L. fermentum* JAC 231, evaluate its safety profile and tolerance under simulated gastrointestinal conditions, and further characterize its antimicrobial activity and ability to inhibit the adherence of bacterial pathogens to eukaryotic host cells.

Materials and Methods

Bacterial Strains and Growth Conditions

The microorganisms used in this study were the *L. fermentum* JAC 231 probiotic strain isolated from the vaginal microbiota of healthy women and strains acquired from the American Type Culture Collection (ATCC, Manassas, VA): *L fermentum* ATCC 23271), *Klebsiella pneumoniae* ATCC 700063, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* ATCC 25923, *Salmonella enteritidis* ATCC 13076, *Enterococcus faecalis* (ATCC 29212), and enteropathogenic *Escherichia coli* O127:H6 E2348/69 (EPEC) from the Basic and Applied Microbiology Laboratory of the Federal University of Maranhão.

L. fermentum JAC 231 and ATCC 23,271 strains were grown De Man, Rogosa, and Sharpe (MRS) agar and broth (Difco Laboratories, Detroit, MI, USA) under anaerobic conditions, while the other bacteria were grown in broth and brain heart infusion (BHI) agar under aerobic conditions. All microorganisms were stored at −80 °C until use.

Sequencing of the Complete Genome of *L. fermentum* JAC 231

Genome sequencing of *L. fermentum* JAC 231 was performed using Neoprospecta Microbiome Technologies genomic services (Florianópolis, SC, Brazil). Genomic DNA was extracted using the PureLink® Genomic DNA Mini Kit (Invitrogen, USA) following the manufacturer's instructions. The complete genome was sequenced using the Illumina MiSeq paired-end library approach and prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), resulting in an estimated genome coverage of approximately 35–50×. Raw Illumina reads (FASTQ format) were used for de novo genome assembly analysis. No long-read sequencing technologies (such as Oxford Nanopore or PacBio) were used. Raw reads exhibited high quality, with a mean Phred score of 30.76 and 71.24% of bases presenting Phred scores ≥ Q30. The OneShotWGS Bacteria pipeline was used to assemble the genome. This pipeline uses A5 [29], which features assembly corrections using the ID-BA-UD [30] and Spades programs [31]. To identify a potential mixing profile of organisms in a sample, the 16 S and 23 S rRNA ribosomal genes were taxonomically assigned using BLASTN algorithm [32]. Protein prediction was performed from the assembled genome using the Prokka program [33].

Genome Analysis

The genome sequence of *L. fermentum* JAC 231 underwent comprehensive annotation and functional analysis using Rapid Annotation using Subsystem Technology (RASTtk v. 1.3.0) pipeline [34, 35] and KEGG database [36]. Protein similarity searches against the NCBI databases were performed using BLAST. To ensure methodological consistency, all strains included in the analysis (*L. fermentum* MCC 2760, AGR 1485, ATCC 23271, CECT 5716, and IFO 3956) were re-annotated using the Prokka pipeline [33] prior to their comparison. Orthologous proteins among these strains were analyzed using OrthoVenn 3 [37]. Phylogenetic analyses were performed using nucleotide sequences from the Type (Strain) Genome Server (TYGS) [38, 39]. The genomic content was visualized using the CGView server [40].

Mobile genetic elements, including plasmids and bacteriophages, were identified using PlasmidFinder v2.1 and PHASTER [41], and CRISPR arrays and Cas proteins were analyzed using CRISPRCasFinder v. 1.1.2 [42]. Pathogenicity and virulence determinants were assessed using PathogenFinder v.1.1 [43] and VirulenceFinder v.2.0 [44]. Antimicrobial resistance genes were detected using ResFinder v.4.0 [45], RAST [34], and CARD v3.2.4 [46]. Bacteriocin-coding genes were investigated using BAGEL5 [47] and manually confirmed using BLASTp 2.17.0 [48].

Safety and Probiotic Properties of *L. fermentum* JAC 231

L. fermentum ATCC 23271 served as the probiotic positive control in all following assays due to its known probiotic characteristics (Itapary Dos Santos et al. 2019).

Antibiotic Susceptibility Testing

Antibiotic susceptibility was evaluated using disk diffusion as described by Charteris et al. [49] and the broth microdilution method according to the CLSI guideline [50]. For the disc diffusion method, antibiotics tested (Oxoid/Thermo Fisher Scientific, Basingstoke, UK) included chloramphenicol (30 µg), tetracycline (30 µg), norfloxacin (10 µg), rifampicin (5 µg), penicillin (10 µg), erythromycin (15 µg), nitrofurantoin (300 µg), gentamicin (120 µg), cefazolin (30 µg), tobramycin (10 µg), clindamycin (2 µg), vancomycin (30 µg), sulfazotrim (25 µg), oxacillin (1 µg), novobiocin (5 µg), ciprofloxacin (5 µg), teicoplanin (30 µg), tigecycline (15 µg), cefoxitin (30 µg), and linezolid (30 µg). Plates were incubated anaerobically at 37 °C for 24 h, and the inhibition zones were measured in mm.

The Minimum Inhibitory Concentration (MIC) of *L. fermentum* JAC 231 against vancomycin (0.031–64 µg/ml). was determined using 96-well microtiter plates. Inocula were prepared from colonies grown for 24 h at 37 °C under anaerobic conditions and standardized using a spectrophotometer (OD600 nm = 0.1). The antibiotic was serially diluted across the wells. The standardized bacterial suspension was then inoculated into the wells and incubated anaerobically at 37 °C for 48 h. MICs were recorded as the lowest concentration of vancomycin at which visible bacterial growth was inhibited.

Tolerance to Gastrointestinal Conditions

Resistance to acidic pH and bile salts (Oxgall, Sigma–Aldrich) was assessed as described by Monteiro et al. [51]. Briefly, aliquots of MRS broth adjusted to pH 2 or 4 or supplemented with 5 g/L or 10 g/L bile salts were inoculated with 100 µL of a 24-hour culture and incubated anaerobically for 3 h at 37 °C. The control consisted of MRS broth without bile salts or was adjusted to pH 7. After incubation, the samples were serially diluted, plated on MRS agar, and incubated at 37 °C for 24 h. The percentage of viable cells relative to the control was calculated from colony counts obtained on MRS agar using the following formula: Survival rate (%) = (CFU test condition/CFU control) × 100.

Mucin Binding Assay

Mucin adhesion capacity was tested using porcine gastric mucin type III (Sigma–Aldrich, Lyon, France), as described by Carmo et al. [10]. Briefly, 100 µL of a 10 mg/mL solution of Porcine Gastric Mucin Type III (Sigma, Saint-Quentin-Fallavier, France) was solubilized in PBS (pH 7.2), added to 24-well microtiter plates, and allowed to bind to the wells for 16 h at 4 °C. The wells were washed with PBS and saturated with 2% (w/v) bovine serum albumin (BSA) solution (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 4 °C and washed again. with PBS. The bacterial inocula in MRS broth were washed with PBS and standardized using a spectrophotometer (OD600 nm = 0.1). Aliquots of 500 µL of the bacterial suspension were added to each well, and the microplates were incubated at 37 °C for 1 h. The wells were then washed thrice with 1 mL PBS to remove non-adherent bacteria. The wells were treated with 500 µL of 0.5% Triton X-100 (Sigma-Aldrich), and the plates were incubated for 30 min at room temperature with orbital shaking. To quantify the number of bacteria capable of binding mucin, the wells were scraped, serially diluted, plated on MRS agar, and incubated at 37 °C for 24 h under anaerobic conditions. Wells containing mucin without bacteria were used as negative controls.

Adhesion to HeLa Cells

Adherence to human cervical epithelial (HeLa) cells was evaluated as described by Carmo et al. [10]. Briefly, human cervical epithelial (HeLa) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing GlutaMAX (Gibco, USA) supplemented with 1× antibiotic-antimycotic solution (Gibco, USA) and 10% fetal bovine serum (Gibco, USA) at 37 °C under 5% CO_2. HeLa cells (5×10^5 cells/mL) were cultured in 24-well plates and incubated at 37 °C in 5% CO_2 atmosphere. Before the adhesion assay, *Lactobacillus* cultures were grown in MRS agar and standardized (5×10^8 CFU/mL) using DMEM. After 24 h, the cells, which had grown to approximately 70% confluence, were washed with PBS, treated with 250 µL of the standard *L. fermentum* JAC 231 suspension plus 250 µL of DMEM, and incubated for 4 h at 37 °C in 5% CO_2. The wells were then washed and treated with 500 µL of 0.1% Triton X-100 (Sigma, USA) for 5 min while shaking. To quantify the number of adherent cells, serial dilutions and counts were performed on MRS agar plates. The results are expressed as Log10 CFU/mL. HeLa cells incubated in the absence of bacteria were used as negative controls. To visualize bacterial adherence to HeLa cells, the cells were fixed with methanol (Amresco, Gymea, Australia) and stained using a rapid panotype kit. The coverslips were mounted on glass slides and visualized using an optical microscope (100× magnification).

Interference with Adhesion to HeLa Cells

Competition, exclusion, and displacement assays were performed to evaluate the effect of *L. fermentum* JAC 231 on the adhesion of pathogenic bacteria to HeLa cell monolayers. After preparing the standard suspensions, the tests proceeded as follows: (I) in the competition assay, *L. fermentum* JAC 231 and the pathogens were inoculated simultaneously with HeLa cells and incubated for 4 h at 37 °C; (II) in the exclusion assay, *L. fermentum* JAC 231 was inoculated and incubated initially for 1 h at 37 °C with HeLa cells, after which the pathogens were added and incubated for another 3 h; (III) in the displacement assay, pathogens were added and incubated for 1 h at 37 °C, after which *L. fermentum* JAC 231 was added and incubated for an additional 3 h. Serial dilutions and plating were performed to quantify the number of adherent cells in each sample. The results are expressed as Log10 CFU/mL.

Antagonism Assay

Antagonism assays were performed using the overlay technique, as described by Chew et al. [52]. Cultures of *L. fermentum* JAC 231 grown in MRS broth for 24 h at 37 °C under anaerobic conditions were standardized (1×10^8 cells/mL), and 10 µL aliquots were deposited in MRS agar medium and incubated for 48 h at 37 °C under anaerobic conditions. After this period, Müeller-Hinton agar medium (Difco Laboratories, Detroit, MI, USA) was added at 50 °C to form a 2 mm thick layer. After solidification, standardized pathogenic bacterial inocula (1×10^8 cells/mL) were prepared, including. *K. pneumoniae* ATCC 700063, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *S. enteritidis* ATCC 13076, *E. faecalis* ATCC 29212, and enteropathogenic *E. coli* O127:H6 E2348/69 (EPEC). The plates were incubated at 37 °C for 24 h under aerobic conditions, and the inhibition zones were measured (in mm).

Antimicrobial Activity of Cell-Free Supernatant

To evaluate whether the antimicrobial activity of the cell-free supernatant (CFS) was attributable to organic acids or proteinaceous substances, *L. fermentum* JAC 231 was cultured in 200 mL of MRS broth for 18 h at 37 °C under anaerobic conditions, following a protocol described by Scillato et al., [53] and Wang and Zang [54]. After incubation, the cultures were centrifuged at 5,000 × g for 15 min at 4 °C, and the supernatant was sterilized by filtration through a 0.22 µm syringe filter (Merk, Millipore, Darmstadt, Germany). The obtained CFS was adjusted to pH 6.0 using 10 M NaOH and subsequently treated with trypsin and proteinase K at a final concentration of 1 mg/mL each, at pH 7.5, for 2 h at 37 °C. The enzymes were then inactivated by heating at 100 °C for 5 min, and the pH was readjusted to the initial value. MRS broth adjusted to pH 6.5 and 4.0 was used as the experimental control.

Following these treatments, the antimicrobial activity of the CFS was evaluated using an agar diffusion assay. Inocula of the pathogenic bacteria used in the antagonism assay were seeded onto Mueller–Hinton agar plates. Wells were created using sterile 1 mL micropipette tips and filled with 100 µL of each treated or untreated CFS. Plates were incubated at 35 °C for 24 h, and antimicrobial activity was assessed by measuring inhibition zones (mm).

Ethical Aspects

The vaginal isolate *L. fermentum* JAC 231 was obtained from a previous study and approved by the Ethics Committee of CEUMA University (n.° 2.519.446/2018/CEP-UNICEUMA).

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism software (version 9.0; GraphPad Software, San Diego, CA, USA). Data normality was assessed using the Shapiro–Wilk test. For inhibition assays, adhesion to HeLa cells and mucin, the unpaired Student's t-test was applied. Two-way ANOVA was used for tolerance tests of gastrointestinal conditions and interference with adhesion to HeLa cells, followed by Dunnett's test. Statistical significance was set at $P<0.05$. All assays were performed in triplicate on three separate days.

Complete Genome Sequence Data Accession Number

This whole-genome shotgun project for *L. fermentum* JAC231 was deposited in the DDBJ/ENA/GenBank under accession number JAVFHY000000000. Raw sequencing reads (FASTQ files) are available in the NCBI Sequence Read Archive (SRA) under the accession number SRR37146884. The BioProject accession number PRJNA1007394 and BioSample is SAMN37068468.

Results

Genome Analysis

General Characteristics of the *L. fermentum* JAC 231 Genome

To elucidate the genetic basis of the probiotic properties, the genome of the vaginal clinical strain of *L. fermentum* JAC 231 was sequenced in this study. Table 1 summarizes the general genomic features obtained using RAST. A genome map was generated using CGVIEW (Fig. 1).

Genetic prediction performed by RAST revealed the presence of 1,993 protein-coding sequences distributed across 292 subsystems (Table 1). Of these, 1,358 were related to non-hypothetical proteins and 635 to hypothetical proteins. By analyzing the distribution of categories in the subsystems, it was found that the subsystems with the greatest number of genes were related to the metabolism of proteins, carbohydrates, amino acids (and derivatives), cofactors, vitamins, prosthetic groups, pigments, and DNA (Fig. 2A). Functional annotation of the genome was also performed using the KEGG database, where 1,801 genes (56.2%) were annotated and divided into six classes of metabolic pathways (Fig. 2B). The categories with the highest gene abundance were global and overview maps (44.5%), carbohydrate metabolism (8.5%), and amino acid metabolism (6.2%). A comprehensive summary of the identified subsystems and functional resources is presented in Tables S1 (RAST) and S2 (KEGG).

Table 1 General features of the genome assembly of *L. fermentum* JAC 231

Attribute	Value
Size of genome (bp)	2,064,918
GC (%)	51.4
N50	36,970
L50	20
No of Contigs	133
No of Subsystems	292
No of Coding Sequences	1993
No of RNAs	62
No of operons de tmRNA	1
No of tRNA	55
No of rRNA	6
No of tmRNA	1
No of CRISPR loci	2

Phylogenetic Analysis

A phylogenetic tree was constructed using reference genomes and other sequences available in the TYGS database showing the evolutionary relationships between strains of the genus *Limosilactobacillus* and related taxa (Fig. 3). The clade formed by *L. fermentum* exhibited a confidence level of 100%. *L. fermentum* JAC231 clustered within the *L. fermentum* clade, presenting high bootstrap support values (>87), which confirm the robustness of its position. Its closest lineage was *L. fermentum* ATCC 23271. *L. cellobiosus* DSM 20055 was positioned within the clade containing JAC231 and other *L. fermentum* strains recognized as probiotics, including AGR 1485, CECT 5716, MCC 2760, and IFO 3956. Comparative visualization demonstrated that JAC 231 shares genomic traits consistent with those of other *L. fermentum* strains, reinforcing its identity and functional potential within the genus.

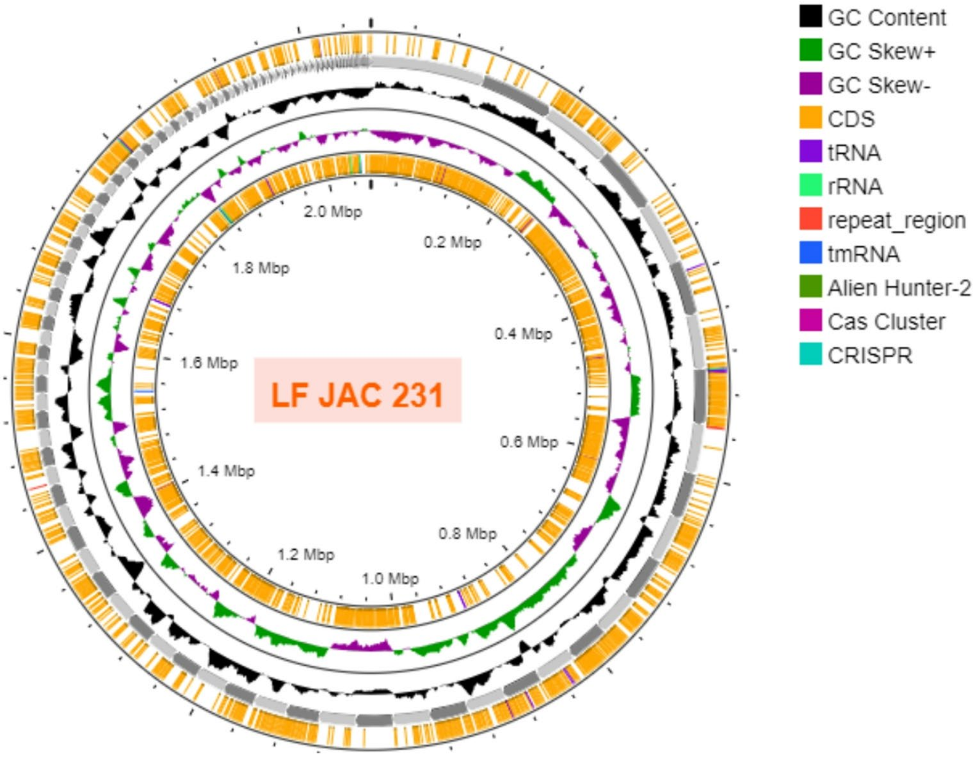


Fig. 1 Circular map of the *L. fermentum* JAC 231 genome. The first and last circles represent genes on the positive and negative strands, respectively, including coding sequences (CDS), transfer tRNA, rRNA, and other genes; the third circle represents the GC content; the fourth circle represents the GC slope, where green indicates GC > 0, purple indicates GC < 0, and the junction of green and purple is the starting point and endpoint of replication, respectively

Orthologous cluster analysis

Orthologous cluster analysis using OrthoVenn3 (Fig. 4) revealed that the strains formed 2,261 clusters, with 42 overlaps and 1,450 single-copy clusters, encoding 12,902 proteins and 459 singletons (3.56%) (no orthologs). Of these, 1,587 were orthologous clusters common to all strains examined, reflecting essential and conserved genetic features of the species. A supplementary analysis of the distribution of orthologous clusters among *L. fermentum* strains is presented in Figure S1 and S2, highlighting the core genome and strain-specific adaptations of *L. fermentum* JAC 231.

OrthoVenn3 analysis revealed that *L. fermentum* JAC 231 comprises 1,983 orthologous (Figure S2) clusters and a lineage-specific cluster (cluster 2261) containing two proteins unique to *L. fermentum* JAC 231 (Figure S1). Although a Swiss-Prot hit (P75980) was detected, no Gene Ontology annotations were available, and both proteins were classified as hypothetical proteins. These findings indicate that *L. fermentum* JAC 231 harbors unique genes with currently uncharacterized functions that may reflect strain-specific genomic adaptations.

The 1,587 shared clusters included 10,049 proteins, with each strain encoding approximately 16% of these proteins (Fig. S1). Most proteins were involved in biological, metabolic, and cellular processes (Table S3). Furthermore, we observed the presence of orthologous clusters among lineages important for probiotic activity, such as those related to vitamin synthesis, biofilms, fermentation, carbohydrate metabolism, and responses to external stimuli. Regarding molecular functions, 14.5% of the identified proteins were involved in transport activities, and 15.6% were related to molecular and oxidoreductase functions. The third group of proteins was related to cellular and membrane components (Table S3).

Analysis of Probiotic Characteristics

We analyzed the probiotic characteristics of the strains through genome annotation using the RAST SEED server, as described above. The following genes involved in vitamin synthesis were screened: thiamine (vitamin B1), riboflavin (vitamin B2), pyridoxine (vitamin B6), biotin (vitamin B7), and folate (vitamin B9) (Table S4). The analysis also revealed the presence of five genes related to adhesion, four related to the biosynthesis of exopolysaccharides (EPSs), and sixty-one involved in the response to oxidative, osmotic, and thermal stress, tolerance to bile salts, and pH. (Table 2).

Identification and Analysis of Bacteriocin Coding Sequences

Genes related to bacteriocin production were identified using BAGEL5. A sequence 48% similar to enterolysin

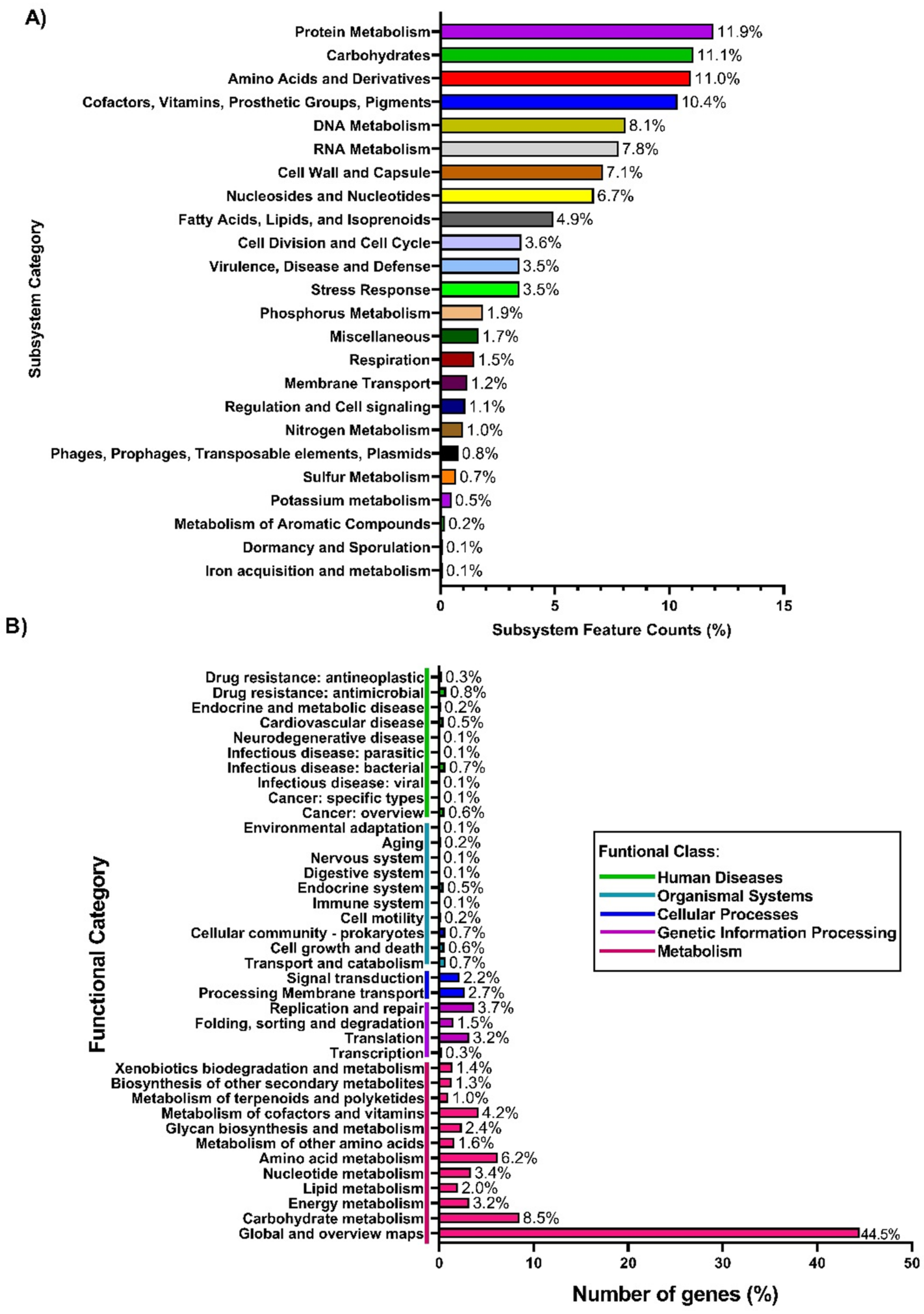


Fig. 2 (**A**) Overview of the categories and subsystems of the *L. fermentum* JAC 231 genome annotated using the RAST. (**B**) Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotation

A, a protein from *Enterococcus faecalis*, was identified. Figure 5 shows the position of the bacteriocin sequence in green.

Analysis of Genes Related to the Presence of Plasmids, Pathogenicity, Virulence and Resistance to Antimicrobials

Analysis of the *L. fermentum* JAC 231 genome using the RAST and PlasmidFinder tools did not reveal the presence of any mobile genetic elements. Using the PathogenFinder and VirulenceFinder algorithms, we did not identify pathogenicity genes or homologous virulence factors of common or clinically relevant pathogens, with the probability of being a human pathogen calculated as 0.203 using the PathogenFinder tool (Table S5).

The presence of resistance genes was assessed using RAST and ResFinder v. 4.0 and CARD. ResFinder v. 4.0 did not identify any resistance genes. The CARD program

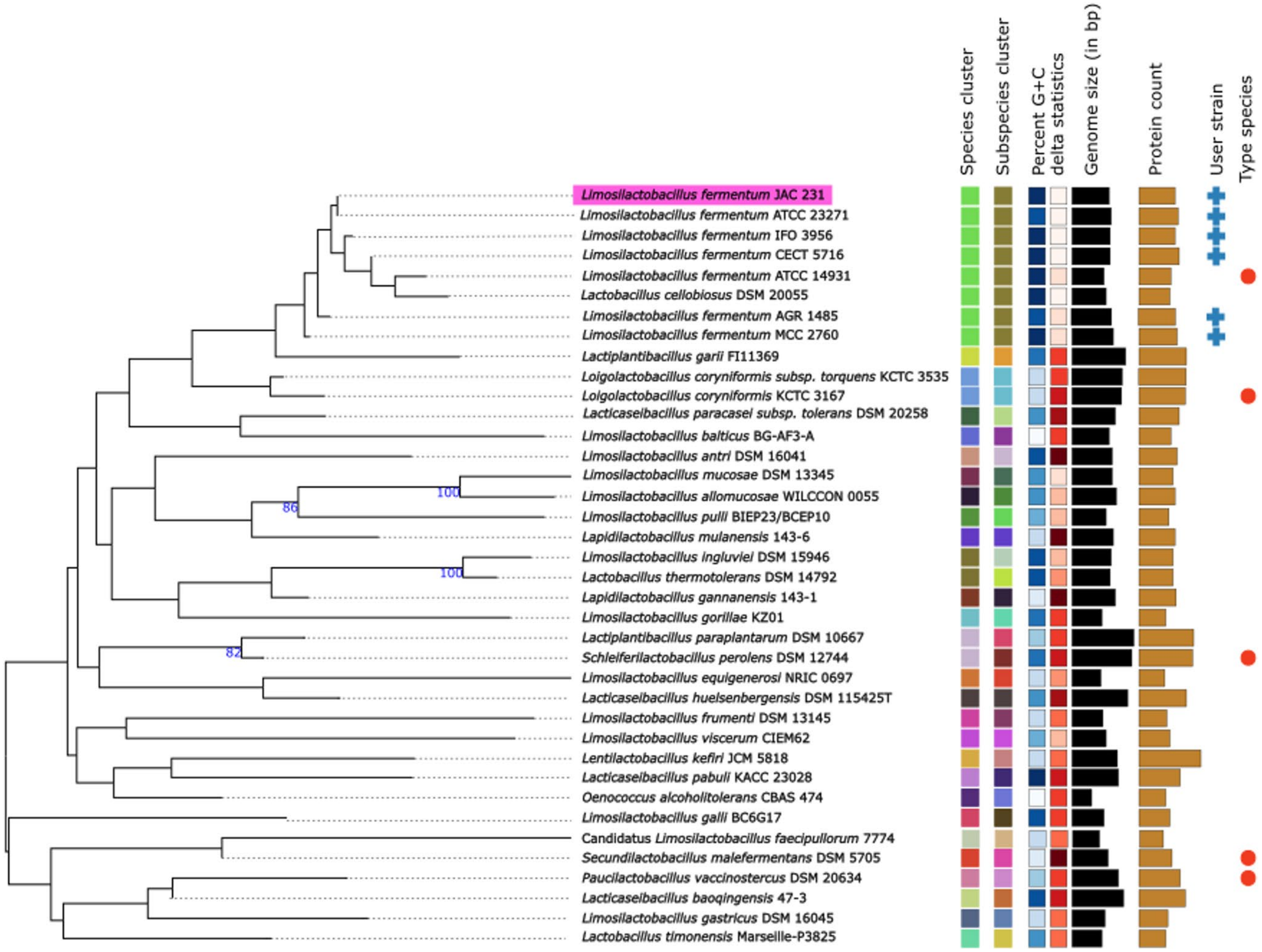


Fig. 3 Phylogenetic tree of *Limosilactobacillus fermentum* strain JAC 231 and related taxa. The tree was inferred with FastME 2.1.6.1 [55] from Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences available in the TYGS database. Branch lengths are scaled in terms of GBDP distance formula d5. Numbers above the branches indicate GBDP pseudo-bootstrap support values > 60% from 100 replicates (average branch support: 22.8%). The tree was rooted at the midpoint [56]. Strain JAC 231 is highlighted in magenta and marked with a blue star. Type strain genomes are indicated by red circles. Additional metadata (species, subspecies, experimental source, genome size, and protein count) are shown

highlighted the presence of antibiotic resistance genes in the glycopeptide class (Table 3). The RAST tool revealed the presence of 20 coding sequences related to intrinsic antibiotic resistance, including two multidrug efflux pumps, one β-lactamase, four fluoroquinolone resistance determinants, and four ribosomal protection proteins associated with tetracycline resistance (Table 4).

The presence of CRISPR-Cas sequences was investigated using the online program CRISPRCasFinder, which identified two hit sequences associated with the *cas* gene (Table S6).

The PHASTER server was used to predict the presence of prophage sequences in the *L. fermentum* JAC 231 genome. Based on oversight and potential prediction, this tool can identify intact, questionable, or incomplete prophage regions. A prophage region was identified throughout the genome, and analysis using the PHASTER tool revealed incomplete sequences.

In Vitro Safety and Probiotic Activity

Antibiotic Susceptibility Assay

Antibiotic susceptibility profiles were assessed using disk diffusion method and the broth microdilution. *L. fermentum* JAC 231 was susceptible to most antibiotics tested. However, it showed resistance the vancomycin, and moderate sensitivity to ciprofloxacin (Table 5).

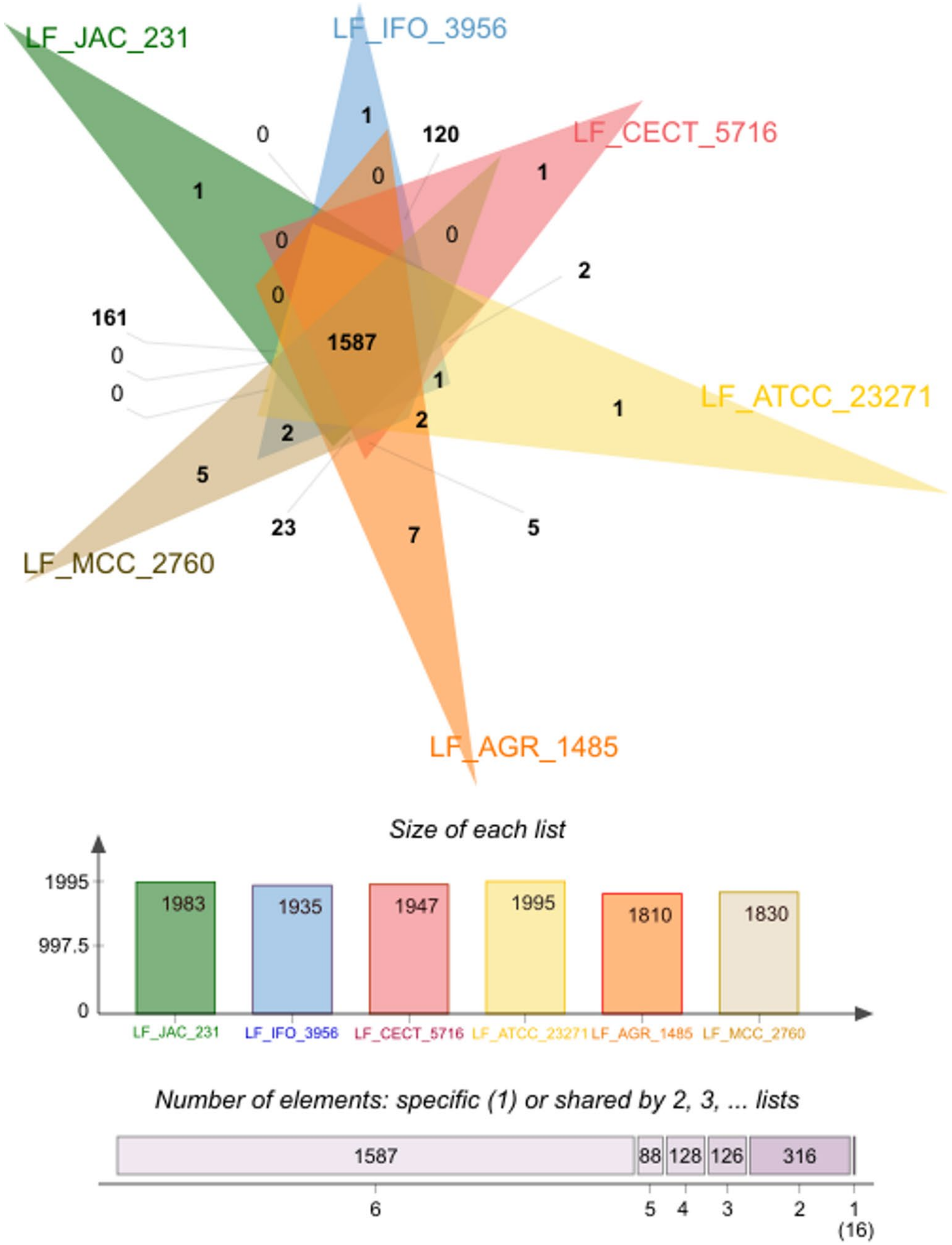


Fig. 4 Venn diagram showing the distribution of orthologous clusters shared among the following *L. fermentum* strains: JAC 231, MCC 2760, AGR 1485, ATCC 23271, CECT 5716, and IFO 3956. The Venn diagram represents the number of unique and shared orthologous clusters, whereas the bar graph represents the number of clusters in each strain

Tolerance to Gastrointestinal Conditions

Host survival under adverse gastrointestinal conditions was evaluated in vitro (Table 6). In the acid pH tolerance test (pH 2 and 4), the strains tested showed greater tolerance to pH 4 than to pH 2, where we observed a 31.15% reduction in the survival rate of the *L. fermentum* JAC 231 strain compared to the control (pH 7), and its growth was lower than that observed for *L. fermentum* ATCC 23,271 ($p = 0.0005$). We also evaluated survival at different concentrations of bile salts (0.5% and 1%) and compared it with that of control (without salts). *L. fermentum* JAC 231 showed a reduction in survival compared to the control without bile salts, but maintained stable viability at the tested concentrations of 5 g/L (87.34%) and 10 g/L (84.76%) (Table 6).

Mucin and HeLa Cell Binding Assay

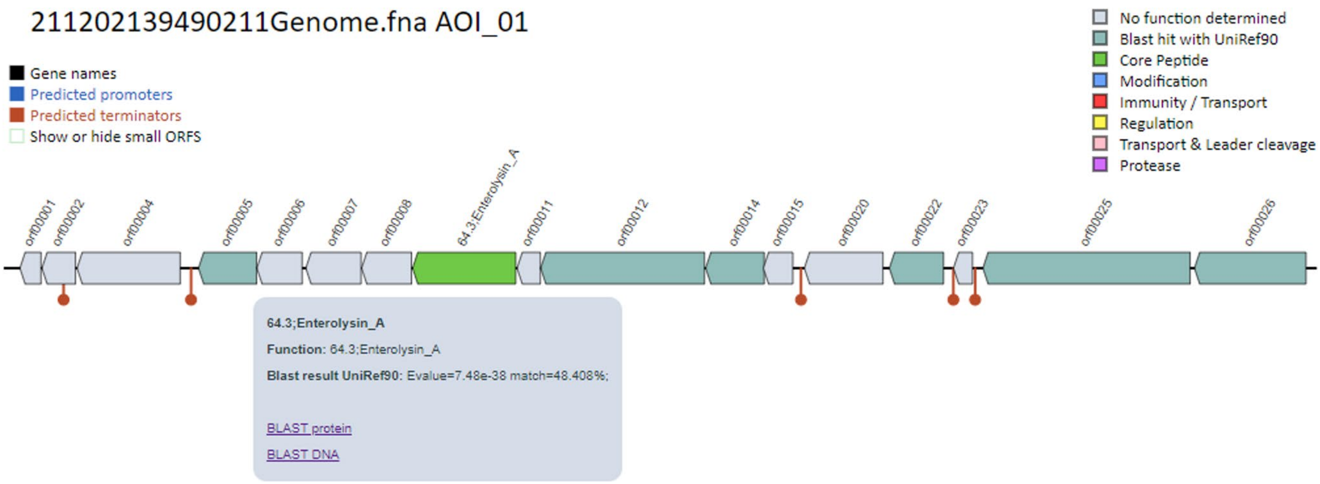
The results of the binding assays for mucin and HeLa cells are presented as $\log_{10}$ CFU/mL (Fig. 6). In the mucin-binding assay (Fig. 6a), the *L. fermentum* ATCC 23,271 strain had a mean of 6.05 Log_{10} CFU/mL, significantly higher than that of *L. fermentum* JAC 231 strain, which had 5.97 Log_{10} CFU/mL ($p < 0.05$). In the HeLa cell adhesion test (Fig. 6b),

Table 2 Subsystems related to probiotic characteristics identified in the genome of *L. fermentum* JAC 231

Gene Function RAST/BLAST	Gene (Abbrev.)	Query Length	Accession Length	Query Cover (%)	E Value	Per Ident (%)	Accession
Adhesion							
Sortase A, LPXTG specific	*SrtA*	678	2,297,851	100	0.0	100	CP011536.1
		703	2,161,679	100	0.0	99.86	CP110798.1
Fibronectin-binding protein	*PrtF*	1693	2,100,449	100	0.0	99.79	CP002033.1
Peptide methionine sulfoxide reductase MsrA	*MsrA*	522	1,955,042	100	0.0	100	CP116618.1
		540	1,905,587	100	0.0	100	CP039750.1
Peptide methionine sulfoxide reductase MsrB	*MsrB*	426	2,100,449	100	0.0	100	CP002033.1
Chaperonin (heat shock protein 33)	*Hsp33*	882	2,224,515	100	0.0	100	CP188318.1
Translation elongation factor Tu	*EF-Tu*	1191	2,068,538	100	0.0	99.83	CP124737.1
Exopolysaccharide							
Manganese-dependent protein-tyrosine phosphatase	*EpsB*	771	2,042,277	100	0.0	98.31	CP021964.1
Tyrosine-protein kinase EpsD	*EpsD*	741	2,089,202	100	0.0	96.22	LT906621.1
T yrosine-protein kinase transmembrane modulator EpsC	*EpsC*	771	2,114,971	100	0.0	99.09	CP121468.1
Undecaprenyl-phosphate galactosephosphotransferase	*EpsE*	666	2,089,202	100	0.0	97.45	LT906621.1
		654	1,905,587	100	0.0	99.69	CP039750.1
Stress Resistance							
Cold shock							
Cold shock protein CspB	*CspB*	213	1,955,042	100	3e-105	100	CP116618.1
Cold shock protein CspA	*CspA*	201	2,161,679	100	1e-98	100	CP110798.1
PTS system, sucrose-specific IIA component	*EIIA*	1959	1,905,587	100	0.0	100	CP039750.1
Heat shock							
Ribosomal RNA small subunit methyltransferase E	*RsmE*	750	2,221,475	100	0.0	99.20	CP116755.1
DNA replication intiation control protein YabA	*YabA*	336	2,215,744	100	2e-173	100	CP118092.1
Heat-inducible transcription repressor HrcA	*HrcA*	1047	1,285,454	100	0.0	100	CP033371.1
Ribosomal protein L11 methyltransferase	*L11 + m*	963	2,173,659	100	0.0	99.79	CP184765.1
Figure 009886009886: phosphoesterase	*Figure 009886009886*	513	2,161,679	100	0.0	100	CP110798.1
tmRNA-binding protein SmpB	*SmpB*	474	2,100,449	100	0.0	99.79	CP002033.1
Translation elongation factor LepA	*Lep35A*	1833	2,315,729	100	0.0	99.95	CP170189.1
rRNA small subunit methyltransferase I	*RsmI*	861	2,297,851	100	0.0	99.88	CP011536.1
Nucleoside 5-triphosphatase RdgB (dHAPTP, dITP, XTP-specific) (EC 3.6.1.15)	*RdgB*	590	2,221,475	100	0.0	100	CP116755.1
		594	1,955,042	100	0.0	100	CP116618.1
Chaperone protein DnaJ	*DnaJ-fam*	1161	2,221,475	100	0.0	100	CP116755.1
Chaperone protein DnaK	*DnaK*	1857	1,285,454	100	0.0	99.95	CP033371.1
Heat shock protein GrpE	*GrpE*	588	1,285,454	100	0.0	100	CP033371.1
GTP-binding protein HflX	*HflX*	1278	2,068,538	100	0.0	99.84	CP124737.1
Heat-inducible transcription repressor HrcA	*HrcA*	1047	1,285,454	100	0.0	100	CP033371.1
Heat shock protein 60 family co-chaperone GroES	*GroES*	282	1,955,042	100	8e-142	99.65	CP116618.1
Heat shock protein 60 family chaperone GroEL	*GroEL*	1632	2,101,878	100	0.0	99.69	CP050919.1
ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)	*ClpP*	591	2,077,177	100	0.0	100	CP172330.1
ATP-dependent Clp protease, ATP-binding subunit ClpC	*ClpC*	2505	2,063,606	100	0.0	99.88	CP021790.1
ATP-dependent Clp protease ATP-binding subunit ClpX	*ClpX*	1251	2,042,277	100	0.0	99.92	CP021964.1
ClpB protein	*ClpB*	1881	2,297,851	100	0.0	100	CP011536.1
DedA protein	*DedA*	651	1,905,587	100	0.0	99.69	CP039750.1
		657	2,161,679	100	0.0	100	CP110798.1
Osmotic stress							
Glycine betaine ABC transport system, permease protein OpuAB	*OpuAB*	645	1,955,042	100	0.0	100	CP116618.1
Choline binding protein A	*ChA*	1122	2,093,653	100	0.0	99.91	CP120668.1
L-proline glycine betaine binding ABC transporter protein ProX	*ProX*	903	2,224,515	100	0.0	100	CP188318.1

Table 2 (continued)

Gene Function RAST/BLAST	Gene (Abbrev.)	Query Length	Accession Length	Query Cover (%)	E Value	Per Ident (%)	Accession
Glycine betaine ABC transport system, glycine betaine-binding protein OpuAC	*OpuAC*	645	1,955,042	100	0.0	100	CP116618.1
Oxidative stress							
Nicotinamidase	*PNC1*	576	2,221,475	100	0.0	100	CP116755.1
NAD-dependent protein deacetylase of SIR2 family	*SIR2*	1752	1,905,587	100	0.0	100	CP039750.1
Nicotinate phosphoribosyltransferase	*NPT1*	699	2,100,449	100	0.0	99.71	CP002033.1
NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	*GAPDH_6*	1014	2,315,729	100	0.0	100	CP170189.1
Glutaredoxin-like protein NrdH, required for reduction of Ribonucleotide reductase class Ib	*NrdH*	222	2,101,878	100	3e-110	100	CP050919.1
Ferroxidase (EC 1.16.3.1)	*Fr*	549	1,905,587	100	0.0	99.82	CP039750.1
		468	2,100,449	100	0.0	100	CP002033.1
Zinc uptake regulation protein ZUR	*ZUR*	450	1,955,042	100	0.0	99.78	CP116618.1
Peroxide stress regulator PerR, FUR family	*PerR2*	456	2,267,305	100	0.0	100	CP044354.1
Redox-sensitive transcriptional regulator (AT-rich DNA-binding protein)	*Rex*	651	1,955,042	100	0.0	100	CP116618.1
Iron-binding ferritin-like antioxidant protein	*IBP*	549	1,954,763	100	0.0	99.82	CP116617.1
		468	2,233,760	100	0.0	100	CP102532.1
Non-specific DNA-binding protein Dps	*Dps*	1017	1,954,763	100	0.0	99.82	CP116617.1
Transcriptional regulator, Crp/Fnr family	*Crp*	645	2,094,354	100	0.0	100	CP065522.1
		636	2,298,221	100	0.0	100	CP034099.1
		651	2,149,913	100	0.0	100	CP082359.1
Peptide methionine sulfoxide reductase MsrA	*MsrA*	522	1,955,042	100	0.0	100	CP116618.1
		540	1,905,587	100	0.0	100	CP039750.1
Bile salt tolerance							
Peptide methionine sulfoxide reductase MsrA	*MsrA*	522	1,955,042	100	0.0	100	CP116618.1
		540	1,905,587	100	0.0	100	CP039750.1
HPr kinase/phosphorylase	*HPrK/P*	964	2,224,515	100	0.0	99.9	CP188318.1
Argininosuccinate synthase	*ArgA*	1291	1,973,978	100	0.0	99.85	AP024320.1
Acid tolerance							
dTDP-glucose 4,6-dehydratase		934	2,086,671	100	0.0	98.61	CP101688.1
		1043	2,032,186	100	0.0	99.32	CP089305.1
dTDP-4-dehydrorhamnose reductase	*rmlD*	859	2,297,851	100	0.0	99.42	CP011536.1
Arginine deiminase	*ArcA*	1223	2,233,760	100	0.0	99.92	CP102532.1



64.3;Enterolysin_A

Function: 64.3;Enterolysin_A

Blast result UniRef90: Evalue=7.48e-38 match=48.408%;

BLAST protein

BLAST DNA

Fig. 5 Prediction of bacteriocin structure in the genome of *L. fermentum* JAC 231 using the BAGEL5 program

Table 3 Resistance genes identified using the CARD tool for *L. fermentum* JAC 231 strain

CARD (v. RGI 5.1.0)

RGI Criteria	**ARO Term**	**Detection criteria**	**AMR gene family**	**Drug Class**	**Resistance mechanism**
Strict	Gene vanT no cluster vanG	Protein Homologous Model	Glycopeptide resistance gene cluster, vanT Glycopeptide antibiotic	Glycopeptide antibiotic	Changing the antibiotic target

we observed a similar result; the *L. fermentum* ATCC 23,271 strain showed 6.22 Log$_{10}$ CFU/mL, while the *L. fermentum* JAC 231 showed 5.79 Log$_{10}$ CFU/mL.

Antagonistic and Antimicrobial Activities of *L. fermentum* JAC 231 and its Cell-Free Supernatant

The inhibitory activity of *L. fermentum* JAC 231 against reference bacterial strains was determined using an antagonistic test with an overlay method. *L. fermentum* JAC 231 inhibited the growth of all bacteria tested, with zones of inhibition ranging from 16.60±2.08 to 28.00±2.88 mm (Table 7).

Table 4 Genes involved in resistance to antibiotics and toxic compounds

Gene Function RAST/BLAST	Gene (Abbrev.)	Query Length	Accession Length	Query Cover (%)	E Value	Per Ident (%)	Accession
Beta-lactamase							
Beta-lactamase class C and other penicillin binding proteins	*BLc*	1021	2,298,221	100	0.0	99.9	CP034099.1
		1017	2,149,913	100	0.0	100	CP082359.1
Cobalt-zinc-cadmium resistance							
Cobalt-zinc-cadmium resistance protein	*CZCR*	552	2,001,184	100	0.0	100	CP091132.1
Cobalt-zinc-cadmium resistance protein CzcD	*CzcD*	907	2,042,277	100	0.0	99.56	CP021964.1
Probable cadmium-transporting ATPase (EC 3.6.3.3)	*PCT*	1931	2,221,475	100	0.0	99.84	CP116755.1
Transcriptional regulator, MerR family	*TRMer*	423	2,149,913	100	0.0	100	CP082359.1
		425	2,298,221	100	0.0	100	CP034099.1
		288	2,146,888	100	2e-141	99.68	CP035054.1
		126	2,298,221	100	1e-55	100	CP034099.1
Copper homeostasis							
Copper chaperone	*CopZ*	231	2,077,616	100	3e-112	100	CP119406.1
		229	2,068,538	100	2e-108	99.56	CP124737.1
Copper-translocating P-type ATPase (EC 3.6.3.4)	*CIA*	2286	2,233,760	100	0.0	99.73	CP102532.1
		2022	2,098,575	100	0.0	99,07	*CP174186.1*
Multicopper oxidase	*MO*	1540	2,098,685	100	0.0	99.68	AP008937.1
Negative transcriptional regulator-copper transport operon	*TR*	459	2,267,305	100	0.0	99.64	CP044354.1
		195	81,588	100	3e-95	100	CP096158.1
Mercuric reductase/Mercury resistance operon							
Mercuric ion reductase (EC 1.16.1.1)	*MerA*	1347	2,149,913	100	0.0	99.78	CP082359.1
PF00070 family, FAD-dependent NAD(P)-disulphide oxidoreductase	*PF00070*	1347	2,149,913	100	0.0	99.78	CP082359.1
Multidrug Resistance Efflux Pumps							
Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps	*MATE_all*	1320	2,001,184	100	0.0	99.92	CP091132.1
Multidrug-efflux transporter, major facilitator superfamily (MFS) (TC 2.A.1)	*MFS*	1693	2,238,401	100	0.0	99.84	CP045034.1
Resistance to fluoroquinolones							
DNA gyrase subunit A (EC 5.99.1.3)	*gyrA*	2511	2,034,355	100	0.0	100	CP095385.1
DNA gyrase subunit B (EC 5.99.1.3)	*gyrB*	1950	2,099,581	100	0.0	100	CP103293.1
Topoisomerase IV subunit A (EC 5.99.1.-)	*parC*	2478	2,267,305	100	0.0	99.96	CP044354.1
Topoisomerase IV subunit B (EC 5.99.1.-)	*parB*	1998	2,267,305	100	0.0	100	CP044354.1
Tetracycline resistance, ribosome protection type							
Ribosome protection-type tetracycline resistance related proteins, group 2	*Tet*-like2	1935	2,221,475	100	0.0	99.9	CP116755.1
Translation elongation factor G	*EF-G*	2085	2,165,664	100	0.0	100	CP160833.1

Table 5 Antimicrobial susceptibility testing results for *L. fermentum* JAC 231

Antimicrobials	*L. fermentum* JAC 231			
	Disk diffusion		Microdilution	
	Inhibition zone in mm (±SD) *	Interpretation*	MIC (µg/mL)	Activity**
Chloramphenicol	34.20 (±0.84)	S		
Tetracycline	28.40 (±1.14)	S		
Rifampicin	30.40 (±3.44)	S		
Penicillin G	41.60 (±1.14)	S		
Erythromycin	37.80 (±1.64)	S		
Nitrofurantoin	39.60 (±1.14)	S		
Gentamicin	14.20 (±1.79)	S		
Cefazolin	41.20 (±0.84)	S		
Clindamycin	30.40 (±0.89)	S		
Sulfazotrim	16.40 (±2.70)	S		
Oxacillin	13.75 (±0.96)	S		
Novobiocin	40.40 (±1.82)	S		
Teicoplanin	10.75 (±1.50)	S		
Tigecycline	31.00 (±1.58)	S		
Cefoxitin	27.80 (±1.92)	S		
Linezolid	29.20 (±1.92)	S		
Ciprofloxacin	14.00 (±1.00)	MS		
Vancomycin	0	R	>64	R

* Interpretation of results was based on Charteris et al. [49]. R: resistant; MS: moderately susceptible; S: sensitive. **Interpretative values for Vancomycin, according to the CLSI [50]: sensitive (S): ≤2 µg/mL; intermediate (I): 4–8 µg/mL; resistant (R): ≥16 µg/mL. All experiments were performed in triplicates in three independent experiments.

Table 6 Tolerance of *L. fermentum* JAC 231 and ATCC 23,271 to acidic pH and bile salts

Conditions	Survival % (±SD)*		*p*-value***
	L. fermentum JAC 231	*L. fermentum* ATCC 23271**	
pH 2.0	68.85 (±0.81)	83.85 (±2.23)	0.0077
pH 4.0	82.70 (±0.71)	92.27 (±1.37)	0.0005
Bile salts 5 g/L	87.34(±0.76)	95.58 (±0.92)	0.0003
Bile salts 10 g/L	84.76 (±0.35)	75.37 (±1.77)	0.0008

*The data represent the percentage survival of probiotic strains after 3 h of exposure to different gastrointestinal conditions compared to standard conditions. ** *L. fermentum* ATCC 23,271 was used as a positive control for the tests. ***Comparative analysis was performed using Student's t test ($p<0.05$).

The antimicrobial activity of the cell-free supernatant (CFS) was evaluated using the agar diffusion assay. The untreated CFS exhibited inhibitory activity against S*taphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, with inhibition zones ranging from 10.00±1.00 to 14.70±0.57 mm (Table 7). Neutralization of CFS resulted in a complete loss of inhibitory activity, whereas treatment with proteinase K or trypsin did not abolish inhibition, indicating that the antimicrobial effect is primarily mediated by acidic compounds rather than proteinaceous substances.

Inhibition of Pathogen Adhesion to HeLa Cells

The effect of *L. fermentum* JAC 231 on the adhesion of bacterial strains to HeLa cells was evaluated using three methods: competition, exclusion, and displacement (Fig. 7a-c). In the competition assays, the tested probiotic strains reduced the adhesion of *S. aureus* ATCC 25923, *P. aeruginosa* ATCC

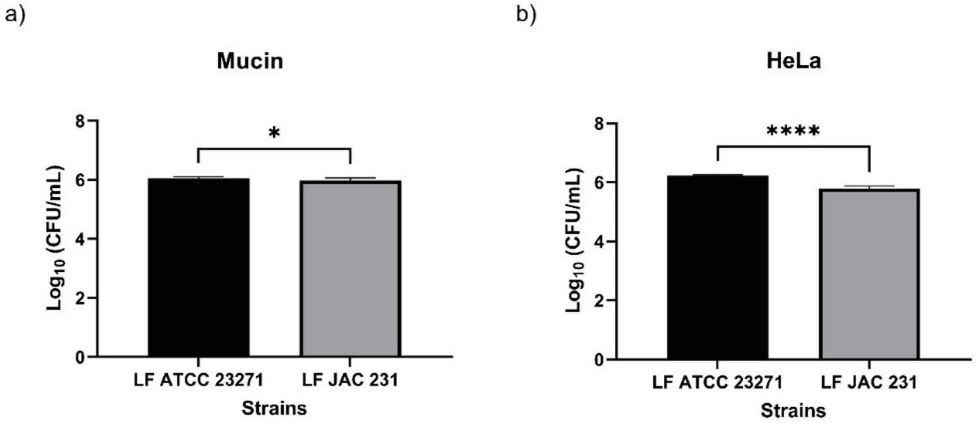


Fig. 6 Quantification of the adhesion of *L. fermentum* JAC 231 (LF JAC 231) to HeLa cells (**a**) and mucin (**b**) relative to that of the control *L. fermentum* ATCC 23271 (LF ATCC 27271). The values are expressed as Log10 CFU/mL and represent the average of the obtained values. Legend: (*) $p< 0.5$ and (****) $p<0.0001$

Table 7 Antimicrobial activity of *L. fermentum* JAC 231 and its cell-free supernatant

Strains	Inhibition zone in mm (±SD) *						
	Overlay method	CFS (untreated)	CFS pH-neutralized	CFS+proteinase K	CFS+Trypsin	MRS pH 4.0	MRS pH 6.0
S. aureus ATCC 25923	28.30 (±2.88)	10.00 (±1.00)	-	9.0 (±1.00)	9.00 (±1.00)	-	-
E. faecalis ATCC 29212	18.60 (±1.15)	-	-	-	-	-	-
K. pneumoniae ATCC 700603	21.00 (±3.21)	-	-	-	-	-	-
P. aeruginosa ATCC 27853	26.00 (±1.73)	14.70 (±0.57)	-	10.33 (±0.57)	10.33 (±1.15)	-	-
EPEC E2348/69	24.40 (±2.88)	-	-	-	-	-	-
E. coli ATCC 25922	16.60 (±2.08)	10.30 (±0.57)	-	8.60 (±0.57)	8.33 (±0.57)	-	-
S. enteriditis ATCC 13076	26.60 (±2.88)	-	-	-	-	-	-

*Values represent the mean in mm (±standard deviation - SD) of triplicate assays performed on three separate days. (–) indicates no detectable inhibition.

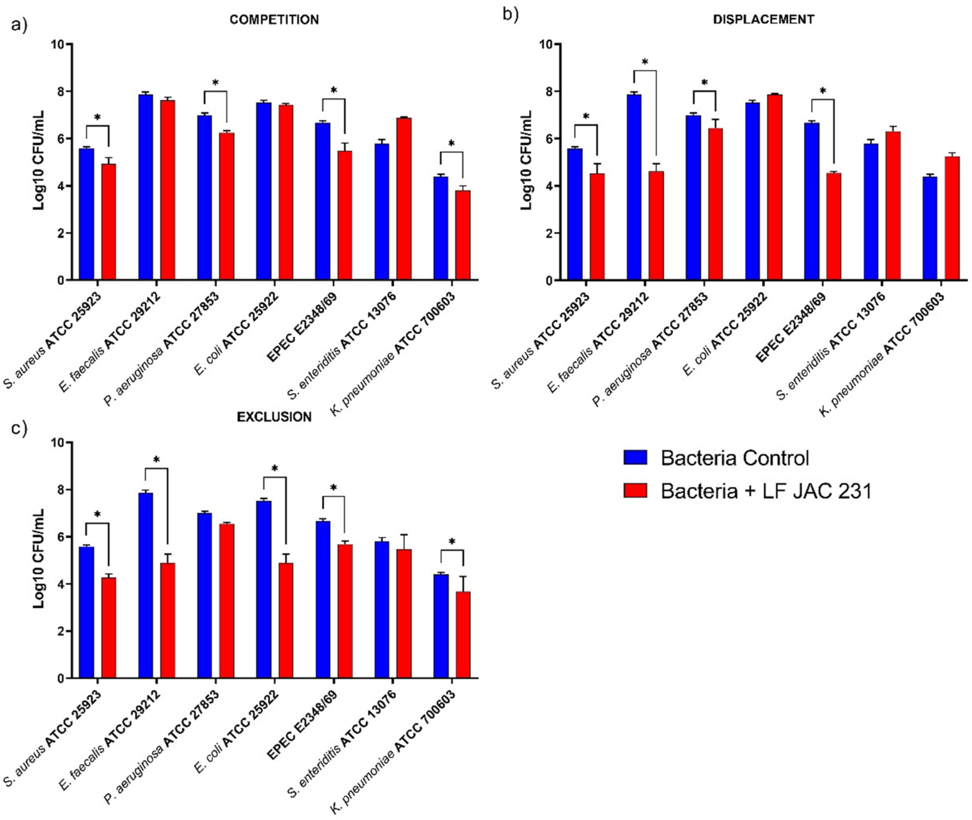


Fig. 7 Inhibition of pathogen adhesion to HeLa cells by *L. fermentum* JAC 231 in three experiments: (**a**) competition, (**b**) displacement, and (**c**) exclusion. Two-way ANOVA followed by Dunnet analysis. * $p < 0.05$

27853, EPEC E2348/69, and *K. pneumoniae* ATCC 700603 (Fig. 7a). In the displacement assays, where the probiotic strain was introduced after the adhesion of pathogenic bacteria, we observed better interference with the adhesion of *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853, and EPEC E2348/69 (Fig. 7b).

In the displacement experiments, we observed a reduction in four bacterial strains (*S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853, and EPEC E2348/69) (Fig. 7c). In all tests, *S. enteritidis* ATCC 13076 adhered more or had a CFU/mL count equivalent to that of the control, which contained only the bacteria, when incubated in the presence of *L. fermentum* JAC 231.

Discussion

This study presents a comprehensive genomic and phenotypic evaluation of *L. fermentum* JAC 231, a strain previously isolated from the vaginal microbiota of an asymptomatic woman [28]. The findings build on earlier evidence of anti-*Candida* activity by demonstrating probiotic potential through genomic insights, stress resilience, adhesion capacity, antimicrobial effects, and safety. Together, these data contribute to the growing evidence base positioning *L. fermentum* strains as promising candidates for therapeutic and prophylactic use as probiotics in humans.

The genomic data of *L. fermentum* JAC 231 revealed a genome of approximately 2.06 Mbp with GC content of 51.4%. These features are consistent with those of previously sequenced *L. fermentum* strains, such as ATCC 23,271 and AGR1485 [1, 57–59]. Phylogenetic analysis based on core genome alignments confirmed that JAC 231 clustered closely with strains known for their probiotic and health-promoting effects, including AGR 1485, CECT 5716, MCC 2760, and IFO 3976. This evolutionary proximity supports the hypothesis that JAC 231 shares conserved probiotic determinants with these strains [37, 60].

Comparative genomics revealed that *Limosilactobacillus fermentum* JAC 231 shares 1,587 orthologous clusters with other probiotic strains of the species, including ATCC 23271 (reported anti-*Candida* activity) and CECT 5716 (known immunomodulatory properties) [10, 21, 61]. Only one lineage-specific cluster encoding two hypothetical proteins was identified, indicating that strain-specific genetic determinants are limited. Therefore, the particular features of JAC 231 are unlikely to be explained by unique genes but rather by its functional phenotype, potentially shaped by its ecological origin and the specific combination of conserved traits it expresses, reinforcing the need to integrate genomic insights with experimental validation.

In contrast to strains such as *L. fermentum* CECT 5716, originally isolated from human milk, and ATCC 23271, obtained from the gastrointestinal tract, JAC 231 was recovered from the vaginal microbiota of a healthy asymptomatic woman [28]. This ecological niche may contribute to adaptive traits associated with persistence and microbial interactions in the urogenital environment. Consistent with this possibility, JAC 231 exhibited a dual functional profile characterized by broad antibacterial activity and marked anti-*Candida* effects. In our previous study, JAC 231 significantly reduced the virulence of *C. albicans*, including hyphal formation, biofilm development, and adhesion to epithelial cells [28].

The present findings extend this functional profile by demonstrating its activity against several bacterial pathogens. In competition, exclusion, and displacement assays, JAC 231 reduced the adhesion of both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Escherichia coli*. This interference likely reflects multiple mechanisms, including organic acid production, the presence of genes potentially associated with bacteriocin-related activity (e.g., a sequence with 48% identity to enterolysin A), and anti-adhesive interactions with epithelial surfaces. Taken together, the vaginal origin of JAC 231 and its combination of antagonistic mechanisms against both fungal and bacterial pathogens highlight a distinct functional profile that may differentiate this strain from other *L. fermentum* isolates and support its potential as a niche-adapted probiotic for urogenital health.

In this study, we used data mining of the genomic sequences of *L. fermentum* JAC 231 to discover genes that encode proteins responsible for adhesion, survival, and adaptation to adverse gastrointestinal environments, traits that are associated with probiotic functions. These include genes involved in the biosynthesis of essential B-complex vitamins (thiamine, riboflavin, pyridoxine, biotin, and folate), which not only support host metabolism but also enhance microbial competitiveness in the gastrointestinal tract [62–64]. The presence of multiple genes related to oxidative stress tolerance, heat shock proteins, osmotic stress resistance, and acid and bile tolerance further suggests that *L. fermentum* JAC 231 can endure harsh conditions encountered during gastrointestinal transit, which is a prerequisite for probiotic efficacy [22, 65].

Importantly, the genome encodes key adhesion-related proteins, such as Sortase A, fibronectin-binding proteins, and LPxTG motif-containing surface proteins. These molecules play essential roles in the adhesion of probiotic bacteria to epithelial surfaces and mucins, facilitating colonization and immunomodulatory interactions in the gut [66, 67]. Although *L. fermentum* JAC 231 exhibited lower in vitro adhesion to mucin and HeLa cells than the reference strain *L. fermentum* ATCC 23,271, this finding does not necessarily contradict its probiotic potential. Adhesion is strain-specific and multifactorial, and *L. fermentum* JAC 231 may exert anti-adhesive effects via competitive exclusion, steric hindrance or modulation of host cell receptors [10].

Indeed, despite modest adhesion, *L. fermentum* JAC 231 significantly inhibited the adhesion of pathogens such as *S. aureus*, *E. faecalis*, *P. aeruginosa*, and EPEC to epithelial cells. This effect was observed in competitive, exclusion, and displacement assays, indicating a robust ability to interfere with pathogen colonization of the host. Such anti-adhesive activity is supported by the presence of adhesin-encoding genes and the production of exopolysaccharides, which may form steric barriers or promote host cell signaling that suppresses pathogen attachment [66–68].

JAC 231 also displayed strong antimicrobial activity in agar diffusion assays against a range of pathogens, including Gram-positive and Gram-negative bacteria. Experimental evaluation using treated CFS demonstrated that this inhibitory effect was predominantly mediated by organic acid production, as antimicrobial activity was abolished after pH neutralization and was not affected by protease treatment. These results indicate that the acidic compounds are responsible for the antimicrobial activity observed under the tested conditions [69]. In the BAGEL 5 database, a cluster of genes encoding bacteriocin enterolysin A was detected with 48% sequence identity. Enterolysin A is a class III bacteriocin initially identified in *Enterococcus faecalis* and other LAB, such as *Lactobacillus plantarum* and *Lactococcus lactis* [70, 71]. Initial analyses revealed that the antimicrobial activity of this bacteriocin against gram-positive bacteria affects cell wall degradation [70, 72]. The presence of this bacteriocin in other *L. fermentum* strains has also been reported. Santos et al. [59] demonstrated that the *L. fermentum* ATCC 23,271 strain presented a coding region for a protein similar to enterolysin A, which, through three algorithms of the CAMPR3 tool, indicated a high probability of possessing antimicrobial activity.

In the evaluation of probiotic candidates, it is crucial to assess potential risks, particularly the possibility of antibiotic resistance mechanisms and horizontal gene transfer to other microorganisms [20, 73]. To address these concerns, in vitro testing and, more recently, genome analysis have been employed to assess the safety and other factors related to pathogenicity and virulence [60, 74]. Analysis of the *L. fermentum* JAC 231 genome using RAST, PlasmidFinder, PathogenFinder, and VirulenceFinder tools did not reveal the presence of mobile genetic elements or pathogenicity-related genes. However, CARD and RAST tools indicated the presence of glycopeptide-class antibiotic resistance genes (vancomycin).

Vancomycin resistance is one of the most well-documented antibiotic resistance mechanisms within the LAB group and is considered intrinsic and non-transferable [73, 75]. In our antibiotic susceptibility testing, *L. fermentum* JAC 231 demonstrated moderate sensitivity to ciprofloxacin and resistance to vancomycin, as previously reported using in silico tools. This finding is consistent with that of Anisimova and Yarullina [76], who studied the antibiotic resistance patterns of 20 strains of *Lactobacillus*, including *L. fermentum*, and found that the majority of the isolates were resistant to vancomycin, ciprofloxacin, and aminoglycosides, with five strains showing resistance to these antibiotics. These results indicate a strong relationship between genotype and phenotype, as previously reported [59, 75, 76], highlighting the need for tools that combine genotypic and phenotypic analyses to evaluate the safety of probiotic candidates [24, 59].

L. fermentum JAC 231 exhibited an incomplete prophage region, which is likely non-functional, as well as two CRISPR sequences connected to the Cas genes. CRISPR–Cas genes serve as an essential defense mechanism for bacterial cells, preventing the spread of infections by bacteriophages and the insertion of foreign DNA into the bacterial genome [77]. Together, these findings strongly support the safety profile and suitability of this strain for probiotic use, especially considering the increasing concern over antimicrobial resistance dissemination.

From a broader perspective, the probiotic profile of JAC 231 reflects the characteristics of other well-studied *L. fermentum* strains. For instance, CECT 5716, a strain used in commercial formulations, has shown efficacy in preventing infections and modulating immune response [64, 78]. MCC 2760, another clinically evaluated strain, exhibits anti-inflammatory, antidiabetic, and cholesterol-lowering effects [79–82]. The genomic and phenotypic similarities between JAC 231 and these strains strengthen the rationale for its development as a multifunctional probiotic.

However, this study had some important limitations. First, all probiotic traits were assessed using in vitro assays and bioinformatic analysis. Although informative, these approaches do not capture the complexity of host-microbe interactions, immune responses, or ecological competition within the human body [83, 84]. Second, gene expression under host-relevant conditions remains unexplored. It is essential to validate whether the identified genes are actively transcribed and translated in vivo in humans. Third, antimicrobial assays, while indicative, do not confirm therapeutic efficacy in situ, where complex variables such as pH, enzymatic degradation, and microbiota interactions play key roles.

In addition, the long-term colonization potential and technological stability of this strain have not been evaluated.

These aspects are crucial for the design of probiotic products and their regulatory approval. Future investigations should incorporate animal models, human clinical trials, and functional studies on gene expression, bioactive compound production and host immune modulation.

Conclusion

L. fermentum JAC 231 displays a compelling combination of genomic, phenotypic, and functional features that support its potential as a probiotic. It possesses a stable genome enriched with genes related to stress tolerance, vitamin biosynthesis, adhesion, and immune system interaction. Phenotypically, the strain survived acidic and bile stress, inhibited the adhesion of major pathogens, and exhibited broad-spectrum antimicrobial activity. Its in silico and in vitro safety profiles further underscore its suitability for therapeutic applications.

The phylogenetic proximity of JAC 231 to other validated probiotic strains, coupled with its multifunctional traits, positions it as a strong candidate for clinical development for the treatment of mucosal infections, biofilm-associated diseases, and possibly metabolic or inflammatory disorders. However, robust in vivo validation, mechanistic studies, and technological assessments are required before clinical application. Addressing these gaps is pivotal in determining the true potential of this strain as a safe, effective, versatile probiotic agent.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12602-026-11025-7.

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Data Availability The genomic data of *L. fermentum* JAC 231 was submitted to NCBI (National Centre for Biotechnology Information) under the accession number JAVFHY000000000. Raw sequencing reads (FASTQ files) are available in the NCBI Sequence Read Archive (SRA) under accession number SRR37146884. The BioProject accession number PRJNA1007394 and BioSample is SAMN37068468.

Declarations

Competing interests The authors declare no competing interests.

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5. CONSIDERAÇÕES FINAIS

Os resultados deste estudo confirmam as características e o perfil de segurança de *L. fermentum* JAC 231 isolado da cavidade vaginal de uma paciente assintomática como uma linhagem probiótica. A análise do genoma demonstrou ser eficaz para avaliar as propriedades benéficas dessa linhagem, mostrando correlação com os dados obtidos *in vitro*. Entre essas propriedades temos a tolerância aos sais biliares e pH, indicando que ela possa apresentar alta estabilidade ao passar pelo trato gastrointestinal, após ser ingerido. Ausência de genes envolvidos em aspectos de virulência, patogenicidade e resistência a antibióticos. Adicionalmente a essas características, a *L. fermentum* JAC 231 também apresentou atividade antagonista e anti-adesiva dos patógenos testados. Estudos posteriores são necessários para elucidar os mecanismos pelos quais essa linhagem exerce seus efeitos antimicrobianos. Além de testes para avaliar a sua eficácia probiótica em ensaios *in vivo*. Em resumo, este estudo indica o uso promissor *L. fermentum* JAC 231 como probiótico de amplo espectro.

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Comprehensive Genomic Characterization and Probiotic Properties Evaluation of *Limosilactobacillus fermentum* JAC 231 Isolated from Vaginal Microbiota

Carmem Duarte Lima Campos[1], Amanda Graziella Goncalves Mendes[1], José Lima Pereira-Filho[1], Viviane da Silva Sousa Almeida[1], Ana Beatriz Santos Sousa[1], Israel Viegas Moreira[1], João Lucas do Carmo Lima[1], Aleania Polassa Almeida Pereira[1], Allysson Kayron Carvalho Silva[2], Laís Araújo Souza Wolff[2], Kátia Regina Assunção Borges[2], Cristianne Roberta Rhoden[3], Cinara Regina Aragão Vieira Monteiro[1], Cristina de Andrade Monteiro[4], Elizabeth Soares Fernandes[5], Valério Monteiro-Neto[1]*.

[1] Programa de Pós-Graduação em Ciências da Saúde – PPGCS, Centro de Ciências Biológicas e da Saúde, Universidade Federal do Maranhão. Avenida dos Portugueses, 1966, campus Bacanga, São Luís, MA, 65080-040, Brazil;

[2] Programa de Doutorado em Biotecnologia – Rede Nordeste de Biotecnologia (RENORBIO). Universidade Federal do Maranhão. Avenida dos Portugueses, 1966, campus Bacanga, São Luís, MA, 65080-040, Brazil;

[3] Laboratório Cedro, Avenida Silva Maia, 81, Centro, São Luís MA, 65020-570, Brazil

[4] Instituto Federal de Educação Ciência e Tecnologia do Maranhão, Avenida Getúlio Vargas, 4, Monte Castelo, São Luís, 65030-005, MA, Brazil.

[5] Department of Animal and Veterinary Sciences - ANIVET Ruminant Nutrition (RUN), Aarhus University, Denmark

* Correspondence to: valerio.monteiro@ufma.br.

Figure S1. Orthologous cluster analysis of the six strains of *L. fermentum*: JAC 231, AGR 1485, ATCC 23271, CECT 5716, IFO 3956, and MCC 2760.

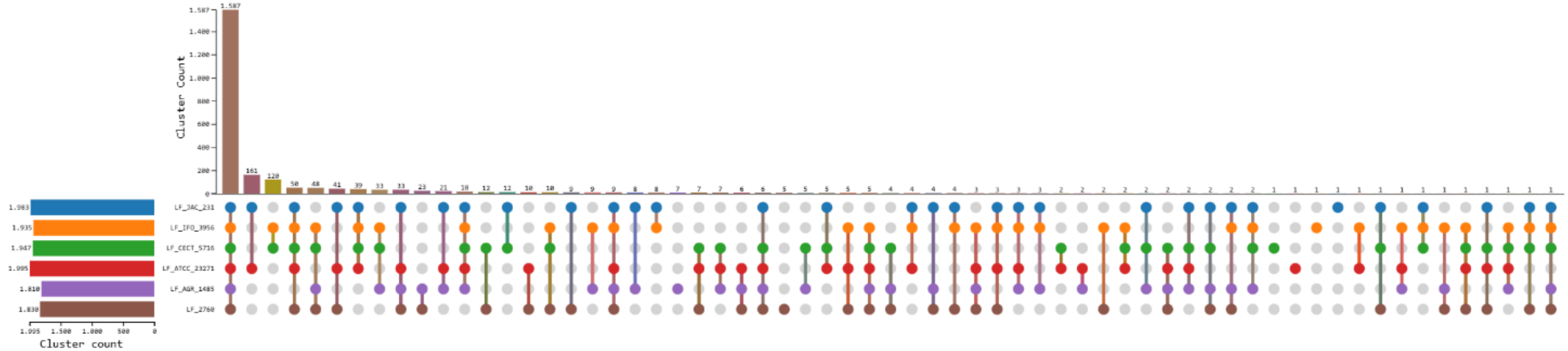

Figure S2. Distribution of orthogroups among *L. fermentum* strains. The plot highlights the core genome, which is composed of 1,587 conserved orthogroups, as well as species-specific adaptations. The strain *L. fermentum* JAC 231 contains 1,983 orthogroups, including one unique cluster. The presence of unique and subset-specific orthogroups suggests functional specialization, reflecting the evolutionary divergence among strains.

Table S1. RAST Subsystems and features of *L. fermentum* JAC 231

Category - subcategory - Subsystem	Gene count
Amino Acids and Derivatives	
Alanine, serine, and glycine	
Alanine biosynthesis	3
Glycine and Serine Utilization	8
Glycine Biosynthesis	1
Serine Biosynthesis	3
Amino Acids and Derivatives - no subcategory	
Creatine and Creatinine Degradation	1
Arginine; urea cycle, polyamines	
Arginine and Ornithine Degradation	7
Arginine Biosynthesis -- gjo	10
Arginine Biosynthesis extended	10
Arginine Deiminase Pathway	5
Aromatic amino acids and derivatives	
Common Pathway For Synthesis of Aromatic Compounds (DAHP synthase to chorismate)	2
Phenylalanine and Tyrosine Branches from Chorismate	3
Glutamine, glutamate, aspartate, asparagine; ammonia assimilation	
Glutamate and Aspartate uptake in Bacteria	2
Glutamine synthetases	1
Glutamine, Glutamate, Aspartate and Asparagine Biosynthesis	7
Lysine, threonine, methionine, and cysteine	
Lysine Biosynthesis DAP Pathway	9
Lysine Biosynthesis DAP Pathway, GJO scratch	9
Methionine Biosynthesis	11
Methionine Degradation	7
S-methylmethionine	2
Threonine and Homoserine Biosynthesis	6
Threonine degradation	1
Proline and 4-hydroxyproline	
Proline Synthesis	3
Carbohydrates	
Central carbohydrate metabolism	
Dehydrogenase complexes	3
Glycolate, glyoxylate interconversions	3
Glycolysis and Gluconeogenesis	8
Pentose phosphate pathway	8
Pyruvate Alanine Serine Interconversions	4
Pyruvate metabolism I: anaplerotic reactions, PEP	5
Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate	8
Di- and oligosaccharides	
Lactose and Galactose Uptake and Utilization	9
Sucrose utilization	5

Trehalose Biosynthesis	1
Trehalose Uptake and Utilization	2
Fermentation	
Acetoin, butanediol metabolism	2
Acetyl-CoA fermentation to Butyrate	4
Fermentations: Lactate	5
Monosaccharides	
Deoxyribose and Deoxynucleoside Catabolism	3
D-galactarate, D-glucarate and D-glycerate catabolism	3
D-galactarate, D-glucarate and D-glycerate catabolism - gjo	3
D-gluconate and ketogluconates metabolism	4
D-ribose utilization	4
Mannose Metabolism	3
Xylose utilization	5
One-carbon Metabolism	
One-carbon metabolism by tetrahydropterines	4
Organic acids	
Alpha-acetolactate operon	2
Glycerate metabolism	5
Lactate utilization	4
Sugar alcohols	
Glycerol and Glycerol-3-phosphate Uptake and Utilization	5
Cell Division and Cell Cycle	
Cell Division and Cell Cycle - no subcategory	
Bacterial Cytoskeleton	16
Control of cell elongation - division cycle in Bacilli	7
Macromolecular synthesis operon	7
YgjD and YeaZ	2
Checkpoint control	
Bacterial checkpoint-control-related cluster	4
Cell Wall and Capsule	
Capsular and extracellular polysacchrides	
dTDP-rhamnose synthesis	4
Exopolysaccharide Biosynthesis	4
Rhamnose containing glycans	5
Sialic Acid Metabolism	5
Cell Wall and Capsule - no subcategory	
Murein Hydrolases	3
Peptidoglycan Biosynthesis	16
Peptidoglycan biosynthesis--gjo	6
Recycling of Peptidoglycan Amino Acids	1
UDP-N-acetylmuramate from Fructose-6-phosphate Biosynthesis	6
YjeE	4
Gram-Positive cell wall components	
D-Alanyl Lipoteichoic Acid Biosynthesis	4
Polyglycerolphosphate lipoteichoic acid biosynthesis	5
Sortase	1

Teichoic and lipoteichoic acids biosynthesis	7
Teichuronic acid biosynthesis	1
Cofactors, Vitamins, Prosthetic Groups, Pigments	
Biotin	
Biotin biosynthesis	3
Coenzyme A	
Coenzyme A Biosynthesis	7
Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	
Thiamin biosynthesis	8
Folate and pterines	
5-FCL-like protein	12
Folate Biosynthesis	10
Folate biosynthesis cluster	7
Molybdenum cofactor biosynthesis	11
Lipoic acid	
Lipoic acid metabolism	1
NAD and NADP	
NAD and NADP cofactor biosynthesis global	8
Pyridoxine	
Pyridoxin (Vitamin B6) Biosynthesis	6
Pyridoxin(Vitamin B6) Degradation Pathway	1
Riboflavin, FMN, FAD	
Flavodoxin	3
riboflavin to FAD	4
Riboflavin, FMN and FAD metabolism	9
Riboflavin, FMN and FAD metabolism in plants	12
Tetrapyrroles	
Heme and Siroheme Biosynthesis	3
DNA Metabolism	
CRISPs	
CRISPRs	7
DNA Metabolism - no subcategory	
DNA structural proteins, bacterial	2
Restriction-Modification System	3
Type I Restriction-Modification	3
YcfH	1
DNA recombination	
RuvABC plus a hypothetical	3
DNA repair	
ATP-dependent Nuclease	2
DNA Repair Base Excision	7
DNA repair system including RecA, MutS and a hypothetical protein	2
DNA repair, bacterial	14
DNA repair, bacterial DinG and relatives	1
DNA repair, bacterial MutL-MutS system	3
DNA repair, bacterial RecBCD pathway	1
DNA repair, bacterial RecFOR pathway	8

DNA repair, bacterial UvrD and related helicases	1
DNA repair, UvrABC system	4
Uracil-DNA glycosylase	2
DNA replication	
DNA topoisomerases, Type I, ATP-independent	2
DNA topoisomerases, Type II, ATP-dependent	4
DNA uptake, competence	
DNA processing cluster	5
Gram Positive Competence	7
Dormancy and Sporulation	
Dormancy and Sporulation - no subcategory	
Sporulation-associated proteins with broader functions	1
Fatty Acids, Lipids, and Isoprenoids	
Fatty acids	
Fatty Acid Biosynthesis FASII	11
Fatty acid metabolism cluster	2
Fatty Acids, Lipids, and Isoprenoids - no subcategory	
Polyhydroxybutyrate metabolism	4
Isoprenoids	
Isoprenoid Biosynthesis	10
Isoprenoinds for Quinones	2
Mevalonate Branch of Isoprenoid Biosynthesis	6
Phospholipids	
Cardiolipin synthesis	1
Glycerolipid and Glycerophospholipid Metabolism in Bacteria	13
Triacylglycerols	
Triacylglycerol metabolism	1
Iron acquisition and metabolism	
Iron acquisition and metabolism - no subcategory	
Encapsulating protein for DyP-type peroxidase and ferritin-like protein oligomers	1
Membrane Transport	
Cation transporters	
Copper Transport System	1
Magnesium transport	2
Transport of Nickel and Cobalt	1
Protein translocation across cytoplasmic membrane	
Bacterial signal recognition particle (SRP)	3
EcsAB transporter affecting expression and secretion of secretory preproteins	4
Uni- Sym- and Antiporters	
Proton-dependent Peptide Transporters	1
Metabolism of Aromatic Compounds	
Peripheral pathways for catabolism of aromatic compounds	
Biphenyl Degradation	2
Miscellaneous	
Miscellaneous - no subcategory	
Broadly distributed proteins not in subsystems	2

DedA family of inner membrane proteins	1
Muconate lactonizing enzyme family	1
Phosphoglycerate mutase protein family	4
Plant-Prokaryote DOE project	
Conserved gene cluster possibly involved in RNA metabolism	2
Iron-sulfur cluster assembly	7
Nitrogen Metabolism	
Denitrification	
Denitrifying reductase gene clusters	4
Nitrogen Metabolism - no subcategory	
Nitrate and nitrite ammonification	5
Nitrosative stress	1
Nucleosides and Nucleotides	
Detoxification	
Housecleaning nucleoside triphosphate pyrophosphatases	4
Nudix proteins (nucleoside triphosphate hydrolases)	2
Nucleosides and Nucleotides - no subcategory	
Ribonucleotide reduction	7
Purines	
De Novo Purine Biosynthesis	15
Purine conversions	15
Purine Utilization	3
Xanthine Metabolism in Bacteria	2
Pyrimidines	
De Novo Pyrimidine Synthesis	9
pyrimidine conversions	11
Phages, Prophages, Transposable elements, Plasmids	
Phages, Prophages	
Phage capsid proteins	2
Phage packaging machinery	3
Phage replication	3
Phosphorus Metabolism	
Phosphorus Metabolism - no subcategory	
High affinity phosphate transporter and control of PHO regulon	7
Phosphate metabolism	10
Polyphosphate	2
Potassium metabolism	
Potassium metabolism - no subcategory	
Potassium homeostasis	5
Protein Metabolism	
Protein biosynthesis	
Glycyl-tRNA synthetase	2
Programmed frameshift	1
Ribosome activity modulation	1
Ribosome biogenesis bacterial	11
Translation elongation factor G family	2
Translation elongation factors bacterial	5

Translation initiation factors bacterial	5
Translation termination factors bacterial	9
tRNA aminoacylation, Ala	1
tRNA aminoacylation, Arg	1
tRNA aminoacylation, Asp and Asn	5
tRNA aminoacylation, Cys	1
tRNA aminoacylation, Glu and Gln	5
tRNA aminoacylation, Gly	2
tRNA aminoacylation, His	1
tRNA aminoacylation, Ile	1
tRNA aminoacylation, Leu	1
tRNA aminoacylation, Lys	1
tRNA aminoacylation, Met	1
tRNA aminoacylation, Phe	3
tRNA aminoacylation, Pro	1
tRNA aminoacylation, Ser	1
tRNA aminoacylation, Thr	1
tRNA aminoacylation, Trp	1
tRNA aminoacylation, Tyr	1
tRNA aminoacylation, Val	1
tRNAs	12
Universal GTPases	17
Protein degradation	
Metallocarboxypeptidases (EC 3.4.17.-)	1
Protein degradation	2
Proteolysis in bacteria, ATP-dependent	7
Protein folding	
GroEL GroES	6
Peptidyl-prolyl cis-trans isomerase	1
Protein chaperones	4
Protein processing and modification	
Lipoprotein Biosynthesis	2
Peptide methionine sulfoxide reductase	2
Signal peptidase	2
Regulation and Cell signaling	
Regulation and Cell signaling - no subcategory	
cAMP signaling in bacteria	3
Cell envelope-associated LytR-CpsA-Psr transcriptional attenuators	1
HPr catabolite repression system	2
LysR-family proteins in Escherichia coli	1
Sex pheromones in Enterococcus faecalis and other Firmicutes	3
Stringent Response, (p)ppGpp metabolism	1
Respiration	
Electron accepting reactions	
Anaerobic respiratory reductases	3
Terminal cytochrome d ubiquinol oxidases	4
Terminal cytochrome oxidases	4

Electron donating reactions	
Respiratory dehydrogenases 1	3
Succinate dehydrogenase	1
RNA Metabolism	
RNA processing and modification	
16S rRNA modification within P site of ribosome	6
Ribonuclease H	3
Ribonucleases in Bacillus	5
RNA methylation	12
RNA processing and degradation, bacterial	4
RNA pseudouridine syntheses	6
tRNA modification Bacteria	19
tRNA nucleotidyltransferase	1
tRNA processing	7
Transcription	
RNA polymerase bacterial	5
Rrf2 family transcriptional regulators	1
Transcription factors bacterial	9
Transcription initiation, bacterial sigma factors	1
Stress Response	
Cold shock	
Cold shock, CspA family of proteins	2
Detoxification	
Uptake of selenate and selenite	1
Heat shock	
Heat shock dnaK gene cluster extended	12
Osmotic stress	
Choline and Betaine Uptake and Betaine Biosynthesis	4
Oxidative stress	
Glutaredoxins	1
Glutathione: Biosynthesis and gamma-glutamyl cycle	1
Glutathione: Redox cycle	1
Oxidative stress	7
Redox-dependent regulation of nucleus processes	4
Stress Response - no subcategory	
Dimethylarginine metabolism	1
Hfl operon	1
Sulfur Metabolism	
Sulfur Metabolism - no subcategory	
Galactosylceramide and Sulfatide metabolism	3
Thioredoxin-disulfide reductase	4
Virulence, Disease and Defense	
Adhesion	
Streptococcus pyogenes recombinatorial zone	2
Invasion and intracellular resistance	
Mycobacterium virulence operon involved in an unknown function with a Jag Protein and YidC and YidD	2

Mycobacterium virulence operon involved in DNA transcription	2
Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	3
Mycobacterium virulence operon involved in protein synthesis (SSU ribosomal proteins)	4
Resistance to antibiotics and toxic compounds	
Beta-lactamase	1
Cobalt-zinc-cadmium resistance	4
Copper homeostasis	4
Mercuric reductase	2
Mercury resistance operon	1
Multidrug Resistance Efflux Pumps	2
Resistance to fluoroquinolones	4
Tetracycline resistance, ribosome protection type	2
Tetracycline resistance, ribosome protection type, too	2
Total	**1014**

Table S2. KEGG orthology (KO) categories of identified protein-coding genes in the *L. fermentum* JAC 231 genome.

CLASS - Category functional - Definition	Gene Count
Cellular Processes	**39**
Cell growth and death	**11**
Cell cycle - Caulobacter	10
Necroptosis	1
Cell motility	**3**
Cytoskeleton in muscle cells	1
Flagellar assembly	2
Cellular community - prokaryotes	**13**
Biofilm formation - Escherichia coli	1
Biofilm formation - Vibrio cholerae	2
Quorum sensing	10
Transport and catabolism	**12**
Efferocytosis	10
Peroxisome	2
Environmental Information	**89**
Processing Membrane transport	**49**
ABC transporters	31
Bacterial secretion system	8
Phosphotransferase system PTS	10
Signal transduction	**40**
HIF- 1 signaling pathway	6
MAPK signaling pathway - plant	2
Phosphatidylinositol signaling system	2
Two-component system	30
Genetic Information Processing	**156**
Folding, sorting and degradation	**27**
Protein export	10
RNA degradation	9
Sulfur relay system	8
Replication and repair	**66**
Base excision repair	9
DNA replication	15
Homologous recombination	19
Mismatch repair	16
Nucleotide excision repair	7
Transcription	**5**
RNA polymerase	5
Translation	**58**
Aminoacyl-tRNA biosynthesis	4
Ribosome	53
Ribosome biogenesis in eukaryotes	1
Human Diseases	**63**
Infectious disease: viral	**1**

Human papillomavirus infection	1
Cancer: overview	**11**
Central carbon metabolism in cancer	7
MicroRNAs in cancer	1
Pathways in cancer	1
Proteoglycans in cancer	1
Viral carcinogenesis	1
Cancer: specific types	**1**
Renal cell carcinoma	1
Cardiovascular disease	**9**
Diabetic cardiomyopathy	6
Fluid shear stress and atherosclerosis	2
Lipid and atherosclerosis	1
Drug resistance: antimicrobial	**15**
beta-Lactam resistance	3
Cationic antimicrobial peptide CAMP resistance	7
Vancomycin resistance	5
Drug resistance: antineoplastic	**5**
Antifolate resistance	4
Platinum drug resistance	1
Endocrine and metabolic disease	**4**
Cushing syndrome	1
Insulin resistance	1
Type I diabetes mellitus	1
Type II diabetes mellitus	1
Infectious disease: bacterial	**13**
Legionellosis	2
Pathogenic Escherichia coli infection	1
Salmonella infection	2
Staphylococcus aureus infection	5
Tuberculosis	3
Infectious disease: parasitic	**2**
Amoebiasis Immune disease	2
Neurodegenerative disease	**2**
Alzheimer disease	1
Parkinson disease	1
Metabolism	**1435**
Amino acid metabolism	**112**
Alanine, aspartate and glutamate metabolism	17
Arginine and proline metabolism	6
Arginine biosynthesis	12
Cysteine and methionine metabolism	21
Glycine, serine and threonine metabolism	11
Histidine metabolism	6
Lysine biosynthesis	14
Lysine degradation	3
Phenylalanine metabolism	2

Phenylalanine, tyrosine and tryptophan biosynthesis	5
Tryptophan metabolism	2
Tyrosine metabolism	6
Valine, leucine and isoleucine biosynthesis	2
Valine, leucine and isoleucine degradation	5
Biosynthesis of other secondary metabolites	**24**
Acarbose and validamycin biosynthesis	1
Biosynthesis of various antibiotics; Including: Kanosamine biosynthesis, Aurachin biosynthesis, Bacilysin biosynthesis, Puromycin biosynthesis, Dapdiamides biosynthesis, Fosfomycin biosynthesis, Cremeomycin biosynthesis, Fumagillin biosynthesis, Pentalenolactone biosynthesis, Terpentecin biosynthesis, Roseoflavin biosynthesis, Cycloserine biosynthesis	1
Biosynthesis of various plant secondary metabolites; Including: Crocin biosynthesis, Ginsenoside biosynthesis, Saponin adjuvant biosynthesis, Cannabidiol biosynthesis, Mugineic acid biosynthesis, Pentagalloylglucose biosynthesis, Benzoxazinoid biosynthesis, Gramine biosynthesis, Coumarin biosynthesis, Furanocoumarin biosynthesis, Hordatine biosynthesis, Podophyllotoxin biosynthesis	1
Carbapenem biosynthesis	2
Glucosinolate biosynthesis	1
Monobactam biosynthesis	4
Neomycin, kanamycin and gentamicin biosynthesis	1
Novobiocin biosynthesis	2
Penicillin and cephalosporin biosynthesis	1
Prodigiosin biosynthesis	3
Streptomycin biosynthesis	6
Tropane, piperidine and pyridine alkaloid biosynthesis	1
Carbohydrate metabolism	**153**
Amino sugar and nucleotide sugar metabolism	21
Butanoate metabolism	11
C-5 Branched dibasic acid metabolism	2
Citrate cycle (TCA cycle)	6
Fructose and mannose metabolism	7
Galactose metabolism	15
Glycolysis / Gluconeogenesis	18
Glyoxylate and dicarboxylate metabolism	5
Inositol phosphate metabolism	2
Pentose and glucuronate interconversions	6
Pentose phosphate pathway	14
Propanoate metabolism	11
Pyruvate metabolism	24
Starch and sucrose metabolism	11
Energy metabolism	**58**
Carbon fixation in photosynthetic organisms	6
Carbon fixation pathways in prokaryotes	11
Methane metabolism	7
Nitrogen metabolism	8

Oxidative phosphorylation	13
Photosynthesis	8
Sulfur metabolism	5
Global and overview maps	**801**
2-Oxocarboxylic acid metabolism	14
Biosynthesis of amino acids	62
Biosynthesis of cofactors	70
Biosynthesis of nucleotide sugars	19
Carbon metabolism	39
Degradation of aromatic compounds	5
Fatty acid metabolism	12
Nucleotide metabolism	28
Biosynthesis of secondary metabolites	148
Metabolic pathways	328
Microbial metabolism in diverse environments	76
Glycan biosynthesis and metabolism	**43**
Glycosaminoglycan degradation	1
Glycosphingolipid biosynthesis - globo and isoglobo series	1
Lipopolysaccharide biosynthesis	1
O-Antigen nucleotide sugar biosynthesis	9
Other glycan degradation	1
Peptidoglycan biosynthesis	20
Teichoic acid biosynthesis	10
Lipid metabolism	**36**
Fatty acid biosynthesis	11
Fatty acid degradation	4
Glycerolipid metabolism	11
Glycerophospholipid metabolism	8
Sphingolipid metabolism	2
Metabolism of cofactors and vitamins	**76**
Biotin metabolism	5
Folate biosynthesis	12
Lipoic acid metabolism	5
Nicotinate and nicotinamide metabolism	6
One carbon pool by folate	9
Pantothenate and CoA biosynthesis	8
Porphyrin metabolism	1
Retinol metabolism	2
Riboflavin metabolism	8
Thiamine metabolism	10
Ubiquinone and other terpenoid-quinone biosynthesis	7
Vitamin B metabolism	3
Metabolism of other amino acids	**28**
Cyanoamino acid metabolism	2
D-Amino acid metabolism	10
Glutathione metabolism	7
Selenocompound metabolism	6

Taurine and hypotaurine metabolism	3
Metabolism of terpenoids and polyketides	**18**
Biosynthesis of ansamycins	1
Biosynthesis of vancomycin group antibiotics	1
Nonribosomal peptide structures	1
Polyketide sugar unit biosynthesis	3
Terpenoid backbone biosynthesis	11
Zeatin biosynthesis	1
Nucleotide metabolism	**61**
Purine metabolism	37
Pyrimidine metabolism	24
Xenobiotics biodegradation and metabolism	**25**
Aminobenzoate degradation	1
Benzoate degradation	4
Chloroalkane and chloroalkene degradation	3
Dioxin degradation	1
Drug metabolism - cytochrome P	2
Drug metabolism - other enzymes	8
Metabolism of xenobiotics by cytochrome P	2
Naphthalene degradation	3
Xylene degradation	1
Organismal Systems	**19**
Aging	**3**
Longevity regulating pathway - worm	3
Digestive system	**2**
Fat digestion and absorption	1
Mineral absorption	1
Endocrine system	**9**
Glucagon signaling pathway	5
PPAR signaling pathway	2
Prolactin signaling pathway	1
Thyroid hormone synthesis	1
Environmental adaptation	**2**
Plant-pathogen interaction	2
Immune system	**1**
NOD-like receptor signaling pathway	1
Nervous system	**2**
GABAergic synapse	1
Glutamatergic synapse	1
Total	**1801**

Table S3. Orthologous clusters among *L. fermentum* strains JAC 231, AGR 1485, ATCC 23271, CECT 5716, IFO 3956, and MCC 2760.

Slimmed GO	Name	Count of unique input accessions
Biological process		
GO:0008152	metabolic process	500
GO:0008150	biological_process	477
GO:0044237	cellular metabolic process	424
GO:0006807	nitrogen compound metabolic process	351
GO:0044238	primary metabolic process	296
GO:0009987	cellular process	260
GO:0046483	heterocycle metabolic process	229
GO:0006725	cellular aromatic compound metabolic process	208
GO:0043170	macromolecule metabolic process	179
GO:0006139	nucleobase-containing compound metabolic process	178
GO:0006082	organic acid metabolic process	152
GO:0006793	phosphorus metabolic process	124
GO:0016070	RNA metabolic process	110
GO:0006412	translation	96
GO:0065007	biological regulation	89
GO:0009117	nucleotide metabolic process	85
GO:0051186	cofactor metabolic process	81
GO:0006810	transport	62
GO:0051234	establishment of localization	62
GO:0050896	response to stimulus	57
GO:0005975	carbohydrate metabolic process	55
GO:0009116	nucleoside metabolic process	53
GO:0016043	cellular component organization	43
GO:0006259	DNA metabolic process	40
GO:0019538	protein metabolic process	40
GO:0006629	lipid metabolic process	38
GO:0006396	RNA processing	37
GO:0006811	ion transport	36
GO:0006518	peptide metabolic process	35
GO:0043603	cellular amide metabolic process	34
GO:0044255	cellular lipid metabolic process	34
GO:0006260	DNA replication	32
GO:0032502	developmental process	31
GO:0051179	localization	30
GO:0042254	ribosome biogenesis	26
GO:0043412	macromolecule modification	26
GO:0006281	DNA repair	25
GO:0032989	cellular component morphogenesis	22
GO:0006091	generation of precursor metabolites and energy	19
GO:0006865	amino acid transport	19
GO:0007154	cell communication	18
GO:0032196	transposition	17
GO:0006766	vitamin metabolic process	15

GO:0051704	multi-organism process	12
GO:0006081	cellular aldehyde metabolic process	11
GO:0008643	carbohydrate transport	11
GO:0015031	protein transport	11
GO:0006464	cellular protein modification process	9
GO:0042180	cellular ketone metabolic process	9
GO:0051189	prosthetic group metabolic process	9
GO:0006457	protein folding	8
GO:0006818	hydrogen transport	8
GO:0005976	polysaccharide metabolic process	7
GO:0044419	interspecies interaction between organisms	7
GO:0051641	cellular localization	7
GO:0065003	macromolecular complex assembly	7
GO:0006066	alcohol metabolic process	6
GO:0006996	organelle organization	6
GO:0007049	cell cycle	6
GO:0009225	nucleotide-sugar metabolic process	6
GO:0009292	genetic transfer	6
GO:0022411	cellular component disassembly	6
GO:0022607	cellular component assembly	6
GO:0043094	cellular metabolic compound salvage	5
GO:0043101	purine-containing compound salvage	5
GO:0045333	cellular respiration	5
GO:0051276	chromosome organization	5
GO:0051301	cell division	5
GO:0006304	DNA modification	4
GO:0006730	one-carbon metabolic process	4
GO:0015074	DNA integration	4
GO:0016032	viral process	4
GO:0000003	reproduction	3
GO:0006662	glycerol ether metabolic process	3
GO:0007059	chromosome segregation	3
GO:0015849	organic acid transport	3
GO:0046490	isopentenyl diphosphate metabolic process	3
GO:0046903	secretion	3
GO:0006113	fermentation	2
GO:0006354	DNA-templated transcription, elongation	2
GO:0008655	pyrimidine-containing compound salvage	2
GO:0017144	drug metabolic process	2
GO:0032392	DNA geometric change	2
GO:0042440	pigment metabolic process	2
GO:0046794	transport of virus	2
GO:0051180	vitamin transport	2
GO:0071555	cell wall organization	2
GO:0001906	cell killing	1
GO:0006508	proteolysis	1
GO:0007005	mitochondrion organization	1
GO:0009308	amine metabolic process	1
GO:0009372	quorum sensing	1
GO:0015931	nucleobase-containing compound transport	1

GO:0015976	carbon utilization	1
GO:0019748	secondary metabolic process	1
GO:0031640	killing of cells of other organism	1
GO:0034622	cellular macromolecular complex assembly	1
GO:0042445	hormone metabolic process	1
GO:0042620	poly(3-hydroxyalkanoate) metabolic process	1
GO:0042710	biofilm formation	1
GO:0045229	external encapsulating structure organization	1
GO:0045230	capsule organization	1
GO:0051181	cofactor transport	1
GO:0051258	protein polymerization	1
Cellular component list		
GO:0044464	cell part	31
GO:0016020	membrane	25
GO:0005622	intracellular	7
GO:0005575	cellular_component	2
GO:0005576	extracellular region	1
GO:0005634	nucleus	1
GO:0005783	endoplasmic reticulum	1
GO:0043226	organelle	1
GO:0043229	intracellular organelle	1
Molecular function list		
GO:0003674	molecular_function	55
GO:0005215	transporter activity	51
GO:0016787	hydrolase activity	44
GO:0043167	ion binding	35
GO:0016740	transferase activity	34
GO:0003676	nucleic acid binding	27
GO:0008233	peptidase activity	19
GO:0016491	oxidoreductase activity	19
GO:0000166	nucleotide binding	18
GO:0005488	binding	14
GO:0001882	nucleoside binding	10
GO:0048037	cofactor binding	9
GO:0004871	signal transducer activity	4
GO:0005515	protein binding	3
GO:0008289	lipid binding	3
GO:0005198	structural molecule activity	2
GO:0016829	lyase activity	1
GO:0016874	ligase activity	1
GO:0019213	deacetylase activity	1
GO:0043021	ribonucleoprotein complex binding	1
GO:0060090	binding, bridging	1

Table S4. Subsystems related to vitamin synthesis identified in the *L. fermentum* JAC 231 genome.

Subsystems	**Gene count**
Biotin	
Biotin biosynthesis	3
Coenzyme A	
Coenzyme A Biosynthesis	7
Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	
Thiamin biosynthesis	8
Folate and pterines	
5-FCL-like protein	12
Folate Biosynthesis	10
Folate biosynthesis cluster	7
Molybdenum cofactor biosynthesis	11
Lipoic acid	
Lipoic acid metabolism	1
NAD and NADP	**8**
NAD and NADP cofactor biosynthesis global	8
Pyridoxine	
Pyridoxin (Vitamin B6) Biosynthesis	6
Pyridoxin(Vitamin B6) Degradation Pathway	1
Riboflavin, FMN, FAD	
Flavodoxin	3
riboflavin to FAD	4
Riboflavin, FMN and FAD metabolism	9
Riboflavin, FMN and FAD metabolism in plants	12
Tetrapyrroles	**3**
Heme and Siroheme Biosynthesis	3
Total	105

Table S5. Genes from non-pathogenic families identified by the PlasmidFinder v2.1 tool for *L. fermentum* JAC 231.

	PathogenFind			
Probability of being a human pathogen	0,203			
Corresponding Pathogenic Families	0			
Corresponding Nonpathogenic Families	10			
Class	Lactobacillares Organisms	Protein function	Identity (%)	Protein ID
	Lactobacillus helveticus DPC 4571	Cation transport protein	98,5	ABX27776
		Purine-cytosine permease	98,89	ABX27803
		Sugar kinase	99,66	ABX27804
		Carbonate dehydratase	98,1	ABX27797
	Lactobacillus reuteri DSM 20016	Transposase, IS605 OrfB family	90,77	ABQ83922
	Lactobacillus casei ATCC 334	Recombinase Transposition	98.12	ABJ69435
	Lactobacillus brevis ATCC 367	Site-specific, DNA invertase pin-related protein	96,26	ABJ65378
	Lactobacillus reuteri JCM 1112	Transposition putative metal-binding protein	89,81	SACO24586
	Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842	(probable CopZ copper chaperone)	97,33	ABJ58075
		Crp-like transcriptional regulator	97,69	ABJ58075

Table S6. Predicted CRISPR-Cas systems in the genome of *L. fermentum* JAC 231.

READ□	Spacers	DR Length	DR Consensus	Direction	Potential Orientation	Cas	CRISPR Start	CRISPR End	Length	CAS Start	CAS End
gnl_Prokka_EPNMGAAP_4	1	46	CRT results	ND	ND	Cas3_0_I	409	525	117	33092	34474
gnl_Prokka_EPNMGAAP_4	1	29	CRT results	ND	ND	Cas3_0_I	10070	10159	90	33092	34474
gnl_Prokka_EPNMGAAP_1	3	53	CRT results	ND	ND	Cas3_0_I	1769	2055	287	13702	15018