Ecole Doctorale des Sciences de la Matière, du Rayonnement et del'Environnement

Unité Mixte de Recherche Transfrontalière BioEcoAgro 1158

**THESIS**

Thesis supported in cotutelle between the Université de Lille and the Universidade Federal de Viçosa

To obtain the degree of:

**DOCTOR OF THE UNIVERSITY OF LILLE**

**Specialty Agri-food biotechnology, food science and physiology**

Presented by: Camila GONÇALVES TEIXEIRA, on December 21, 2022

# ANTIMICROBIAL PROPERTIES OF *Weissella cibaria* STRAINS ISOLATED FROM CAMPOS DAS VERTENTES, MINAS GERAIS, BRAZIL, AND ITS POTENTIAL USE AS PROBIOTICS AND BACTERIOCINS PRODUCERS

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**THÈSE DE DOCTORAT**

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**DOCTEUR DE L'UNIVERSITE DE LILLE**

**Spécialité: Biotechnologies agroalimentaires, sciences de l'aliment, physiologie**

Présentée par: Camila GONÇALVES TEIXEIRA, le 21 Decembre 2022

# PROPRIÉTÉS ANTIMICROBIENNES DES SOUCHES DE *Weissella cibaria* ISOLÉES DE CAMPOS DAS VERTENTES, MINAS GERAIS, BRÉSIL ET SON UTILISATION POTENTIELLE COMME PRODUCTEURS DE PROBIOTIQUES ET DE BACTÉRIOCINES

Composition du Jury:

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# Scientific valorization

Published papers

[1] **Teixeira, Camila Gonçalves**, Andressa Fusieger, Evandro Martins, et al. 2021. "Biodiversity and Technological Features of *Weissella* Isolates Obtained from Brazilian Artisanal Cheese-Producing Regions." *LWT - Food Science and Technology* 147(April): 111474.

[2] **Teixeira, Camila Gonçalves**, Raiane Rodrigues da Silva, et al. 2021. "The *Weissella* Genus in the Food Industry: A Review." *Research, Society and Development* 10(5): 1–15.

[3] **Teixeira, Camila Gonçalves**, Andressa Fusieger, Gustavo Leite Milião, et al. 2021. "*Weissella*: An Emerging Bacterium with Promising Health Benefits." *Probiotics and Antimicrobial Proteins* 13(4): 915–25.

[4] **Teixeira, Camila Gonçalves** et al. 2022. "Genomic Analyses of *Weissella Cibaria* W25, a Potential Bacteriocin-Producing Strain Isolated from Pasture in Campos Das Vertentes, Minas Gerais, Brazil." *Microorganisms* 10(2): 314.

Conference abstracts

**Camila Gonçalves Teixeira**, Evandro Martins, Andressa Fusieger, Rosângela De Freitas, Tatiana Santos Lima, Júlia Oliveira Lopes, Luis Gustavo Lima Nascimento, Antônio Fernandes De Carvalho. "Technological Potential of *Weissella* Strains Isolated from Artisanal Cheeses from Different Regions of Brazil" XXI Encontro Nacional e VII Congresso Latino Americano de Analistas de Alimentos, May, 26 to 30, 2019, Florianópolis/SC (Brasil).

**Camila Gonçalves Teixeira,** Antônio Fernandes de Carvalho, Djamel Drider. "Brazilian *Weissella* strains with antimicrobial activity against Gram-positive and Gram-negative foodborne pathogens." Congrès de la Société Française de Microbiologie, September, 22 to 24, 2021, Nantes (France).

Papers to be published

**Camila Gonçalves Teixeira,** Rafaela da Silva Rodriguesa, Ricardo Seiti Yamatogi, Anca Lucau-Danila, Djamel Drider, Luís Augusto Nero, Antônio Fernandes de Carvalho.

“Genome characterization of *Weissella* strains isolated from Campos das Vertentes, Minas Gerais, Brazil focused revealed new bacteriocins with a large spectrum of activity”

**Camila Gonçalves Teixeira**, Yanath Belguesmia, Rafaela da Silva Rodrigues, Anca Lucau-Danila, Luís Augusto Nero, Antônio Fernandes de Carvalho, Djamel Drider. “Biosecurity of *Weissella* strains isolated from Campos das Vertentes, Minas Gerais, Brazil focused on its potential as probiotic”.

## Abstract

TEIXEIRA, Camila Gonçalves, D.Sc., Thesis supported in cotutelle betweenthe Université de Lille and the Universidade Federal de Viçosa, October 2022. **Antimicrobial Properties of *Weissella cibaria* Strains Isolated from Campos Das Vertentes, Minas Gerais, Brazil and its Potential use as Probiotics and Bacteriocins Producers**. Advisor: Antônio Fernandes de Carvalho and Djamel Drider. Co-advisors: Luis Augusto Nero and Anca Lucau-Danila.

The genus *Weissella* is composed of bacteria belonging to the group known as lactic acid bacteria (LAB), are autochthonous to both dairy and non-dairy environments and many strains have the ability to produce various compounds of interest to the food industry. This work began with deep research for two literature reviews regarding the use of *Weissella* strains for the food and pharmaceutical industry and the probiotic potential of this genus. A large number of scientific studies reported and support the use of *Weissella* strains in this field due to their health-promoting properties. From the point of view of food technology, some strains have the potential production of exopolysaccharides, non-digestible oligosaccharides, which is beyond their probiotic potential. This work continuous with the study of the biodiversity of *Weissella* strains isolated in the regions producing artisanal cheeses in Brazil. For this, the analysis of PFGE and Rep-PCR were carried out. With these analyses, it was observed that each study region represents a specific ecological niche. After selecting the representatives of each group the antimicrobial potential of these strains was analyzed against the most common foodborne pathogens. Most of the isolates tested showed good inhibition of the pathogens. Also, the technological potential of thirteen strains of these cultures was evaluated. Some strains showed positive results for most of the tests performed. After that, we selected three strains with good inhibitory activity, the first one we worked with was *Weissella cibaria* W25, and we sequenced and assembled the draft genome sequence. Then we carried out comprehensive a comparative genomic analysis with *W. cibaria* 110, known to pro-duce the weissellicin 110 bacteriocin, and four other non-bacteriocin-producing *W. cibaria* strains. The analyses showed that the strain W25 has its unique protein cluster genes which can be related to bacteriocins genes indicated by AntiSmash confirming the possibility of producing two different bacteriocins. Based on these results we look further into the genome of the three strains of *W. cibaria* (W21, W25, and W42). After, we investigated the presence of genes for secondary metabolites in the genome of the strains and discovered that they have a common region in the genome with a putative

bacteriocin identified as Bacteriocin_IIc. Also, we compared the nucleotides and the amino acids of this putative bacteriocin in the NCBI platform and there was no similarity with no other so far described bacteriocin from the genus *Weissella*. Finally, we analyze the safety of using these three strains for human and/or animal consumption and their capacity to be used as probiotics. All three strains have no acquired antimicrobial resistance genes and no mobile elements in the genome. *W. cibaria* strains ware resistant to one antibiotic with intrinsic resistances confirmed by genomic analysis, no hemolytic activity and no one of the known virulence factor ware detected. They possess the defense system CRISPR-Cas. Besides they didn't present any inflammation or cytotoxicity capacity. *W. cibaria* W21 presents higher potential, once it demonstrates excellent results of adhesion to intestinal cells and exclusion of *S. aureus* MRSA SA1. However none of the three strains survived completely through the simulated gastro intestinal tract, but some protective ways can be further study. In conclusion, this work allowed us to understand the diversity and the distribution of *Weissella* species found in the different regions producing artisanal cheeses in Brazil, besides helping to understand the role of these strains in artisanal cheeses. It also showed that some of them have a favorable technological and safety potential for future uses in the food industry or as a probiotic culture.

## Résumé

TEIXEIRA, Camila Gonçalves, D.Sc., Thèse soutenue en cotutelle entre l'Université de Lille et Universidade Federal de Viçosa, octobre, 2022. **Propriétés antimicrobiennes des souches de *Weissella cibaria* isolées de Campos Das Vertentes, Minas Gerais, Brésil et son utilisation potentielle en tant que producteurs de probiotiques et de bactériocines**. Directeur: Antônio Fernandes de Carvalho et Djamel Drider. Co-advisors: Luis Augusto Nero et Anca Lucau-Danila.

Le genre *Weissella* est composé de bactéries appartenant au groupe de bactéries lactiques, sont autochtones aux environnements laitiers et non laitiers et ont la capacité de produire divers composés d'intérêt pour l'industrie alimentaire. Ce travail a débuté par deux revues de littérature concernant l'utilisation des souches *Weissella* pour l'industrie alimentaire et pharmaceutique et le potentiel probiotique de ce genre. Un grand nombre d'études scientifiques rapportent et soutiennent l'utilisation des souches *Weissella* dans ce domaine en raison de leurs propriétés bénéfiques pour la santé. Du point de vue de la technologie alimentaire, certaines souches ont un potentiel de production d'oligosaccharides non digestibles, qui dépasse leur potentiel probiotique. Ce travail se poursuit par l'étude de la biodiversité des souches isolées dans les régions productrices de fromages artisanaux au Brésil. Pour cela, les analyses PFGE et Rep-PCR ont été réalisée et il a été observé que chaque région d'étude représente une niche écologique spécifique. Après avoir sélectionné les représentants de chaque groupe, le potentiel antimicrobien de ces souches a été analysé contre les agents pathogènes d'origine alimentaire. La plupart des isolats testés ont montré une bonne inhibition des pathogènes. Aussi, le potentiel technologique de treize souches de ces cultures a été évalué. Certaines souches ont montré des résultats positifs pour la plupart des tests effectués. Après cela, nous avons sélectionné trois souches avec une bonne activité inhibitrice, la première avec laquelle nous avons travaillé était *Weissella cibaria* W25, nous avons séquencé et assemblé le projet de séquence du génome. Ensuite, nous avons effectué une analyse génomique comparative complète avec *W. cibaria* 110, connu pour produire la bactériocine weissellicine 110, et quatre autres souches de *W. cibaria* non productrices de bactériocine. Les analyses ont montré que la souche W25 possède ses gènes de groupe de protéines uniques qui peuvent être liés aux gènes de bactériocines indiqués par AntiSmash confirmant la possibilité de produire deux bactériocines différentes. Sur la base de ces résultats, nous avons approfondi le génome des trois souches de *W. cibaria* (W21, W25 et W42). Ensuite, nous avons étudié la présence

de gènes pour les métabolites secondaires dans le génome des souches et avons découvert qu'elles avaient une région commune dans le génome avec une bactériocine putative identifiée comme Bacteriocin_IIc. De plus, nous avons comparé les nucléotides et les acides aminés de cette bactériocine putative dans la plateforme NCBI et il n'y avait aucune similitude avec autre bactériocine décrite jusqu'à présent du genre *Weissella*. Enfin, nous analysons la sécurité d'utilisation de ces trois souches pour l'alimentation humaine et/ou animale et leur capacité à être utilisées comme probiotiques. Les trois souches n'ont pas de gènes acquis de résistance aux antimicrobiens ni d'éléments mobiles dans le génome. Les souches étaient aucune activité hémolytique et aucun facteur de virulence connu. Ils possèdent le système de défense CRISPR-Cas. De plus, ils n'ont présenté aucune capacité d'inflammation ou de cytotoxicité et les trois souches de Weissella n'étaient pas censées être des agents pathogènes humains. W21 démontre d'excellents résultats d'adhésion aux cellules intestinales et d'exclusion de *S. aureus*. Cependant, aucune des trois souches n'a survécu complètement à travers le tractus gastro-intestinal simulé, mais certaines méthodes de protection peuvent être étudiées plus avant. En conclusion, ce travail nous a permis de comprendre la diversité et la distribution des espèces de *Weissella* présentes dans les différentes régions productrices de fromages artisanaux au Brésil. Elle a également montré que certains d'entre eux ont un potentiel technologique et de sécurité favorable pour des utilisations futures dans l'industrie alimentaire ou comme culture probiotique.

## Resumo

TEIXEIRA, Camila Gonçalves, D.Sc., Tese em cotutela entre l'Université de Lille e Universidade Federal de Viçosa, outubro de 2022. **Propriedades Antimicrobianas de Estirpes de *Weissella cibaria* Isoladas dos Campos das Vertentes, Minas Gerais, Brasil e seu Potencial uso como Probióticos e Produtoras de Bacteriocinas**. Orientadores: Antônio Fernandes de Carvalho e Djamel Drider. Co-orientadores: Luis Augusto Nero e Anca Lucau-Danila.

O gênero *Weissella* é composto por bactérias pertencentes ao grupo conhecido como bactérias láticas (BAL), portanto é capaz de produzir ácido lático através da fermentação de carboidratos. Bactérias desse gênero são autóctones tanto para ambientes lácteos quanto não lácteos e muitas linhagens têm a capacidade de produzir diversos compostos de interesse para a indústria alimentícia, como exopolissacarídeos, bacteriocinas e peróxido de hidrogênio, por exemplo. Este trabalho começou com uma profunda pesquisa culminando em duas revisões de literatura compreendendo o uso de cepas de *Weissella* para a indústria alimentícia e farmacêutica e o potencial probiótico deste gênero. Um grande número de estudos científicos relataram e apoiam o uso de cepas de *Weissella* neste campo devido às suas propriedades promotoras da saúde. Do ponto de vista da tecnologia de alimentos, algumas cepas apresentam potencial na produção de exopolissacarídeos, oligossacarídeos não digeríveis, que está além de seu potencial probiótico. É importante notar que a maioria das cepas de *Weissella* com propriedades interessantes para uso para consumo humano são seguras, devido à ausência ou baixa ocorrência de genes de virulência ou de resistência a antibióticos. Em seguida, realizou-se o estudo da biodiversidade de cepas de *Weissella* isoladas em regiões produtoras de queijos artesanais no Brasil. Para isso, foram realizadas as análises de PFGE e Rep-PCR, que permitiram o agrupamento das cepas estudadas. Com essas análises, observou-se que cada região de estudo representa um nicho ecológico específico e, além disso, observou-se também que a técnica de PFGE possibilita obter um melhor resultado de heterogeneidade entre as linhagens de uma mesma espécie. Após a seleção dos representantes de cada grupo foi analisado o potencial antimicrobiano dessas cepas por meio de testes antagônicos e produção de bacteriocinas contra os patógenos alimentares mais comuns. A maioria dos isolados testados apresentou boa inibição dos patógenos, com percentual de inibição superior a 60%. No entanto, não houve inibição significativa de patógenos quando o teste de produção de bacteriocina foi realizado. Também foi avaliado o potencial tecnológico

dessas culturas. Treze cepas foram selecionadas e testadas para determinar a capacidade de coagulação, produção de diacetil, produção de exopolissacarídeos, capacidade de acidificação, atividade proteolítica extracelular e características do coágulo formado. Algumas cepas apresentaram resultados positivos para a maioria dos testes realizados, exceto para a produção de exopolissacarídeos, com destaque para a cepa número 16 isolada de queijos do sul do Pará, apresentando boa capacidade de acidificação e diminuição do pH do leite, e resultados positivos para os testes de produção de diacetil, atividade proteolítica e formação de coágulos homogêneos. Logo depois selecionamos três cepas com boa atividade inibitória dos patógenos, a primeira com a qual trabalhamos foi *Weissella cibaria* W25, sequenciamos e montamos a sequência preliminar do genoma, que consiste em 41 contigs totalizando ~2,4Mbp, com um teor de G+C de 45,04%. Em seguida, realizamos uma análise genômica comparativa abrangente com *W. cibaria* 110, conhecida por produzir a bacteriocina weissellicina 110, e quatro outras cepas de *W. cibaria* não produtoras de bacteriocina. *W. cibaria* W25 tem grande potencial para ser utilizada para consumo humano, uma vez que não foi prevista como um patógeno para humanos. Além disso, as análises mostraram que esta cepa possui genes únicos de agrupamento de proteínas que podem ser relacionados a genes de bacteriocinas indicados pelo AntiSmash, confirmando a possibilidade de produzir duas bacteriocinas diferentes. Com base nesses resultados, analisamos o genoma das três linhagens de *W. cibaria* (W21, W25 e W42), e o genoma de W21 e W42 também foram sequenciados. Seguidamente, investigamos a presença de genes para metabólitos secundários no genoma das cepas e descobrimos que eles possuem uma região comum no genoma com uma putativa bacteriocina identificada como Bacteriocin_IIc. Posteriormente, testamos a atividade antimicrobiana in vitro do sobrenadante dessas cepas. Os resultados mostraram que a cepa W21, que estava faltando um gene relacionado ao transporte na análise do genoma, também não apresentou diferença no teste antimicrobiano in vitro, comparando os resultados após e antes da neutralização do sobrenadante e tratamento com proteinase K. Além disso, comparamos os nucleotídeos e os aminoácidos desta putativa bacteriocina na plataforma NCBI e não houve similaridade com nenhuma outra bacteriocina do gênero *Weissella* até agora descrita. Por fim, analisamos a segurança do uso dessas três cepas para consumo humano e/ou animal e sua capacidade de uso como probióticos. A sequência completa do genoma foi avaliada para genes relacionados à segurança. Além disso, a suscetibilidade aos antibióticos, citotoxicidade, capacidade de inflamação e atividade hemolítica foram testados. Para os testes probióticos, analisamos a sobrevivência através da simulação do

trato gástrico, adesão à cultura de células intestinais e exclusão de S. aureus MRSA SA1 e E. coli 184 também em cultura de células intestinais. Todas as três estirpes não possuíram genes de resistência antimicrobiana adquiridos e nenhum elemento móvel no genoma. As cepas de *W. cibaria* foram resistentes a um antibiótico com resistências intrínsecas confirmadas por análise genômica, nenhuma atividade hemolítica e nenhum fator de virulência conhecido foi detectado. As estirpes possuem o sistema de defesa CRISPR-Cas. Além de não apresentarem nenhuma capacidade de inflamação ou citotoxicidade e as três estirpes de *Weissella* não foram preditas como patógenos humanos. *W. cibaria* W21 apresentou maior potencial probiótico, pois demonstrou excelentes resultados de adesão às células intestinais e exclusão de *S. aureus* MRSA SA1. No entanto, nenhuma das três cepas sobreviveu completamente à simulação a passagem pelo trato gástrico, mas algumas formas de proteção para esse fim podem ser mais estudadas. Em conclusão, este trabalho permitiu compreender a diversidade de espécies de *Weissella* encontradas nas diferentes regiões produtoras de queijos artesanais no Brasil e como estão distribuídas nesses ambientes, além de ajudar a compreender o papel dessas linhagens nos queijos artesanais. Também mostrou que alguns deles têm um potencial tecnológico e de segurança favorável para usos futuros na indústria de alimentos ou como cultura probiótica.

# Summary

# GENERAL INTRODUCTION

## 1. Context

The study of microbial diversity in dairy and non-dairy environments has great importance for the understanding of the presence of different microorganisms in these environments, as well as the impact of these microorganisms on the final product when we refer to artisanal dairy products. Each environment has unique characteristics that favor and allow the development of different bacterial species.

Handmade cheeses and raw milk are considered potential sources of new strains of lactic acid bacteria. The way of making these cheeses causes the fermentation to be conducted by bacteria contaminating the grazing, animal skin, utensils, surfaces, and other environments that may come into contact with the cheese during manufacture. The study of the bacterial community present in artisanal cheeses has revealed the presence of species that had not yet been related to cheeses and a great diversity of lactic bacteria with differentiated technological characteristics. In addition, non-dairy environments such as grass, different types of silage, and even animal skin have also been an important source of new strains that have adapted and therefore, can provide interesting features to be explored.

The diversity of *Weissella* isolated from dairy and non-dairy environments is of great interest for the understanding of this microorganism in the final products and can be studied by molecular techniques such as PFGE and Rep-PCR that can phylogenetically relate the strains. In addition, phenotypic characterization is necessary to understand the characteristics and potential of each of them.

## 2. Objectives

This study aims to understand the microbial diversity in different environments (dairy and non-dairy) seeking the application of the strains with the potential to be used as a bacteriocin producer for the food industry or the pharmaceutical industry as a probiotic.

## 3. Manuscript organization

### CHAPTER I: Literature review

This chapter is divided into two parts, the first part presents overall information about the *Weissella* genus and its potential application for the food and pharmaceutical industry.

The physiological and metabolic characteristics, ecology, and application in the food industry are part A. In the second part (pat B), the *Weissella* strains are approached for their

species identification and characterization, beneficial properties, health benefits and exopolysaccharides production, and possibility for being used as probiotic culture. The first part of this review is published as "*The Weissella Genus in the Food Industry: A Review"* in the *Research, Society, and Development.* The second part of this review was published as "*Weissella*: An Emerging Bacterium with Promising Health Benefits" in the *Probiotics and Antimicrobial Proteins.*

**CHAPTER II: Biodiversity and technological features of *Weissella* isolates obtained from Brazilian artisanal cheese-producing regions**

This part aimed to identify the microbial biodiversity and evaluate the technological potential of *Weissella* spp. isolates obtained from different Brazilian regions that produce artisanal cheeses, such as Campo das Vertentes (Minas Gerais), Southern Pará, Guanambi (Bahia), and Marajó Island. The contents of this chapter are published as "Biodiversity and Technological Features of *Weissella* Isolates Obtained from Brazilian Artisanal Cheese-Producing Regions" in *LWT - Food Science and Technology*.

**CHAPTER III: *Weissella cibaria* W25: Comparative genomic analyses**

This part of the thesis aimed to announce and analyze the sequencing and annotation of the *Weissella cibaria* W25 genome and carry out a comprehensive comparative genomic analysis with *W. cibaria* 110, known to produce the weissellicin 110 bacteriocin, and four other non-bacteriocin-producing *W. cibaria* strains. The contents of this chapter are published as "Genomic Analyses of *Weissella Cibaria* W25, a Potential Bacteriocin-Producing Strain Isolated from Pasture in Campos Das Vertentes, Minas Gerais, Brazil." In *Microorganisms.*

**CHAPTER IV: *Weissella cibaria* strains isolated from Campo das Vertentes (MG), a potential bacteriocin producers**

This part aims to identify the antimicrobial compounds produced by *Weissella* strains isolated from Campos das Vertentes, Minas Gerais, Brazil, scheduling their future application as pathogens inhibitors in the food industry and preservation. The content of this chapter will be submitted as "*Genome characterization of Weissella strains isolated from Campos das Vertentes, Minas Gerais, Brazil focused revealed new bacteriocins with a large spectrum of activity"*.

**CHAPTER V: *Weissella cibaria* strains isolated from Campo das Vertentes (MG), biosecurity and probiotics analyses**

In this last chapter, we evaluated the safety capacity of three strains of *Weissella cibaria*, isolated from different producing artisanal cheese regions of Campos das Vertentes, in Minas Gerais, Brazil to be used as probiotics for human consumption or animal feed. This chapter will be valorized as “Biosecurity of *Weissella* strains isolated from Campos das Vertentes, Minas Gerais, Brazil focused on its potential as probiotic”

## 4. General conclusions and perspectives

The genus *Weissella* has many interesting properties and can be explored to aggregate more to the food and pharmaceutical industries. The main conclusions derived from the study are lined out with the future research needed to increment the knowledge about the strains regarding their use as probiotics or as an ingredient in the food industry.

# **CHAPTER I:** Literature Review

**PART A: The *Weissella* genus in the food industry: a review**
**O gênero *Weissella* na indústria de alimentos: uma revisão**
**El género *Weissella* en la industria alimentaria: una revisión**

The content of this part has been published in:

Research Society and Development

Camila Gonçalves Teixeira, Raiane Rodrigues da Silva, Andressa Fusieger, Evandro Martins, Rosângela de Freitas, Antônio Fernandes de Carvalho



**Abstract**

The genus *Weissella* is composed of bacteria classified as Gram-positive, catalase-negative, non-spore-forming, coccoid morphology, or short bacilli. They belong to the group of lactic acid bacteria (LAB), mainly by the production of lactic acid from the fermentation of carbohydrates. *Weissella* species are distributed in different habitats, such as soils, milking machines, and sugar cane and some strains with interesting technological features can be isolated from fermented foods, such as cheeses made from raw milk, fermented vegetables, and fermented milk. From the point of view of food technology, some strains have potential in the production of exopolysaccharides, non-digestible oligosaccharides, which is beyond their probiotic potential. Therefore, the bacteria belonging to the genus *Weissella* might have great technological importance, being also involved in the control of foodborne diseases by the production of bacteriocins and hydrogen peroxide. This genus has great potential for use in the food industry.

**Keywords:** *Weissella*; Food industry; Metabolism; Ecology; Technological potential; Bacteriocins.

**Resumo**

O gênero *Weissella* é composto por bactérias classificadas como Gram-positivas, catalase negativas, não formadoras de esporos, morfologia cocóide ou bacilos curtos. Pertencem ao

grupo das bactérias lácticas (BAL), principalmente pela produção de ácido láctico a partir da fermentação de carboidratos. As espécies de *Weissella* estão distribuídas em diferentes habitats, como solos, ordenhadeiras, cana-de-açúcar e algumas cepas com características tecnológicas interessantes podem ser isoladas de alimentos fermentados, como queijos de leite cru, vegetais fermentados e leite fermentado. Do ponto de vista da tecnologia de alimentos, algumas cepas apresentam potencial na produção de exopolissacarídeos, oligossacarídeos não digeríveis, que está além de seu potencial probiótico. Portanto, as bactérias pertencentes ao gênero *Weissella* podem ter grande importância tecnológica, estando também envolvidas no controle de doenças de origem alimentar pela produção de bacteriocinas e peróxido de hidrogênio. Este gênero possui grande potencial para uso na indústria alimentícia.

**Palavras-chave:** *Weissella*; Indústria alimentícia; Metabolismo; Ecologia; Potencial tecnológico; Bacteriocinas.

**Resumen**

El género *Weissella* está compuesto por bacterias clasificadas como gram-positivas, catalasa negativas, no formadoras de esporas, de morfología cocoide o bacilos cortos. Pertenecen al grupo de las bacterias del ácido láctico (BAL), principalmente por la producción de ácido láctico a partir de la fermentación de carbohidratos. Las especies de *Weissella* se encuentran distribuidas en distintos hábitats, como suelos, ordeñadoras, caña de azúcar y algunas cepas con interesantes características tecnológicas pueden aislarse de alimentos fermentados, como quesos elaborados a partir de leche cruda, vegetales fermentados y leche fermentada. Desde el punto de vista de la tecnología de los alimentos, algunas cepas tienen potencial en la producción de exopolisacáridos, oligosacáridos no digeribles, que está más allá de su potencial probiótico. Por tanto, las bacterias pertenecientes al género *Weissella* podrían tener una gran importancia tecnológica, estando también implicadas en el control de enfermedades transmitidas por alimentos mediante la producción de bacteriocinas y peróxido de hidrógeno. Este género tiene un gran potencial para su uso en la industria alimentaria.

**Palabras clave:** *Weissella*; Industria de alimentos; Metabolismo; Ecología; Potencial tecnológico; Bacteriocinas.

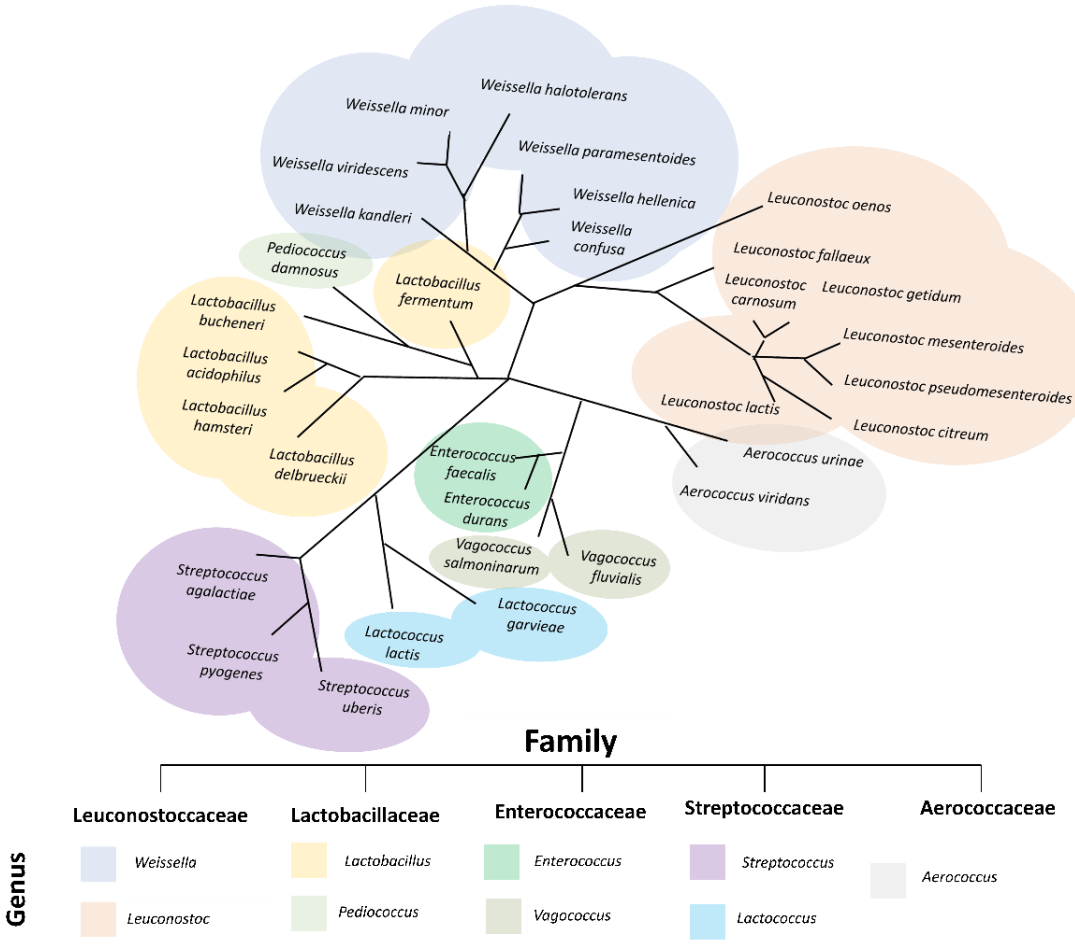
## 1. Introduction

At first, microbiology techniques for the identification of genera and species were based only on phenotypic tests such as morphological and biochemical tests for typing, subtyping, and identification of microbial genera, species, and subspecies. However, despite presenting atypical characteristics, many microorganisms were mistakenly grouped due to the low discriminatory power of these techniques when it comes to microorganisms with little genetic variability. Nevertheless, since the '80s, the techniques of manipulation of the genetic material, allow the evaluation of the bacterial genome. Because of the advancement in the studies of molecular biology, it is now possible to construct more and more precise phylogenetic techniques, such as the evaluation of rDNA (Fakruddin & Mannan, 2013, Gandra, Gandra, de Mello & da Godoi, 2008).

In 1993, using the rDNA technique, Collins found that strains with very close phenotypic characteristics, previously classified as *Leuconostoc*, presented profiles of bands very different from others belonging to the same genus. This was discovered after the study of atypical *Leuconostoc* cultures present in fermented and dried sausages produced in Greece. These isolated microorganisms resembled the bacterium *Leuconostoc* sp. for producing D (-) lactic acid but differed in several biochemical tests. The name of this genus was proposed by Collins *et al*. (Collins, Samelis, Metaxopoulos & Wallbanks, 1993) in honor of the German microbiologist Norbert Weiss due to his many contributions in the field of research related to LAB.

Collins *et al*. (1993) used genetic sequencing of the 16S rDNA to investigate the relationship between isolated bacteria and recognized strains of the genus *Leuconostoc*. They showed that the strains analyzed were phylogenetically closer to the *Leuconostoc paramesenteroides* than to the other *Leuconostoc* species (Figure 1). They proposed that *L. paramesenteroides*, the new species isolated from the fermented sausages, and some heterofermentative *Lactobacillus,* should belong to the new genus, named *Weissella*. The name *Weissella hellenica* was proposed to name the new species.

**Figure 1.** Phylogenetic tree showing the proximity of the genus *Weissella* with other genera of lactic acid bacteria.




Source: Adapted from Collins (1993).

Bacteria belonging to the *Weissella* genus are hardly differentiated from *Leuconostoc* strains and heterofermentative *Lactobacillus* by phenotypic characteristics; thus, the description was only possible through molecular taxonomic analyzes. Collins *et al*. (1993) proposed the reclassification of *L. paramesenteroides, Lb. confusus, Lb. halotolerans, Lb. kandleri, Lb. minor*, and *Lb. viridescens* to *W. paramesenteroides, W. confusa*, *W. halotolerans, W. kandleri, W. minor,* and *W. viridescens*, respectively. Subsequently, other studies have identified new species, and currently 25 species are validated: *W. viridescens,* (Niven & Evans, 1956)*, W. paramesenteroides* (Garvie, 1967), *W. confusa* (Holzapfel e Kandler, 1969 apud Collins et al., 1993), *W. kandleri* (Holzapfel & Van Wyk, 1982), *W. halotolerans* (Kandler, Schillinger & Weiss, 1983), *W. minor* (Kandler *et al.*, 1983), *W. hellenica* (Collins *et al*., 1993), *W. thailandensis* (Tanasupawat, Shida, Okada, & Komagata,

2000), *W. soli* (Magnusson, Jonsson, Schnurer & Roos, 2002), *W. cibaria* (Björkroth, K. J. et al., 2002), *W. koreensis* (Lee, *et al.*, 2002), *W. ghanensis* (De Bruyne, Camu, Lefebvre, De Vuyst & Vandamme, 2008), *W. beninenses* (Padonou, *et al.* 2010), *W. fabaria* (De Bruyne, Camu, De Vuyst, & Vandamme, 2010), *W. ceti* (Vela *et al.*, 2011), *W. fabalis* (Snauwaert, Papalexandratou, De Vuyst, & Vandamme, 2013), *W. oryzae* (Tohno *et al.*, 2013), *W. diestrammenae* (Oh, *et al*., 2013), *W. uvarum* (Nisiotou, Dourou, Filippousi, Banilas, & Tassou, 2014), *W. cryptocerci* (Heo, *et al.* 2019), *W. bombi* (Praet, *et al.*, 2015), *W. jogaejeotgali* (Lee *et al.* 2015), *W. kimchi* (Choi *et al*., 2002), *W. muntiaci* (Lin *et al*., 2020) and *W. sagaensis* (Li, Tian & Gu, 2020).

The genus *Weissella* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales,* and family *Leuconostocaceae*. The bacteria belonging to this genus may have different morphologies, being short rods with rounded to tapered ends or coccoid in shape, which is the morphology of microorganisms belonging to the genus *Leuconostocs, Oenococcus,* and *Streptococci* (Collins *et al*., 1993). In some species, a tendency to pleomorphism occurs depending on the stress condition to which the bacterium is submitted. Concerning the organization, they can also be found in pairs, alone, or in small chains (Gandra, *et al.*, 2008, Collins *et al*., 1993). *Weissella* is a relatively new genus, which is not yet used as a starter or adjunct culture by the food industry, as there are still few studies on its use as such. The purpose of this review is to bring the most relevant research in this genus to the food industry, presenting the potential application of the strains of the genus *Weissella* in food.

## 2. Methodology

This paper is a bibliographic research (Pereira, Shitsuka, Parreira & Shitsuka, 2018). Therefore, the objective is the verification of scientific data and the discussion about them on the proposed theme. This article was done descriptively and qualitatively to present how *Weissela* sp. can be applied in the food industry.

To obtain the theoretical foundation it was used database websites: such as *Google academic, Scielo, PubMed,* and *Science Direct,* and for the research was used keywords such as *Weissela*, bacteriocin, exopolysaccharides, and lactic acid bacteria. The period used was from the year 1993, when the species was recognized, until the year 2021 as a form to compare the evolution of research and application of the *Weisella* genus and their compounds. Also for this research, there was no limitation to language as a way to gather

as much information as possible. By the end, was select 90 sources of information.

## 3. Physiological and metabolic characteristics

Bacteria from the genus *Weissella* are chemoorganotrophic, facultatively anaerobic, Gram-positive, non-spore-forming, catalase-negative (Collins *et al*., 1993) and have no motility except to *W. beninenses* that has peritrichous flagella (Padonou, *et al.* 2010). All the microorganisms of this genus are compulsory hetero-fermenters producing lactic acid, carbon dioxide, ethanol, and/or acetic acid from carbohydrate fermentation. They use the phosphoketolase and the hexose monophosphate pathways to perform carbohydrate fermentation (Garvie, 1967, Holzapfel & Van Wyk, 1982).

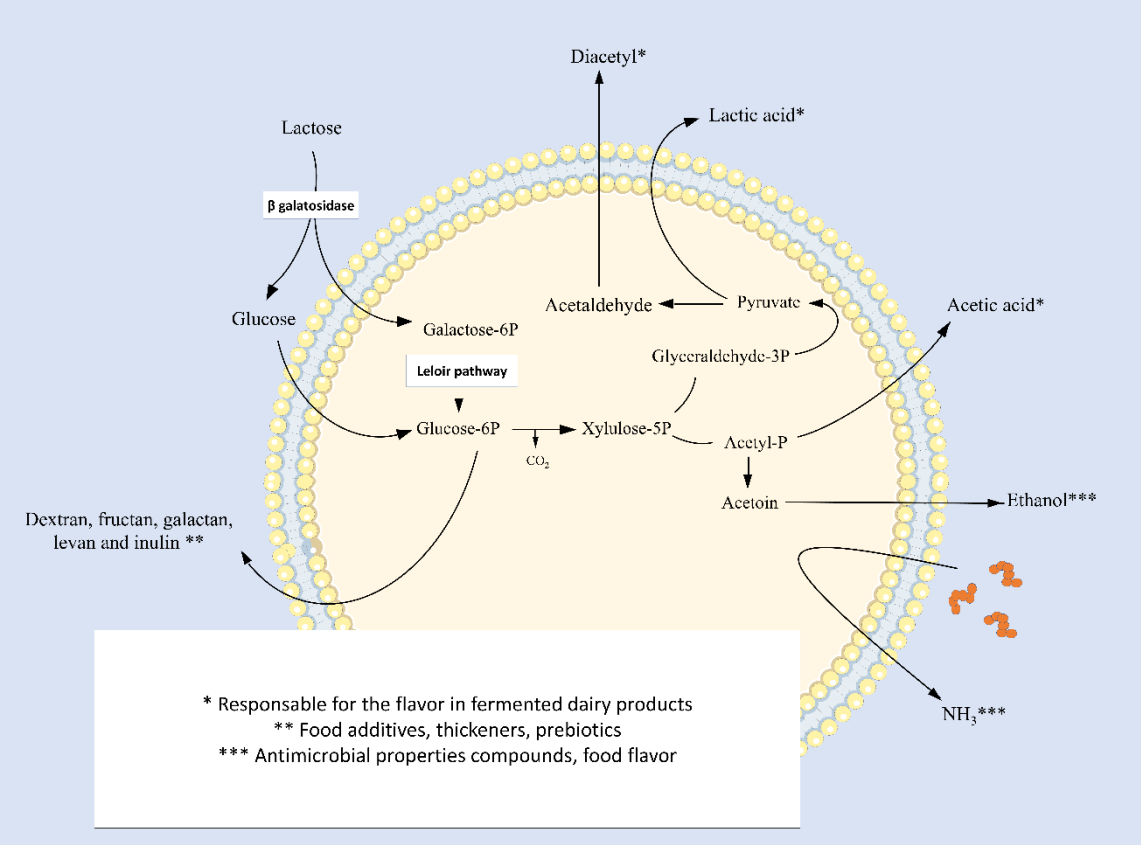
*Weissella* sp. has very complex nutritional needs requiring peptides, amino acids (arginine, aspartic acid, cystine, glutamic acid, histidine, isoleucine, phenylalanine, serine, threonine, tryptophan, and valine), fatty acids, nucleic acids, fermentable carbohydrates (glucose, fructose, mannose, maltose, sucrose, trehalose) and vitamins (riboflavin, pyridoxal, folic acid, biotin, nicotine, thiamine, pantothenic acid) for their development (Gandra, *et al.*, 2008, Björkroth, Dicks, & Endo, 2014, Kandler *et al.*, 1983). However, culture media used as Man Rogosa and Sharp (MRS) and M17 for lactic acid bacteria are easily employed for the multiplication of these microorganisms. Moreover, all the nutritional requirements can be found in many raw materials used in the food industry such as milk, meat, and vegetables, providing the use of such cultures for fermentation thereof.

As part of the LAB group, the species of the genus *Weissella* are chemoorganotrophic microorganisms with strictly fermentative metabolism being unable to synthesize porphyrinic groups (e.g. heme). All species can multiply at 15 °C while some multiply at temperatures between 42 °C and 45 °C, having an optimum temperature of multiplication between 20 °C and 30 °C (Garvie, 1967). These bacteria can be applied in fermented dairy products like fermented milk which has the temperature of incubation of the microorganisms around 40 °C and fermented meat sausages with an incubation temperature of 25 °C.

Different species produce dextran, hydrolyzed esculin, and produces ammonia from arginine. Dextran is an exopolysaccharide important for the manufacture of products such as yogurt, in which it is desired to have viscosity (Mende, Rohm, & Jaros, 2016). In addition, these bacteria can ferment several sugars such as cellobiose, fructose, galactose, maltose, raffinose, ribose, trehalose, and xylose (Fusco *et al.*, 2015). they can be used as starter cultures for the manufacture of products with different sensory characteristics.

*W. cibaria* MG1 possesses all the necessary genes for the use of the phosphoketolase pathway and metabolizes the galactose via the Leloir pathway in figure 2 is possible to understand how it is the metabolism. It can also use maltose, fructose, ribose, xylose, sucrose and gluconate as carbon sources. In addition, a β-galactosidase has been known to indicate that lactose can also be metabolized (Lynch *et al.*, 2015). which suggests that this strain can be used in the fermentation of dairy products that have lactose as their main sugar.

**Figure 2.** Metabolic pathways used by bacteria of the genus *Weissella.*




Source: Own authorship (2021).

Most EPS produced by strains of the genus *Weissella* are homopolysaccharides (HoPS) such as dextran, fructan, galactan, levan, and inulin for example (Björkroth, et al., 2002, Lee, *et al.*, 2002). Strains of the genus *Weissella* use sucrose as the obligatory substrate for the extracellular synthesis of HoPS. The enzymes used to hydrolyze sucrose are glycosyltransferase, glucan-sucrase (GS), or fructan-sucrase (FS), these being highly specific (Zeidan, *et al.,* 2017). The GS and FS enzymes cleave the glycosidic linkage of sucrose and couple a glucose (GS)/fructose (FS) unit to a glucan (GS)/fructan (FS) chain, water, sucrose, or other acceptors (Meng, *et al.*, 2016).

The *Weissella* strains are dependent on the proteolytic system to obtain essential amino acids, which are precursors of peptides, proteins, and aromatic compounds. Lynch et al. (2015) studied the metabolic traits of *W. cibaria* and noted that the secretome of the genus *Weissella* includes both extracellular proteins, and cell wall proteins, that are secreted. It has been found that many of the secretome proteins were large, containing multiple domains, and greater than 1000 amino acids. They also showed that *W. cibaria* and *W. confusa* have a similar number of secretory proteins as *Lb. rhamnosus* GG, which is a known probiotic strain.

The strain *W. cibaria* MG1 has the production capacity of acetate, via the pyruvate oxidase pathway in the presence of oxygen, and the production of lactic acid, diacetyl, or acetoin via the diacetyl/acetoin pathway in anaerobiosis (Lynch *et al.*, 2015). Some strains of *W. confusa, W. paramesenteroides,* and *W. cibaria* can metabolize D-xylose, glucose, D-fructose, D-mannose, sucrose, D-maltose and cellobiose and also showed β-glucosidase and β-galactosidase activity (López-Hernández, Rodríguez-Alegría, López-Munguía, & Wacher, 2017).

## 4. Ecology

Although *Weissella* sp. has a very complex nutritional requirement, it is found as autochthonous bacteria in different ecosystems. Due to this fact, they may contaminate food since it can spread easily in the processing environment. Different strains were isolated from soil (Padonou, *et al.* 2010), sediments of swamps (De Bruyne, *et al.*, 2010), and lake water (Vela *et al.*, 2011), being identified, mainly, in fermented foods such as cheese made with raw milk (Snauwaert *et al.*, 2013, Tohno *et al.*, 2013), fermented milk (Oh, *et al*., 2013, Nisiotou, *et al.*, 2014, 85], vegetables (Wouters, Grosu-Tudor, Zamfir, & De Vuyst, 2013). and sausages (Huys, Leisner, & Björkroth, 2012, Mende, Rohm, & Jaros, 2016) in Table 1 those species and their habitat are better explained.

**Table 1.** Occurrence of *Weissella* species in different ecosystems.

| Species | Habitat or source | Reference |
|---|---|---|
| *W. cibaria* | Orange, pineapple, banana | Endo, *et al*., 2009 |
| | Tomato | Di Cagno, *et al*., 2009 |
| | Wheat flour | Alfonzo, *et al.*, 2013 |
| | Blackberry, papaya | Di Cagno, Minervini, Rizzello, De Angelis & Gobbetti, 2011 |
| *W. confuse* | Rhizosphere of the olive trees and surrounding soil | Fhoula *et al*., 2013 |
| | Red and yellow raw pepper | Di Cagno, *et al*., 2009 |
| *W. halotolerans* | Rhizosphere of the olive trees and surrounding soil | Alfonzo, *et al.*, 2013 |
| | Fermented sausage | Tenea & Lara, 2019 |
| *W. hellenica* | Vegetable forage crops | Tohno, Kobayashi, Nomura, Uegaki & Cai, 2012 |
| | Croatian cheese fermented from raw milk | Fuka, *et al*., 2013 |
| *W. kandleri* | Desert plants | Holzapfel & Van Wyk, 1982 |
| *W. uvarum* | Wine grapes | Nisiotou, *et al.*, 2014 |
| *W. sagaensis* | Chinese yogurt | Li, *et al.*, 2020 |
| *W. paramesenteroides* | Raw milk cheeses | Masoud, *et al.,* 2012 |
| | Fermented sausage | Juárez-Castelán, *et al.,* 2019 |

Source: Own authorship (2021).

Lynch and colleagues investigated the genome of the *W. cibaria* species and according to the study of pan-proteome and core-proteome at the species level. Pan-proteome is all the proteins that are present in a given condition for all the species of a given life branch and the core-proteome are the proteins that are conserved in all the species of a given life branch and produced for a given condition (Trapp, *et al.*, 2016). Lynch and colleagues noted that the pan-proteome was much smaller and the core-proteome much

larger in a level of genus. The fact that the core-proteome is much larger (corresponding to 69% of the pan-proteome of the species) may explain the ability of *W. cibaria* to survive in several ecological niches where they were found since they have a higher number of proteins (729 proteins) that help in their adaptation (Lynch *et al.*, 2015).

Some strains of *Weissella* such as *W. cibaria* MG1 (Lynch *et al.*, 2015) and *W. cetti* (Ortega, *et al*., 2018) can hydrolyze arginine, which favors its survival in environments where it is subjected to stress. A good example of this is providing a greater amount of ATP when the carbon source is scarce or producing ammonia by protecting it from acid stress (Lynch *et al.*, 2015). A technological benefit of arginine deamination is the production of ornithine which is an important precursor of crust aroma compounds in sourdough (De Angelis, *et al*., 2002).

Because they are autochthonous in many places, some strains of *Weissella* can be important in the characterization of traditional products of certain regions. As an example, strains of *W. thailandensis* and *W. cibaria* have been related to Thai fermented fish (Björkroth, Dicks & Endo, 2014, Mende, Rohm, & Jaros, 2016), while the strains *W. cibaria, W. confusa,* and *W. koreensis* were detected in fermented foods of vegetal origin (Fusco *et al.*, 2015, Lynch *et al.*, 2015). In addition, the bacterium *W. beninensis* was isolated from the submerged fermentation of cassava (Padonou, *et al.* 2010) and the bacteria *W. ghanensis* and *W. fabaria* were detected in piles of fermented Ghana cocoa beans (De Bruyne, *et al.*, 2010). These products may not have the same characteristics if they were manufactured without the presence of these strains.

## 5. Some applications of bacteria from genus *Weissella* in food

*Weissella* sp. has great potential in its application in food and for this reason, it has been studied. Some strains show antagonistic activity against pathogens due to the production of several compounds like bacteriocins, organic acids, and hydrogen peroxide, among others (Fusco *et al.*, 2015, Meng, *et al.*, 2016, López-Hernández, Rodríguez-Alegría, López-Munguía, & Wacher, 2017, Goh, & Philip, 2015, Yu *et al*., 2019, Trias, Bañeras, Montesinos & Badosa, 2008). In table 2, the types of bacteriocin and what species can produce these compounds are demonstrated. *W. cibaria* TM 128 presented the production of organic acids and hydrogen peroxide, acting as inhibitors of the growth of phytopathogenic and deteriorating fungi and bacteria in fruits and vegetables (Trias, *et al.*, 2008). Some research demonstrates the antimicrobial capacity of the compounds produced

by *Weissella* against Gram-positive and Gram-negative bacteria growth (Trias, *et al.*, 2008, Kariyawasam, Jeewanthi, Lee & Paik, 2019).

**Table 2.** Bacteriocins produced by *Weissella* strains.

| **Bacteriocin** | **Producing species** | **Reference** |
|---|---|---|
| Weissellicin 110 | *W. cibaria* 110 | Srionnual, Yanagida, Lin, Hsiao, & Chen, 2007 |
| Weissellin A | *W. paramesenteroides* DX | Di Cagno, Minervini, Rizzello, De Angelis, M. & Gobbetti, 2011 |
| Weissellicin L | *W. hellenica* 4-7 | Leong, K. H. *et al.*, 2013 |
| Weissellicin D | *W. hellenica* D1501 | Chen, *et al.*, 2014 |
| Weissellicin M | *W. hellenica* QU 13 | Masuda, *et al.*, 2012 |
| Weissellicin Y | *W. hellenica* QU 13 | Kariyawasam, *et al.*, 2019 |

Source: Own authorship (2021).

Kariyawasam *et al*. (2019), used the strain *W. cibaria* D30 as an adjunct culture in cottage cheese manufacture and the strain increases the prevention of the growth of *Listeria monocytogenes* and ensures the microbial safety of ready-to-eat soft cheeses. Nam, Ha, Bae & Lee (2014), showed that *W. confusa* has antagonistic activity against the pathogen *Helicobacter pylori* a gram-negative microorganism that causes gastritis and gastric carcinoma, infects through the intake of food, and attaches to gastric and duodenal mucous membranes. *W. confusa* strain PL9001 inhibited the binding of *H. pylori* to human gastric-cell line MKN-45 cells by more than 90%. The results suggest that *Weissella* strains can be used as probiotics added in fermented milk, for example, to fight *H. pylori*. Besides that, *W. confusa* DD-A7 has antagonistic activity against the multidrug-resistant *Escherichia coli* which is resistant to almost all antibiotics used for its treatment. The strain *W. confuse* DD-A7 was capable to trigger an oxidative attack and limiting the growth of the pathogen (Dey, Khan & Kang, 2019).

The first new bacteriocin produced by *Weissella* strains to be discovered was Weissellicin 110 in the year 2007. This compound is produced by the strain *W. cibaria* 110 isolated from the Thai fermented fish product plaa-som. This bacteriocin has antimicrobial activity against some Gram-positive microorganisms and it is resistant to high temperatures

and catalase, but loses its activity when exposed to proteinase K and trypsin (Srionnual *et al.*, 2007).

In the year 2014, Chen and colleagues (Chen, *et al.*, 2014) discovered a new bacteriocin called Weissellicin D produced by the strain *W. hellenica* D1501 associated with Chinese Dong fermented meat. This bacteriocin has antimicrobial activity against the pathogenic bacteria *Staphylococcus aureus, L. monocytogenes,* and *E. coli*. This same strain has already been tested for its antagonistic capacity against the pathogens *Kurthia gibsonii, S. aureus,* and *E. coli* in soybean milk and was subsequently used in the manufacture of a new type of Tofu with increased shelf life due to the presence of volatile antimicrobial compounds and bacteriocins (Chen, Rui, Lu, Li & Dong, 2014).

Besides Weissellicin D, another isolate of *W. hellenica* demonstrated the production of Weissellicins L, M, and Y which showed antagonist activity against *L. monocytogenes* and *Bacillus coagulans* (Ayeni, *et al*., 2011, Masoud, *et al.,* 2012). Bacteriocins are natural antimicrobial compounds, there is an interest in their use by the food industry with the purpose of application as bioprotective, i.e. natural preservatives and possible substitutes for chemical preservatives (O'Connor, *et al.,* 2012). Some characteristics that make bacteriocins that are produced by lactic acid bacteria safe when used at the industrial level are their non-toxicity to eukaryotic cells inactivation by digestive proteases, little influence on the intestinal microbiota (Jawan *et al.*, 2019, Wouters *et al.,* 2013) tolerance to different temperatures and pHs, action against pathogens and microorganisms spoilage of food, and do not generate cross-resistance to antibiotics (Wouters *et al.,* 2013, Juárez-Castelán, *et al.,* 2019, Tenea & Lara, 2019).

Bacteriocins are also of great importance in the food industry, for example in the production of biodegradable food packaging with antimicrobial properties (Teixeira, *et al*., 2021). By incorporating the Bacteriocin 7293 produced by the strain *W. hellenica* BCC 7293, it was possible to control pathogenic bacteria in fillets of pangasius fish. The film produced inhibited the multiplication of both Gram-positive bacteria such *as L. monocytogenes* and *S. aureus* as well as Gram-negative bacteria such as *Pseudomonas aeruginosa, Aeromonas hydrophila, E. coli* and *Salmonella Typhimurium* (Woraprayote, *et al.* 2018).

*Weissella* can also be useful for the food industry due to its capacity of some strains to produce EPS in a sucrose culture medium (Woraprayote, *et al.* 2018). EPS are structures of high molecular mass, composed of carbohydrates, which when added to the food, behave mainly as a thickening and emulsifying agent. The polysaccharides that add thickening and gelling properties are irreplaceable in the food industry formulations. In addition to its ability

as a probiotic, EPS imposes highly desirable rheological changes in the food matrix, such as viscosity increase, improved texture, and reduced syneresis (Lakra, Domdi, Tilwani & Arul,2020). They can be used to replace corn starch in manufacturing puddings, for example, where these characteristics are desired. Some studies have tested strategies to improve the EPS characteristics to enlarge its application in the food industry (Kavitake, *et al.*, 2019). Most of the strains of *Weissella* can produce the EPS dextran and can also produce other EPS as shown in Table 3.

**Table 3.** Exopolysaccharides produced by *Weissella* strains.

| **Species** | **Product** | **Reference** |
|---|---|---|
| *Weissella cibaria* RBA12 | Dextran | Baruah, Maina, Katina, Juvonen & Goyal, 2017 |
| *W. confusa* | Dextran | Kajala I. *et al.*, 2016 |
| *Weissella confusa* KR780676 | Linear exopolysaccharide galactan | Devi, Kavitake & Shetty, 2016, Kavitake, Devi & Shetty, 2016 |
| *W. confusa* and *W. cibaria* | Dextran, fructan from sucrose, ropy capsular polysaccharide, levan, and inulin | Malang, Maina, Schwab, Tenkanen & Lacroix, 2015 |
| *W. confusa* XG-3 | Dextran | Zhao, *et al.*, 2021 |
| *W. cibaria* MD2 | Fructan | Lakra,, Ramatchandirane, Kumar, Suchiang & Arul, 2021 |

Source: Own authorship (2021).

The EPS dextran has great efficacy as soluble dietary fiber since it can be fermented by the probiotic intestinal microbiota due to its low digestibility when compared to commercial prebiotic inulin (Teixeira, *et al*., 2021). The basis for EPS production by *Weissella* strains is diverse, some studies use wheat flour and rye as the basis for fermentation. *W. confusa* presented higher rye meal production due to the higher optimum pH period for the synthesis of dextran during fermentation (Cotter, Ross & Hill, 2013, Kavitake, *et al.*, 2019).

The strains that can produce more than one exopolysaccharide are of great interest when it comes to the food industry because of their synergistic effect on texture and also

nutritional improvement (Malang, *et al.*, 2015). Some EPS that are being produced, such as galactan, has good emulsifying and stabilizing capacities which provide its future use in cosmetic and food emulsions (Ortega, *et al*., 2018, De Angelis, *et al*., 2002, Kavitake, Balvan, Devi & Shetty, 2020). The high molecular weight dextran produced by *W. confusa* QS813 can form hydrogens bonds and steric interactions with proteins. When this EPS is used in the frozen dough it's maintained the structural integrity of gluten during the freeze-thaw-cycles being appropriated to use as a cryoprotectant in wheat gluten-based frozen food (Tang, *et al.*, 2019).

Because of the production of exopolysaccharide, the application of *W. cibaria* as an assistant in the manufacture of cheddar cheese was performed resulting in a product with higher retention of humidity and without alteration of the proteolysis degree (Lynch, *et al*., 2014). In addition, *W. cibaria* MG1 produces exopolysaccharides (dextran) and oligosaccharides (glucooligosaccharides) and because of that was studied with the intention of producing a new fermented drink from wort sucrose-supplemented barley-malt-derived (Zannini, *et al.*, 2013). The oligosaccharides are being applied in some foods as prebiotics when it is impossible to use probiotics, an example of this is infant formulas. In developing countries, infant formulas are heated before consumption, because of their dubious quality of water. In this way, the addition of probiotics is impossible, and prebiotics is added instead. The oligosaccharides produced by *Weissella* can be a source of prebiotics for the food industry.

Rosca *et al*. (2018), verified that *W. confusa* produced a dextran with high structural stability and purity that have pharmaceutical importance due to its antifungal characteristics against the pathogenic yeast *Candida albicans*. Some strains such as the *W. cibaria* strain isolated from goat's milk also have been shown to have great potential as probiotics, such as resistance to 1% bile salt and tolerance to pH 3.0 (Elavarasi, Pugazhendhi, Poornima Priyadharsani, Valsala, & Thamaraiselvi, 2014). In addition, *W. cibaria*, jointly with *Lb. plantarum*, showed *in vitro* tests of high antioxidant capacity, survived simulated gastric and intestinal transit, and tolerated bile acids and salts (Yu *et al*., 2019). Furthermore, the two strains were administered to male Wistar albino rats and showed an improvement in liver and kidney functions, damaged by heavy metals compared to rats that received only the heavy metals in their diet (Ojekunle, Banwo, & Sanni, 2017).

In addition, the *Weissella* species-pair *W. cibaria/confusa*, considered potentially probiotic by Immerzeel *et al*. (2014), were studied concerning the use of xylooligosaccharides (XOS) as carbon source, since these are considered prebiotic. The

study showed that strains absorbed XOS, both xylobiose, and xylotriose, and gave an increase in the production of lactic acid when xylan hydrolyzed were used. Not only but some stains can also use xylooligosaccharides (XOS) to produce short-chain fatty acids a feature only observed in *Leuconostoc lactis* and a few strains of the established probiotics *Lactobacillus* (Månberger, *et al*., 2020).

Adesulu-Dahunsi *et al*. (2018) suggest that EPS produced by *W. cibaria* GA44 may be a commercial alternative for the food industry once it presents strong properties as an antioxidant when compared to commercial antioxidant ascorbic acid, especially scavenging of superoxide anions and hydroxyl radicals.

On the other hand, the EPS glucan-sucrase produced by *Weissella* sp. TN610 can solidify semi-skimmed milk supplemented with sucrose which shows its potential in the application as a safe additive food to improve the texture of dairy products (Bejar, *et al*., 2013). Besides that, a novel quinoa-based yogurt fermented with dextran producer *W. cibaria* MG1 was developed and the concentration of EPS (40 mg/L) ensured the high-water retention capacity and viscosity (0.57 mPa s) of the final product (Zaninni, Jeske, Lynch & Arendt, 2018).

The EPS dextran, levan, and ropy capsular polysaccharide, produced by *W. confusa* were evaluated in bread when it comes to the delaying of the deterioration by fungus, as well as improving the texture was observed (Tinzl-Malang, Rast, Grattepanche, Sych, & Lacroix, 2015). In addition to the production of EPS, some strains of *W. cibaria* and *W. confusa* as well as producing lactic acid also can produce folate (vitamin B9), which allows the nutritional improvement of fermented products that use these strains in the fermentation process (Deatraksa *et al*., 2018).

## 6. Conclusion

*Weissella* sp. may have a wide range of potential applications in food products since they can produce a high variety of compounds from the production of EPS, bacteriocins, and even vitamins such as B9. However, a few studies have been conducted and the industrial application of *Weissella* strains is still not a reality. There are still some obstacles that prevent the use of *Weissella* as a starter culture in the food industry, such as the lack of knowledge about the pathogenicity of some strains of the genus and its antagonistic capacity against other microorganisms of industrial interest due to its production of bacteriocins. Because some strains have a potential for opportunistic infection in humans,

the food industry should always be vigilant for the safety testing of any strain before its technological application.

Thereby, for the future use of this potential species in the food industry, is necessary more researches about how *Weissella* sp. and their compounds can be applied, and with this review article, it’s possible to understand that it’s necessary to research the safety use of this strains in food.

## 7. References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## PART B: *Weissella*: An Emerging Bacterium with Promising Health Benefits

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### Abstract

*Weissella* strains have been the subject of much research over the last 5 years because of the genus' technological and probiotic potential. Certain strains have attracted the attention of the pharmaceutical, medical, and food industries because of their ability to produce antimicrobial exopolysaccharides (EPSs). Moreover, *Weissella* strains are able to keep foodborne pathogens in check because of the bacteriocins, hydrogen peroxide, and organic acids they can produce; all listed have recognized pathogen inhibitory activities. The *Weissella* genus has also shown potential for treating atopic dermatitis and certain cancers. *W. cibaria*, *W. confusa*, and *W. paramesenteroides* are particularly of note because of their probiotic potential (fermentation of prebiotic fibers) and their ability to survive in the gastrointestinal tract. It is important to note that most of the *Weissella* strains with these health-promoting properties have been shown to be safe, due to the absence or the low occurrence of virulence or antibiotic-resistant genes. A large number of scientific studies continue to report on and support the use of *Weissella* strains in the food and pharmaceutical industries. This review provides an overview of these studies and draws conclusions for future uses of this rich and previously unexplored genus.

**Keywords:** *Weissella,* Technological potential, Exopolysaccharide, Probiotic

## 1. Introduction

Advances in molecular biology can be credited with the discovery of the *Weissella* genus. Collins et al. [1] reported that strains with very similar phenotypic characteristics that had previously been included in the *Leuconostoc* genus exhibited different DNA profiles from other bacteria in the group. The authors used 16S rRNA sequences to study *Weissella*. They found that certain strains demonstrated specific features that were phylogenetically closer to *Leuconostoc paramesenteroides* than others. Collins et al. [1] recommended that *L. paramesenteroides*, a new species isolated from fermented sausages, and certain heterofermentative lactobacilli be classified as a new genus, *Weissella*.

The *Weissella* genus belongs to the Firmicutes phylum, the Bacilli class, the Lactobacillales order, and the *Leuconostocaceae* family. *Weissella* bacteria are closely related to other lactic acid bacteria (LAB) genera. Fusco et al. [2] reported significant difficulty in separating the *Weissella* genus from other LAB, particularly from *Leuconostoc* species. *Weissella* species morphology may differ within the genus. The short rods with rounded to tapered ends or coccoid shapes that typify *Weissella* species are similar to *Leuconostoc*, *Oenococcus*, and streptococci [1]. Nevertheless, some *Weissella* species can present pleomorphisms under stress conditions. Cells can be present alone, in pairs, or even in small chains [1, 3]. Currently, 25 *Weissella* species have been determined: *W. viridescens*, [4], *W. paramesenteroides* [5], *W. confusa* [1], *W. kandleri* [6], *W. halotolerans* [7], *W. minor* [7], *W. hellenica* [1], *W. thailandensis* [8], *W. soli* [9], *W. cibaria* [10], *W. koreensis*[11], *W. ghanensis* [12], *W. beninenses* [13], *W. fabaria* [14], *W. ceti* [15], *W. fabalis* [16], *W. oryzae*[17], *W. diestrammenae* [18], *W. uvarum* [19], *W. cryptocerci* [20], *W. bombi* [21], *W. jogaejeotgali* [22], *W. kimchi* [23], *W. muntiaci* [24], and *W. sagaensis* [25]. A clear, well-documented summary of *Weissella* taxonomy and ecology can be found in Fusco et al. [2]. Although *Weissella* remains a relatively new genus compared with other LAB, it has been the focus of research in the past 5 years and has attracted strong interest from the food industry. Specific *Weissella* strains have been studied for their ability to produce (i) antimicrobial compounds like bacteriocins and hydrogen peroxide and (ii) exopolysaccharides (EPSs) and diacetyl. The latter is of major importance to dairy applications [26–29]. Different strains have been isolated from artisanally produced foods, which demonstrates the contributing role *Weissella* plays in the foods' characteristic features. Other studies have highlighted *Weissella* strains' ability to survive in the

gastrointestinal tract (GIT) and even grow in the gut. Both of these attributes suggest the use of *Weissella* species as beneficial applied probiotics [30–32].

The purpose of this review is to provide an overview of the *Weissella* genus and present the latest research and findings from the past 5 years. The paper presents the most recent techniques and approaches used in *Weissella* identification and characterization. It also covers the bacteria genus' potential health benefits and its main technological features which are of interest to the food industry.

## 2. *Weissella* Species Identification and Characterization

The past 5 years have seen the development of new methods for identifying *Weissella* species. They now make it possible to distinguish *Weissella* species from other closely related LAB. The polyphasic identification method and sequencing and analysis of the 16S and 23S rRNA genes are two new molecular-based identification methods that allow for rapid differentiation between *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus*, and *Weissella confusa* [33]. Complete genome sequence analysis using bioinformatic analysis can be used to describe metabolic routes and to predict potential uses for the identified strains [34–38]. These methods are the foundation of modern microbial taxonomy, as are the matrix-assisted laser desorption ionization-time of flight method (MALDI-TOF) and database mass spectrometry [39, 40]. One recent study has shown that the colony morphology of *W. confusa* can change according to the adopted culture media and incubation conditions. *W. confusa*'s morphology can vary from rod-shaped coccobacilli—with characteristic, small, cream-colored colonies gathered in single, short or long chains—to a large, irregularly shaped, transparent morphology that lacks characteristic chain cell organization [41]. *Weissella* strains can also thrive in high-sugar niches and produce large amounts of dextran [41]. *Weissella* strains' behavior in different substrates is key to differentiating them from other LAB.

Genomic and metatranscriptomic methods have been used to assess the metabolic and fermentative traits of *W. koreensis* during the kimchi fermentation process. This has led to the characterization of metabolic pathways for certain carbohydrates fermented by *W. koreensis*. These include D-glucose, D-mannose, D-lactose, L-malate, D-xylose, L-arabinose, D-ribose, N-acetyl-glucosamine, and gluconate [42]. The determination of carbohydrate metabolic pathways makes it possible to predict how specific *Weissella* substrates will ferment, as well as pinpoint those which promote higher cell multiplication rates. It also allows for the estimation of type and quantity of products generated by the

fermentation process. With this information, it is possible to predict the ecosystems where *Weissella* will survive and thus potential food and pharmaceutical applications for specific species.

Rizzello et al. [43] delved into *Weissella* genus metabolism by focusing on *W. cibaria* and *W. confusa* strains. The resulting data showed that *W. cibaria* and *W. confusa*. were able to both produce phytase and use phytic acid, thus counteracting an anti-nutritional factor in legumes. The two strains were also able to reduce the raffinose oligosaccharide (RFO) concentrations of α-galactosides raffinose, verbascose, and stachyoserafinose. RFO concentrations represent another anti-nutritional factor in vegetables, especially fava beans. When *W. cibaria* and *W. confusa* strains undergo the formation process, RFO hydrolysis enables galactose production. [43]. Legumes such as fava beans, peas, and lentils are good sources of vegetable proteins. Their anti-nutritional factors include saponins, condensed tannins, and protease inhibitors. α-Galactosides and phytic acid [44] have been shown to inhibit them. Fermentation and its subsequent reduction of these anti-nutritional factors would allow these foods to be used as new protein sources for both humans and animals. These new protein sources could in turn be used to develop new products in the food industry.

Many *Weissella* strains have been isolated from fermented products such as kimchi, pozol, jeotgal, and ogi [33, 39, 42, 45]. These strains have been characterized in terms of the carbon source they use and the compounds they can produce. While studying carbon source metabolism, Lopez-Hernandez et al. [45] observed that strains *W. cibaria*, *W. confusa*, and *W. paramesenteroides* were able to metabolize D-xylose, glucose, D-fructose, D-mannose, sucrose, and D-maltose. Some strains were also able to use ribose and esculin ferric citrate. Others were able to use galactose, metabolize cellobiose, and demonstrate β-glucosidase and β-galactosidase activity. The latter is a desirable characteristic in probiotic microorganisms, as β-glucosidase breaks down certain compounds and makes them easier for the body to absorb. Anthocyanins, which have antioxidant and anti-inflammatory properties, are among these compounds [46].

New identification methods have allowed for rapid and accurate identification of *Weissella* strains and reduced the risk of misidentification. Characterizing *Weissella* strains based on their metabolic pathways opens up the possibilities for their use in the medical and food production sectors.

## 3. Beneficial Properties of *Weissella*

Once the metabolic pathways of *Weissella* have been characterized, isolated strains can be studied for use in the pharmaceutical and food industries. *Weissella* isolates have beneficial, probiotic features and are able to produce EPS. These make them important bacteria in both pharmaceutical and food production sectors. Abriouel et al. [47] described the technological, functional, and pathogenic potential of *Weissella* genus applications in the food industry, but their review only covered research carried out before 2015. Below, our review presents and discusses the primary benefits of *Weissella* based on these areas of study.

## 4. Health Benefits of *Weissella*

Few studies in the past 5 years have examined the effects of *Weissella* strains on human health [48–50]. Some research has evaluated their effects on mice [51–55], gerbils [56], and beagles [57]. Most studies have demonstrated how *Weissella*'s probiotic characteristics and antimicrobial properties may offer health benefits. Only two strains of *W. cibaria* have been studied for their probiotic potential for humans. *W. cibaria* JW15 was studied because of how it affects cytokine and immunoglobulin natural killer cell activity, and *W. cibaria* CMU has been the subject of research because of its oral epithelial cell adhesion and oral colonization; *W. cibaria* CMU's antibacterial activity against *Fusobacterium nucleatum* and *Streptococcus mutans* [48–50] represents another important species feature. A recent study found that immune functions were enhanced by an increase in natural killer cell activity for subjects who consumed probiotic *W. cibaria* capsules [50]. Moreover, oral ingestion of *W. cibaria* CMU can help to reduce halitosis and microbiota numbers in the gingival sulcus, thus improving oral health [48, 49].

Fonseca et al. [56] tested strains of *Bifidobacterium longum* and *W. paramesenteroides* to assess their probiotic potential and their impact on parasite load in gerbils. They observed the positive effect these two strains had in reducing the parasitic load of animals infected with *Giardia*. Strains of *W. cibaria* have been studied as a way to reduce cancer treatment drug side effects in mice [55]. *W. cibaria* strains were shown to assist in the recovery of lymphocytes, hemoglobins, and platelet levels that drop when cyclosporamide (an anti-cancer agent) is administered. Cyclosporamide is widely used in the treatment of acute and chronic leukemia, lymphoma, and several autoimmune diseases [55]. *W. cibaria* strains also proved to be efficient in treating atopic dermatitis when

administered orally to mice. *W. cibaria* improved the clinical symptoms of lesions, such as erythema/hemorrhage, edema/excoriation, erosion, scarring/dryness, and lichenification [54]. In yet another study, *W. cibaria* was shown to reduce the destruction of periodontal tissue in mice and also protected against alveolar bone destruction in mice. *W. cibaria* co-aggregates with periodontal bacteria to produce hydrogen peroxide and bacteriocins that inhibit the production of proinflammatory cytokines [58]. Although clinical studies using mice are an effective research technique, these studies cannot reliably predict the outcome of human studies [59]. Further study is needed to determine the health benefits of *Weissella* strains for humans.

Although there have been few human or animal studies using *Weissella* strains in vitro research indicates a potential for future use as antimicrobial agents (Table 1) and probiotics (Table 2). Further study on animals and/ or humans is necessary to prove their efficiency as a functional species. The *W. confusa* DD_A7 strain has been shown to be a natural alternative prophylactic agent against multidrug-resistant (MDR) ESBL (extended-spectrum β-lactamase) positive *E. coli* bacteria [27, 29]. *W. confusa* has also been shown to be effective in destroying *Listeria monocytogenes* (ATCC 7644), *Staphylococcus aureus* (ATCC 12600), *Salmonella* Typhimurium (ATCC 43174), *Bacillus cereus* (ATCC 13061), and *E. coli* O157: H7 (ATCC 43889) pathogens. Several studies have shown that the supernatant from *W. confusa* culture weakened the membrane of the pathogen *E. coli* O157: H7 (ATCC 43889) and damaged the integrity of its DNA [27, 29]. Another study that paired *W. viridescens* with high-pressure processing showed strong results when it came to protecting ready-to-eat salads from *L. monocytogenes*. The research showed that high pressure was applied to the weakened pathogen membranes which then facilitated the antimicrobial activity produced by the *W. viridescens* strain [60].

**Table 1.** *Weissella* species with antimicrobial effects and the respective pathogens that each one inhibits.

| Species | Pathogen | Reference |
|---|---|---|
| *W. viridescens* | *L. monocytogenes* | Stratakos et al. [60] |
| *W. paramesenteroides* | *Giardia lamblia* | Fonseca et al. [56] |
| | *E. coli*<br>*S. aureus* | Pabari et al. [62] |
| *W. cibaria* | *Porphyromonas gingivalis*<br>*Prevotella intermedia*<br>*Fusobacterium nucleatum* | Do et al. [57] |
| | *S. enterica subsp. enterica*<br>*Escherichia coli* | Tenea et al. [108] |
| *W. confusa* | *S. enterica* Typhi | Pelyuntha et al. [109] |
| | *E. coli* | Dey et al. [29] |
| | *Candida albicans* | Rosca et al. [80] |
| | *S. enterica*<br>*S. enterica* Typhi<br>*L. monocytogenes*<br>*S. aureus* | Lakra et al. [61] |
| | *L. monocytogenes*<br>*S. aureus*<br>*S. enterica* Typhimurium<br>*B. cereus*<br>*E. coli* | Dey et al., [27] |

**Table 2.** *In vitro* and *in vivo* evaluation of *Weissella* species as a probiotic candidate.

| Species | Probiotic property | References |
|---|---|---|
| *Weissella* spp | Cholesterol-reducing potential | Anandharaj et al. [30] |
| *W. cibaria* | Enhance immune functions | Lee et al. [50] |
| | Reduce the side effects of drugs | Park and Lee [55] |
| | Improve atopic dermatitis | Lim et al. [54] |
| | Protective effects against alveolar bone destruction | Kim et al. [58] |
| | Short-chain fatty acids production and adhesion to HT-29 cells. | Silva et al. [64] |
| *W. confusa* | inhibition of –α-amylase activity | Xia et al. [32] |
| | Thermostability, cholesterol removal, β-galactosidase production, and proteolytic activity | Sharma et al. [63] |
| *W. confusa* and *W. cibaria* | reduction in cholesterol, DPPH free radical and inhibition of linoleic acid peroxidation. | Xia et al. [32]<br>Lakra et al. [61] |
| *W. paramesenteroides* | Formation of short-chain fatty acids and antimicrobial compounds. | Pabari et al. [62] |

Recent in vitro studies have shown that *W. cibaria*, *W. koreensis*, *W. confusa*, and *W. paramesenteroides* strains are strong candidates for probiotic applications [30, 32,61–64]. The survival of these strains in the GIT and their tolerance levels when in contact with bile and acids are important factors for intestinal colonization and pathogen protection. *W. paramesenteroides* has also been shown to form a biofilm that facilitates mucin adherence. It uses fructooligosaccharides (FOS) and galactooligosaccharides (GOS) as prebiotic fibers to form compounds that can survive the gut microbiome. *W. paramesenteroides* has also demonstrated antimicrobial activity against *E. coli* and *S. aureus* [62]. In another study, Anandharaj et al. [30] demonstrated that *Weissella* species may help reduce cholesterol due to its ability to use cholesterol from de Man, Rogosa, and Sharpe (MRS) agar with added Oxgall and water-soluble cholesterol. Cholesterol reduction is common in probiotic microorganisms isolated from the intestine [65–67]. Interestingly, Kim et al. [58] found that using the *W. cibaria* D30 probiotic strain in fermented *Inula britannica* created a product with improved anti-inflammatory capacity. *W. confusa* and *W. cibaria*—isolated from Plaa Som Fug—have also been shown to produce folate. Folate, or Vitamin B9, plays an important

role in DNA replication, repair, and methylation. It can also act as an antioxidant [68]. Folate is important for immune and nervous system functions because it plays an integral part in the synthesis of neurotransmitters in the central nervous system [69]. This is especially the case during the early stages of uterine growth and development.

Extensive research has been carried out on the *Weissella* genus's antimicrobial activity and its potential use of strains as probiotics. However, other beneficial compounds produced by *Weissella*, including vitamins, have only been touched on in research thus far.

## 5. EPS Production by *Weissella*

EPSs are extracellular macromolecules that have potential applications in the food, medical, and pharmaceutical industries. It has been shown that *Weissella* strains can produce these molecules, and EPS production has been the focus of several *Weissella* studies (Table 3). EPS production has been primarily documented for *W. confusa* and *W. cibaria* strains, with dextran as the dominant EPS produced by the strains. Structurally, dextran molecules are highly linear. They are formed by α-1,6- linked glucose residues with branches linked to α-1,2, α-1,3, or α-1,4. The α-1,2 bonds are representative of prebiotic properties because they are resistant to adverse GIT conditions and can help to modulate the intestinal microbiota [41, 70–72]. EPS produced by LAB are generally recognized as safe (GRAS), which means it can be used in foods, prebiotics, and medical applications.

**Table 3.** *Weissella* species and its EPS products.

| Species | EPS produced | References |
|---|---|---|
| *W. confusa* | EPS | Benhouna et al. [28] |
| | Dextran | Heperkan et al. [41] |
| | | Rosca et al. [80] |
| | | Tang et al. [86] |
| | Galactan | Devi et al. [73] |
| | | Kavitake et al. [81] |
| | Mannan | Lakra et al. [110] |
| | Lactose- and cellobiose-derived branched trisaccharides Isomelezitose | Shi et al. [78] |
| | Homopolysaccharide with glucose monomers | Adesulu-Dahunsi et al. [33] |
| Wild and mutant *W. confusa* | EPS with eight sugar moieties | Adebayo-Tayo et al. [51] |
| *W. cibaria* | Dextran | Xu et al. [87] |
| | | Zannini et al. [84] |
| | | Baruah et al. [77,82] |
| | | Kanimozhi et al. [72] |
| | | Yu et al. [75] |
| | Linear dextran | Ye et al. [74] |
| | Dextran Oligosaccharides | Hu, Gänzle [71] |
| | Heteropolysaccharides | Zhu et al. [76] |
| | Isomaltooligosaccharide | Baruah et al. [77,82] |
| | | Rolim et al. [85] |
| | EPS mainly composed of glucose and rhamnose sugar units | Adesulu-Dahunsi et al. [26] |

Of the LAB *Weissella* species, *W. cibaria* and *W. confusa* are the most important EPS producers, which is why research has focused on them and their production, purification, and characterization [41, 71–76]. Some studies have proposed ways to optimize EPS production and test different parameters, such as the effects of different temperatures and substrates. Hu and Ganzel [71] showed that *W. cibaria* strains grew faster at 30 °C, but

oligosaccharide production was higher at temperatures at or below 20 °C. The greatest dextran production occurred at 15 °C.

The physicochemical properties of bacteria-produced EPS are an important factor for functionality and use. Devi et al. [73] carried out physicochemical characterizations of galactan EPS produced by a *W. confusa* strain. They showed that the EPS displayed a high oil absorption capacity, strong emulsifying activity, and an emulsion kinetic stability of up to 15 days. These results supported the theory that galactan EPS may be a good candidate as an emulsifier in forming long-term colloidal systems in food, pharmaceutical, and cosmetic products. Other studies have shown that several *Weissella* strains use a dextransucrase enzyme to produce dextran. This enzyme has been the focus of isolation and purification studies for the production of EPS [77, 78] and caffeic acids [79]. Dextransucrase produced by a *W. confusa* strain has been shown to synthesize a rare oligosaccharide called iso-melezitose. This oligosaccharide has a potent nutraceutical effect due to its ability to promote bifidobacteria growth in the colon [78].

The EPS produced by LAB has antifungal [80], antiinflammatory, and immunomodulatory properties [51] that may be of great interest to the medical sector. EPS can also act as antioxidants [26, 28, 51, 76] and help probiotics survive passage through the GIT [81]. The linear EPS galactan produced by the *W. confusa* strain shows strong potential for encapsulation technology that is used to deliver bioactive compounds, probiotics, and drugs. The EPS produced by a *W. confusa* strain was shown to inhibit the multiplication of *Candida albicans* and was able to destroy up to 70% of the biofilm formed by this pathogen [80]. This suggests that it could be a promising candidate for use in antifungal treatments. Another recent study showed that *W. confusa* can produce mannan, an EPS made up of only monomeric units of mannose. This EPS displayed antibiofilm activity against pathogenic bacteria such as *S. aureus*, *L. monocytogenes*, *S. enterica*, and *S. typhi*. It also demonstrated potential as an antioxidant compound [61].

EPS may also have prebiotic properties. This is the case for the EPS produced by the *W. cibaria* strain isolated from pomelos (*Citrus maximum*) grown in India [82]. To be categorized as a prebiotic compound, the EPS carbohydrates must demonstrate higher metabolization levels from probiotic bacteria than from enteric bacteria present in the intestine. Dextran produced by *W. cibaria* was shown to stimulate the growth of *L. plantarum*, *L. acidophilus*, *B. animalis*, *B. bifidum*, and *B. infantis* more than *E. coli* and *E. aerogenes* [82]. This activity confirmed its classification as a prebiotic compound.

In the food industry, EPS can be used to improve the rheological characteristics and texture of fermented products. EPS can also play a role as an emulsifier and stabilizer [28, 74, 81]. EPS-producing LABs can be used as starter or secondary cultures in the production of fermented products such as yogurt [83]. The use of these cultures leads to the production of EPS in situ, where they act as natural thickeners and can eliminate the need for artificial additives [76]. *Weissella* strains have been used to ferment quinoa-based yogurt [84] and fruit juices [85, 86]. The EPS produced can improve the sensory characteristics of such products. One example: Orange juice concentrate was used to produce oligosaccharides. Then, after a fermentation process involving the sucrose present in the juice itself, a drink was obtained with reduced sugar content and specific acidity and sweetness levels [85, 86].

EPS are also of interest to the frozen dough industry, as it can modify the texture and improve the end product characteristics of frozen and thawed doughs. Studies have shown that dextrans produced by different microorganisms have different functionalities. For instance, dextran produced by the *W. cibaria* strain demonstrated a greater ability to reinforce gelling properties compared with dextran produced by *L. pseudomesenteroides* when the strains were mixed with fava bean (*Vicia faba L*.) protein isolate [87, 88]. *Weissella* EPS has also been shown to improve the characteristics of wheat gluten during dough freezing and thawing. In general, the freezing-thawing process leads to an increase in water loss, a decrease in gluten viscoelasticity, and changes in water mobility and distribution. During freezing and thawing, the continuous structure of gluten is destroyed by mechanical damage caused when ice recrystallizes. An addition of *W. confuse*-produced EPS to doughs helped the gluten to maintain its qualities and structural integrity during the freezing-thawing process. This makes *Weissella* EPS promising cryoprotectant candidates for the frozen dough industry [86].

## 6. *Weissella* as an Emerging Pathogen

*Weissella* isolates with potential health benefits must also be assessed for any adverse effects. Testing is standard procedure for isolates to be used as food supplements and starter cultures to determine if they harbor virulent or antibiotic-resistant genes, even when the negative effects are not active or apparent. [89]. If or when any noxious genetic properties are present in the isolate genome, they represent a health risk because they may be transferred to other bacteria present in the food matrix or gut [90–92].

Although many LAB cultures are found in foods and are considered safe, the biosafety of new cultures must be established before they can be used in food production

[93]. Most of the studies carried out to determine *Weissella* strains' probiotic potential also evaluate the strains' health risks. These assessments include tests for hemolytic capacity and resistance to antibiotics, PCR detection of virulence genes, and biogenic amine production [94–96]. Kang et al. [97] assessed the safety of *W. cibaria* CMU and CMS1 when used for oral care probiotics. Their results confirmed the strain is resistant to vancomycin and kanamycin (antibiotics) and that the resistance is intrinsic to the genus. They also demonstrated that the antibiotic resistance of *W. cibaria* CMU was not transferred to recipient strains. This was confirmed by the fact that no homology for a conjugative transposon integron-specific gene was present, i.e., there was no trace of antibiotic resistance genes in either chromosomal or plasmid DNA samples. They found that the strain had negative results for β-hemolysis, mucin degradation, and platelet aggregation tests. The strain also tested negative for various toxic secondary metabolites [97].

Until now, *Weissella* strains have only been associated with "weissellosis," a disease that affects rainbow trout (*Oncorhynchus mykiss*). The disease leads to septicemia in the fish and can cause important economic losses on fish farms. "Weissellosis" is caused by a specific species, *W. ceti* [98–100]. Because certain *Weissella* strains are resistant to antibiotics such as vancomycin, teicoplanin, ceftazidime, and sulfamethoxazole, human infections remain a possibility. These would primarily affect immunocompromised patients [3, 101]. It should be noted, however, that even generally recognized probiotic strains may cause health complications. *Lacticaseibacillus rhamnosus* GG and *L. helveticus* are two examples of this [102, 103].

Even though there are few cases of this genus causing disease in humans or animals, recent studies have evaluated the safety of *Weissella* use in foods. Any strain considered for use in fermented foods must be approved by government regulatory commissions. Although Brazil, the USA, and European Union countries do not have specific legislation for the production of new types of yeast, they do have recommendations. In the USA, for example, some cultures are classified as safe and suitable for human consumption while others are classified as GRAS and must apply for approval from the FDA (Food and Drug Administration) [104].

In 2011, some *Weissella* species, including *W. confusa*, were added to the International Dairy Federation (IDF) inventory, a list of microorganisms allowed in food fermentation processes. The German Committee for Biological Agents has also placed *W. confusa* in Risk Group 1 for microorganisms that are unlikely to cause human diseases [105]. *W. confusa*'s important technological potential as a source of probiotics and other

health benefits has led to two studies that address the microorganism's safety [105, 106]. Cupi and Elvig-Jorgensen [106] carried out a series of toxicity tests which included genotoxicity, skin irritation, eye irritation, and sub-chronic oral toxicity in rats. They found that the *W. confusa* strain did not show any toxic effects and therefore can be used as a safe, direct-feed microbial product for animals. Kang et al. [97] showed that *W. cibaria* strains could be granted GRAS status in the future because: (i) they do not have antibiotic-resistant genes, (ii) they lack antibiotic resistance transferability, (iii) their genomic sequences do not include virulent genes related to pathogenic bacteria, and (iv) they tested negative for most virulent factors (β-hemolysis, mucin degradation, etc.) and toxic metabolic production (ammonia production, β-glucuronidase activity, etc.).

Although many studies have demonstrated the safety of *Weissella*, the genus has not yet been included in the Qualified Presumption of Safety (QPS) list published by the European Food Safety Authority (EFSA) [107]. Research on *W. cibaria* and *W. confusa* has been carried out to this end [96]. To be granted the QPS status, a microorganism must meet the following criteria: (i) its taxonomic identity must be well defined, (ii) the available body of knowledge must be enough to establish its safety, (iii) the lack of pathogenic properties must be established and substantiated, and (iv) its intended use must be clearly described [107].

## 7. Conclusion

Over the past 5 years, the *Weissella* genus has been the focus of many studies due to its strong potential as a probiotic that could be used in both the food and pharmaceutical industries. *Weissella* strains' probiotic potential is attributed to their remarkable ability to survive passage through the GIT, produce antimicrobial substances for a variety of pathogens, and promote the formation of compounds that stimulate the gut microbiome. The ability of some *Weissella* strains to produce large amounts of EPS represents another major attribute, as EPS has prebiotic properties. *Weissella* EPS production also demonstrates the strains' potential as a natural thickener for foods, among other possibilities.

Moreover, recent studies have demonstrated that *Weissella* strains do not have antibiotic-resistant genes and they generally test negative for virulent factors. Thus, there is a low probability that the genus could cause foodborne diseases or carry on virulent genes that may be transferred to other bacteria pathogens.

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**CHAPTER II:** Biodiversity and technological features of *Weissella* isolates obtained from Brazilian artisanal cheese-producing regions

**Biodiversity and technological features of *Weissella* isolates obtained from Brazilian artisanal cheese-producing regions**

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**Abstract**

*Weissella* spp. strains (n = 57) were isolated from different Brazilian food-related environments and characterized based on their genetic profiles and technological potential. PFGE and Rep-PCR revealed the high level of biodiversity among isolates: PFGE grouped the isolates in three major profiles (similarity from 60 to 80%), while rep-PCR characterized the isolates as belonging to a single profile. Based on PFGE, isolates with identical pulsotypes were found within the same geographical region (Campo das Vertentes, Minas Gerais state). Based on these profiles, 26 isolates were selected and characterized based on their inhibitory and bacteriocinogenic activity against Listeria monocytogenes ATCC 15313, and Staphylococcus aureus subsp. aureus ATCC 6538, Salmonella enterica subsp. enterica serovar Typhimurium ATCC 14028 and Escherichia coli ATCC 11229. Most isolates (n = 20) were able to inhibit the targets through organic acids. Most of the isolates (n = 12) were able to produce diacetyl, proteases, and/or coagulating milk, but none were able to produce exopolysaccharides. Isolate 16, in particular, was characterized as possessing high acidification ability, diacetyl, and protease production, with promising technological potential in the dairy industry. The obtained results allowed the understanding of the *Weissella* spp. strains' role as starter cultures in the dairy industry.

**Keywords:** *Weissella*; dairy microbiota; diacetyl profile

## 1. Introduction

Lactic acid bacteria (LAB) is a microbial group naturally present in a wide variety of foods. LAB play an important role in the production of fermented foods due to their ability to produce aroma, flavor, and texture. *Weissella* spp. are a LAB known for lactic acid production acid through carbohydrate fermentation and for its phylogenetic position characterized as Gram-positive, catalase-negative, non-spore-forming, with coccoid morphology or short bacilli (Bj¨orkroth, Dicks, & Holzapfel, 2009; Collins, Samelis, Metaxopoulos, & Wallbanks, 1993).

The genus *Weissella* is made up of 25 validated species, most of which have already been identified in fermented foods such as vegetables, sausages, and dairy products, particularly raw milk cheeses (Jans, Bugnard, Njage, Lacroix, & Meile, 2012; Masoud et al., 2012; Pereira, San Rom˜ao, Lolkema, & Barreto Crespo, 2009; Wouters, Ayad, Hugenholtz, & Smit, 2002). In Brazil, Weissella spp. were previously been isolated from artisanal cheeses produced in Par´a, Minas Gerais, and Paraíba states (Martins et al., 2018; Medeiros et al., 2016; Perin, Savo Sardaro, Nero, Neviani, & Gatti, 2017).

*Weissella* species present many positive attributes for the food industry. Indeed, its wide array of characteristics has been attributed to the strains of this genus. Studies have shown that Weissella spp. Strains can be used as starter cultures and to produce antimicrobial substances and exopolysaccharides (Ayeni et al., 2011; Choi, Kim, Hwang, Kim, & Yoon, 2012; Srionnual, Yanagida, Lin, Hsiao, & Chen, 2007; Zannini et al., 2013). Specifically, *W. paramesenteroides* and *W. hellenica*, usually found in dairy products, have been widely studied for their antimicrobial potential, since certain strains have already been characterized as bacteriocinogenic (Chen et al., 2014; Leong et al., 2013; Masuda et al., 2012; Papagianni & Papamichael, 2011). *W. hellenica* was detected in dairy products such as raw milk Croatian cheese, while *W. paramesenteroides* was previously isolated from raw milk artisanal cheeses from different countries, thus demonstrating the relevance of this genus for dairy production (Coppola et al., 2006; Fuka et al., 2013; Gerasi, Litopoulou-Tzanetaki, & Tzanetakis, 2003; Masuda et al., 2012).

Although the phenotypic characteristics of *Weissella* spp. have been studied and *Weissella* spp. strains have been isolated and identified in artisanal cheeses, there is little information on this genus found in Brazilian artisanal cheeses. *Weissella* spp. function in such products and this genus biodiversity from region to region in Brazil remain unknown. Thus, the present study aimed to identify microbial biodiversity and evaluate the

technological potential of *Weissella* spp. isolates obtained from different Brazilian regions that produce artisanal cheeses.

## 2. Material and methods

### 2.1. Microorganisms and culture conditions

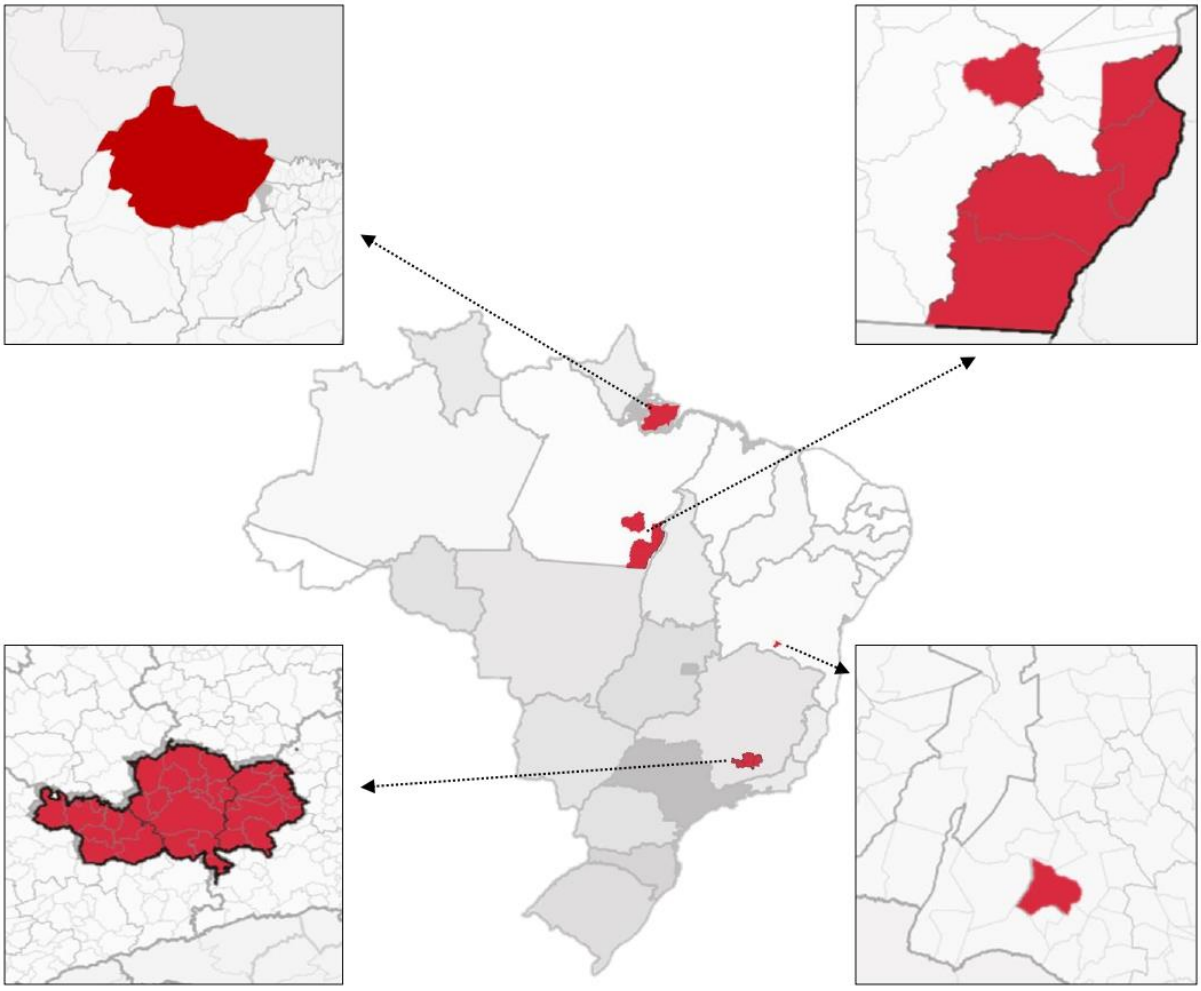
Fifty-seven (n = 57) *Weissella* spp. isolates were obtained from the InovaLeite (Laboratory of Milk and Dairy Products, Universidade Federal de Viçosa) bacterial culture collection and included in this study. The isolates were taken from samples from four different Brazilian regions that produce artisanal cheeses (Supplementary Table 1). The isolates have been previously identified by 16S rRNA sequencing (Felske, Rheims, Wolterink, Stackebrandt, & Akkermans, 1997) as *W. cibaria* (n = 38), *W. paramesenteroides* (n = 11) and *W. confusa* (n = 8) (Fig. 1). The isolates obtained were stored at -60 °C in de Man Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, England) and supplemented with 30% (v/v) glycerol (Synth, São Paulo, SP, Brazil). Prior to analysis, stock cultures (1% v/v) were transferred to MRS broth (Oxoid) and incubated at 30 °C for 14 h.

**Marajó island, Pará state**

| id | origin | species |
|---|---|---|
| 1 to 4 | utensil | *W. confusa* |
| 5, 6 | fermented curd | *W. confusa* |
| 7, 8 | cow milk | *W. confusa* |
| 9 | utensil | *W. paramesenteroides* |
| 10 | artisanal cheese | *W. paramesenteroides* |

**Southern Pará, Pará state**

| id | origin | species |
|---|---|---|
| 11 to 17 | artisanal cheese | *W. paramesenteroides* |



**Campo das Vertentes, Minas Gerais state**

| id | origin | species |
|---|---|---|
| 20 to 30 | pasture | *W. cibaria* |
| 31 to 45 | soil | *W. cibaria* |
| 46 to 50 | silage | *W. cibaria* |
| 51 to 57 | cow milk | *W. cibaria* |

**Guanambi, Bahia state**

| id | origin | species |
|---|---|---|
| 18 | Requeijão do sertão | *W. paramesenteroides* |
| 19 | Cow milk | *W. paramesenteroides* |

**Fig 1.** Strains, biotope, and geographical origins and identification of *Weissella* spp. isolates obtained from Brazilian artisanal cheese-producing regions.

## 2.2. Biodiversity determination using molecular cluster analysis

### 2.2.1. PFGE characterization of *Weissella* spp.

Pulsed-Field Gel Electrophoresis (PFGE) was performed as described by Chuat and Dalmasso (2015), with modifications. *Weissella* spp. isolates were grown to an optical density (λ = 650 nm) of 0.3 in MRS broth (Oxoid). Cells were obtained by centrifuging (3500×*g* for 10 min at 20 °C) 10 mL of culture. The supernatant was discarded and 5 mL of a TES buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0, 0.5 mol/L sucrose) was added to the pellet, then homogenized. The pellet was mixed with a lysis solution to obtain a final concentration of 10 g/L of lysozyme (Sigma, St. Louis, MO, USA) and the mixture was centrifuged once more. After an incubation period at 37 °C for 1 h, the suspension was heated at 55 °C for 1 min and 700 µL of agarose solution (ultrapure agarose, Gibco-BRL, Paisley, United Kingdom) 1% (w/v) in 125 mmol/L EDTA (pH 7.0) at the same temperature were added to the suspension. The mixture was placed in the molds and solidified at 4 °C for 10 min. The blocks were placed in a deproteinization buffer solution (10 mmol/L Tris, 100 mmol/L EDTA, pH 8.0, 10% SDS, 20 g/L proteinase K, Sigma) for 2 h at 55 °C. The agarose blocks were rinsed in sterile water at 55 °C for 10 min and four times in TE buffer (pH 8.0), for 10 min per wash. The agarose blocks were stored at 4 °C in TE Buffer (pH 8.0) until digestion.

For digestion, the DNA in the agarose blocks was cleaved with 10 U of restriction enzyme SmaI (Promega Corp., Madison, WI, USA) for 4h at 24 °C, and transferred to 1% (w/v) agarose gel (PFGE certified agarose, Bio-Rad, Hercules, CA, USA). DNA fragments were separated by PFGE in 0.5 x TBE buffer (45 mmol/L Tris, 45 mmol/L borate, 1.0 mmol/L EDTA, pH 8.3) at 14 °C in a CHEF DR II apparatus (Bio-Rad) with a pulse of 2–25 s, voltage 6 V/cm, for 18 h at 14 °C. The gel was stained with GelRed (3x in 0.1 M NaCl solution) (Biotium Inc., Hayward, CA, USA) for 15 min and the images were taken under ultraviolet light. PFGE gel images were obtained and the band profiles were analyzed using BioNumerics software, version 6.6 (Applied Maths, Kortrijk, Belgium). The normalized band profiles were compared using the Dice similarity coefficient (1%). Cluster analysis for the compiled matrix was established using the unweighted pair group method and an arithmetic average (UPGMA) clustering algorithm.

### 2.2.2. Rep-PCR characterization of *Weissella* spp.

Aliquots of 1 mL of *Weissella* spp. cultures were centrifuged at 10,000×g for 10 min at 4 °C, and the obtained pellets were subjected to DNA extraction using a DNA Purification Wizard® Genomic kit (Promega). DNA concentration was standardized at 100 ng/µL (NanoDrop™ Lite Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA).

Rep-PCR analysis was performed according to the protocol described by Dal Bello et al. (2010) using a single universal primer (GTG)5 (5′-GTGGTGGTGGTGGTG-3′ ). PCR reactions contained 12.5 µL of Go Taq Green Master Mix 2x (Promega), 0.5 µL of the primer (100 mol/L), 100 ng DNA, and ultra-pure water (Promega) for a final volume of 25 µL. PCR amplification was carried out in a thermal cycler, with the following conditions: 95 °C for 5 min as an initial step, 95 °C for 30 s, annealing at 40 °C for 30 s, then 65 °C for 8 min for the next 30 cycles, and 65 °C for 16 min to conclude the amplification.

The PCR products were electrophoresed on 2% (w/v) agarose gel for 2 h at a constant voltage of 75 V in 0.5x TBE buffer. The gels were stained using GelRed (Biotium Inc.) and developed using an LPIX transilluminator (Loccus Biotechnology, São Paulo, SP, Brazil). The band profile was analyzed using BioNumerics software 6.6 (Applied Maths). The similarities between the profiles were calculated using the Dice similarity coefficient (1%). Dendrograms were constructed using the UPGMA method.

### 2.3. Antagonism analysis and bacteriocin production

Based on the fingerprinting profiles, a representative number of isolates (n = 26) were selected for antimicrobial activity evaluation. *Weissella* spp. isolates were cultured in MRS broth (Oxoid) at 30 °C for 24 h and a cell-free supernatant (CFS) was obtained by centrifugation (10,000×g for 10 min at 4 °C) and filtration (Ø = 0.45 µm). The strains' CFS levels were tested using microdilution assays (Arena et al., 2016; Vijayakumar & Muriana, 2015) to determine antagonism against *Listeria monocytogenes* ATCC 15313, *Staphylococcus aureus* subsp. *aureus* ATCC 6538, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and *Escherichia coli* ATCC 11229. Target strains were cultured in a brain heart infusion (BHI, Oxoid) at 37 °C for 24 h and aliquots (100 µL, at a concentration of about $10^4$ CFU/mL) were transferred to 96-well microtiter plates. Then, 50 µL aliquots of the selected Weissella spp. isolates' CFS were added to each well. The microplates were incubated at 37 °C for 24 h in a Multiskan™ GO Microplate

Spectrophotometer (Thermo Fisher). The cultures' optical densities (OD) were measured every 30 min (λ = 600 nm) (Arena et al., 2016; Vijayakumar & Muriana, 2015). The CFS pH was measured both before (pH0h) and after incubation (pH24h), and results were presented as the difference of both records (ΔpH = pH0h – pH24h). Growth controls were prepared in wells added with the target culture (100 µL) and MRS broth (50 µL, Oxoid). Negative controls were prepared in wells added with BHI (150 µL, Oxoid) alone. Inhibition assays were conducted in triplicate and over two independent repetitions. Recorded ODs were corrected based on negative controls, and OD mean values were calculated over the reading time; OD from positive controls (ODc) was compared with OD from CFS-treated cultures (ODt) and inhibition values were calculated based on the following equation:

$$\text{Inhibition} = 100 - (ODt \times 100/ODc)$$

Based on the antagonism test results, isolates that presented antimicrobial activity higher than 60% were selected to verify their bacteriocinogenic activity. CFS of the selected isolates was obtained as previously described. Furthermore, it was treated as follows: pH was adjusted to 6.5 with 1 mol/L NaOH to neutralize the medium and a heat treatment was applied for 10 min, at 80 °C, to eliminate peroxides (Cavicchioli, Camargo, Todorov, & Nero, 2017). The treated CFS were tested using the same target strains as described above. The same positive and negative controls were used, and the same result interpretation methods were used. This assay was conducted in triplicate and over two independent repetitions.

### 2.4. Technological potential

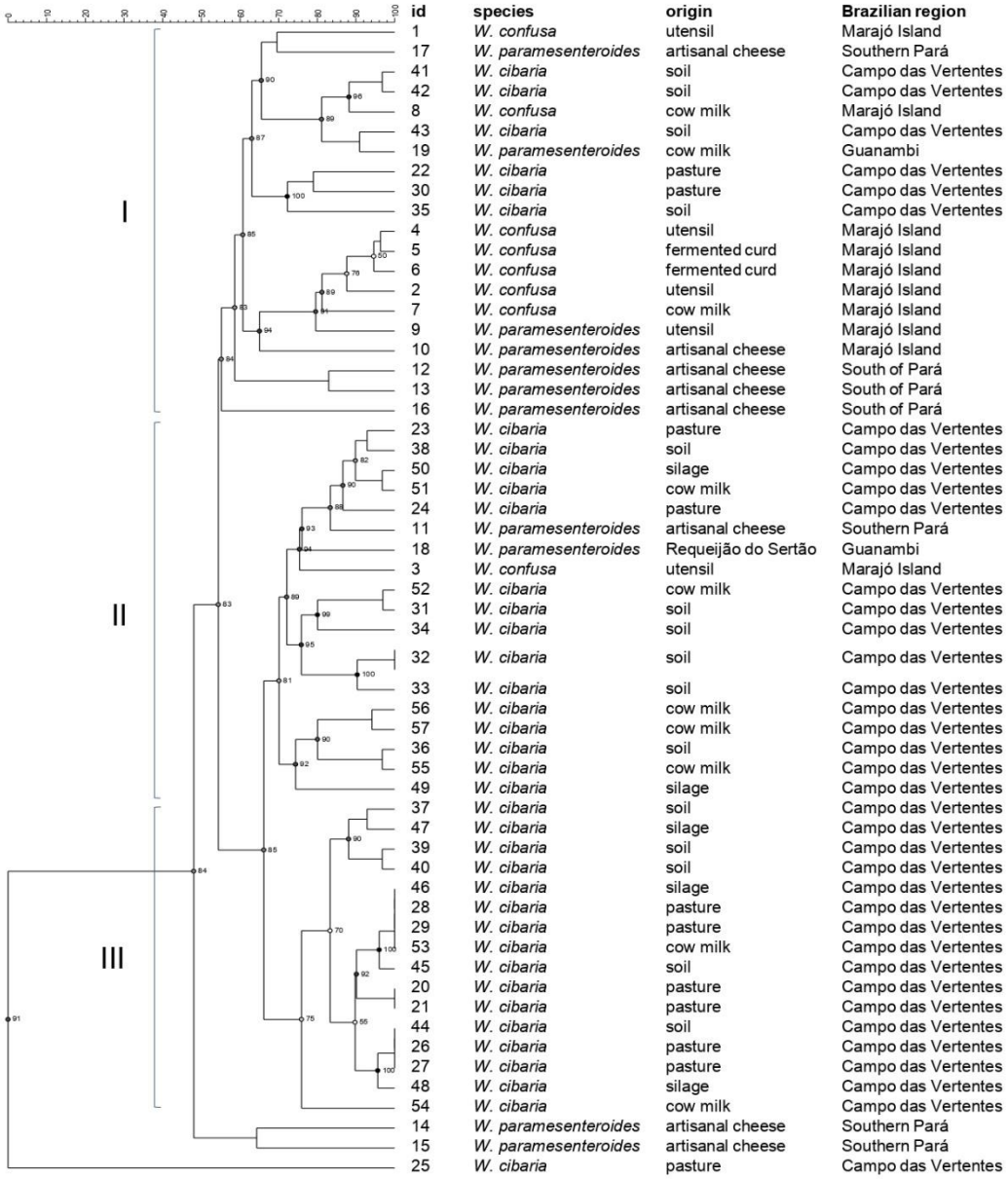
Selected isolates (n = 13) were also subjected to phenotypical analysis in order to characterize the following technological features: diacetyl production, coagulation, proteolysis, acidifying activity, and exopolysaccharide (EPS) production. Before each assay, the isolates were cultured in MRS (Oxoid) at 30 °C for 14 h and centrifuged at 14,000×*g* for 2 min at 4 °C. After the supernatant was discarded, the cell pellets were suspended in NaCl 0.85% (w/v) until a turbidity similar to McFarland tube 1 (approximately $3 \times 10^8$ CFU/mL). The obtained cell suspension of the cultures was used in the assays described below, which were conducted in triplicate and over two independent repetitions. MilliQ water was used as a negative control. To assess diacetyl production, 0.1 mL aliquots of the cell suspension of the cultures were transferred to 10 mL of sterile reconstituted skim milk (10% w/v, Nestlé, São Paulo, SP, Brazil), and incubated at 30 °C for 24 h. Then, 1 mL aliquots of the obtained

cultures were added to 0.5 mL of α-naphthol (1% w/v) and KOH (16% w/v) and incubated at 30 °C for 10 min: the formation of a red ring at the top of the tubes indicated diacetyl production (Franciosi, Settanni, Cavazza, & Poznanski, 2009). *Lactococcus lactis* subsp. *lactis* bv. diacetylactis ATCC 13675 was used as a positive control. Milk coagulation patterns were assessed by inoculating 0.1 mL aliquots of the cell suspension of the cultures into 10 mL of sterile reconstructed skim milk (10% w/v, Nestlé), followed by incubation at 30 °C for 24 h. Based on the formed clot characteristics, the coagulation patterns were described using empirical analysis and classified as: uniform, uniform with the presence of serum, uniform and fragile (appearance), broken with the presence of serum, and absence of clot (Fusieger, Martins, de Freitas, Nero, & de Carvalho, 2020). *L. lactis* subsp. *lactis* bv. diacetylactis Q1C5 was used as a positive control. A screening assay was conducted to identify the presumed extracellular proteolytic activity of the isolates as described by Franciosi et al. (2009). Two µL aliquots of bacterial cultures were spotted on the surface of a Plate Count Agar (PCA, HiMedia, Mumbai, MH, India) added to skim milk (10% w/v, Nestlé), and the cultures were incubated at 30 °C for 4 days. Presumed proteolytic activity was indicated by a clear zone around the colonies and *Pseudomonas fluorescens* 07A (Alves, Salgado, Eller, Vidigal, & Carvalho, 2016) was used as a positive control. EPS production from lactose present in milk was evaluated according to the protocol described by Dal Bello et al. (2012). 0.1 mL aliquots of selected cell suspension of the cultures were inoculated into 10 mL of sterile reconstituted skim milk (10% v/v, Nestlé) and incubated at 30 °C for 48 h. Then, the EPS production was determined by the quality and degree of stringiness: isolates were characterized as EPS producers when the coagulated cultures could be tied to a string with an inoculating loop. Acidifying activity was assessed by adding 100 µL of the selected cell suspension of the cultures to skim milk (10% w/v, Nestlé), followed by incubation at 30 °C for 24 h pH values were measured at the time of inoculation (T0), then after 6 (T6), 12 (T12), and 24 h (T24) using a digital pHmeter (Hanna Instruments, São Paulo, SP, Brazil). Also, titratable acidity was expressed as lactic acid (Dal Bello et al., 2012). Results have been shown as means of the triplicates and repetitions.

## 3. Results

### 3.1. Biodiversity by molecular analysis

Following SmaI macro-restriction, the *Weissella* spp. isolates presented 51 pulsotypes. These were grouped in three major clusters with similarity indexes among the grouped isolates varying from 60 to 80% (Fig. 2).



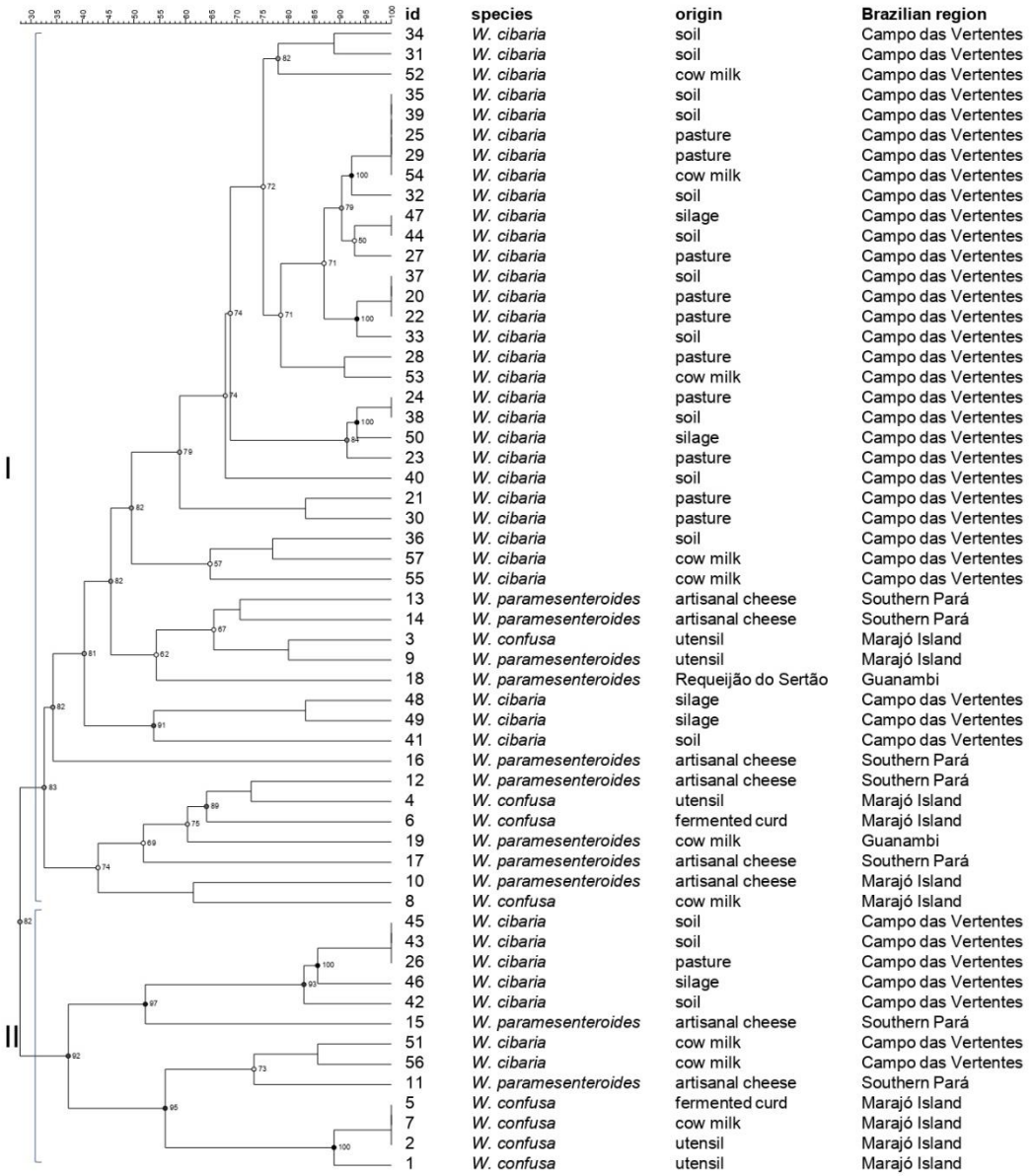

| id | species | origin | Brazilian region |
|---|---|---|---|
| 1 | *W. confusa* | utensil | Marajó Island |
| 17 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 41 | *W. cibaria* | soil | Campo das Vertentes |
| 42 | *W. cibaria* | soil | Campo das Vertentes |
| 8 | *W. confusa* | cow milk | Marajó Island |
| 43 | *W. cibaria* | soil | Campo das Vertentes |
| 19 | *W. paramesenteroides* | cow milk | Guanambi |
| 22 | *W. cibaria* | pasture | Campo das Vertentes |
| 30 | *W. cibaria* | pasture | Campo das Vertentes |
| 35 | *W. cibaria* | soil | Campo das Vertentes |
| 4 | *W. confusa* | utensil | Marajó Island |
| 5 | *W. confusa* | fermented curd | Marajó Island |
| 6 | *W. confusa* | fermented curd | Marajó Island |
| 2 | *W. confusa* | utensil | Marajó Island |
| 7 | *W. confusa* | cow milk | Marajó Island |
| 9 | *W. paramesenteroides* | utensil | Marajó Island |
| 10 | *W. paramesenteroides* | artisanal cheese | Marajó Island |
| 12 | *W. paramesenteroides* | artisanal cheese | South of Pará |
| 13 | *W. paramesenteroides* | artisanal cheese | South of Pará |
| 16 | *W. paramesenteroides* | artisanal cheese | South of Pará |
| 23 | *W. cibaria* | pasture | Campo das Vertentes |
| 38 | *W. cibaria* | soil | Campo das Vertentes |
| 50 | *W. cibaria* | silage | Campo das Vertentes |
| 51 | *W. cibaria* | cow milk | Campo das Vertentes |
| 24 | *W. cibaria* | pasture | Campo das Vertentes |
| 11 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 18 | *W. paramesenteroides* | Requeijão do Sertão | Guanambi |
| 3 | *W. confusa* | utensil | Marajó Island |
| 52 | *W. cibaria* | cow milk | Campo das Vertentes |
| 31 | *W. cibaria* | soil | Campo das Vertentes |
| 34 | *W. cibaria* | soil | Campo das Vertentes |
| 32 | *W. cibaria* | soil | Campo das Vertentes |
| 33 | *W. cibaria* | soil | Campo das Vertentes |
| 56 | *W. cibaria* | cow milk | Campo das Vertentes |
| 57 | *W. cibaria* | cow milk | Campo das Vertentes |
| 36 | *W. cibaria* | soil | Campo das Vertentes |
| 55 | *W. cibaria* | cow milk | Campo das Vertentes |
| 49 | *W. cibaria* | silage | Campo das Vertentes |
| 37 | *W. cibaria* | soil | Campo das Vertentes |
| 47 | *W. cibaria* | silage | Campo das Vertentes |
| 39 | *W. cibaria* | soil | Campo das Vertentes |
| 40 | *W. cibaria* | soil | Campo das Vertentes |
| 46 | *W. cibaria* | silage | Campo das Vertentes |
| 28 | *W. cibaria* | pasture | Campo das Vertentes |
| 29 | *W. cibaria* | pasture | Campo das Vertentes |
| 53 | *W. cibaria* | cow milk | Campo das Vertentes |
| 45 | *W. cibaria* | soil | Campo das Vertentes |
| 20 | *W. cibaria* | pasture | Campo das Vertentes |
| 21 | *W. cibaria* | pasture | Campo das Vertentes |
| 44 | *W. cibaria* | soil | Campo das Vertentes |
| 26 | *W. cibaria* | pasture | Campo das Vertentes |
| 27 | *W. cibaria* | pasture | Campo das Vertentes |
| 48 | *W. cibaria* | silage | Campo das Vertentes |
| 54 | *W. cibaria* | cow milk | Campo das Vertentes |
| 14 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 15 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 25 | *W. cibaria* | pasture | Campo das Vertentes |

**Fig 2.** Dendrograms based on UPGMA clustering of *SmaI* PFGE profile of *Weissella* spp. isolates obtained from Brazilian artisanal cheese-producing regions.

Clusters I and II included isolates from different species and from all four geographical regions, while cluster III was composed only of isolates from Campos das Vertentes and Southern Pará. Also, cluster III presented the highest index similarity among isolates (around 80%), and included isolates from different origins that shared identical pulsotypes:

isolates 28, 29, 46, and 53, obtained from silage, pasture, and cow milk (*W. cibaria*), respectively, and isolates 26, 27 and 44, obtained from soil and pasture samples (*W. cibaria*).

Based on the rep-PCR tests, the isolates presented genetic profiles with bands ranging from 500 to 5000 bp. This allowed the characterization of 45 different profiles (Fig. 3).




| id | species | origin | Brazilian region |
|---|---|---|---|
| 34 | *W. cibaria* | soil | Campo das Vertentes |
| 31 | *W. cibaria* | soil | Campo das Vertentes |
| 52 | *W. cibaria* | cow milk | Campo das Vertentes |
| 35 | *W. cibaria* | soil | Campo das Vertentes |
| 39 | *W. cibaria* | soil | Campo das Vertentes |
| 25 | *W. cibaria* | pasture | Campo das Vertentes |
| 29 | *W. cibaria* | pasture | Campo das Vertentes |
| 54 | *W. cibaria* | cow milk | Campo das Vertentes |
| 32 | *W. cibaria* | soil | Campo das Vertentes |
| 47 | *W. cibaria* | silage | Campo das Vertentes |
| 44 | *W. cibaria* | soil | Campo das Vertentes |
| 27 | *W. cibaria* | pasture | Campo das Vertentes |
| 37 | *W. cibaria* | soil | Campo das Vertentes |
| 20 | *W. cibaria* | pasture | Campo das Vertentes |
| 22 | *W. cibaria* | pasture | Campo das Vertentes |
| 33 | *W. cibaria* | soil | Campo das Vertentes |
| 28 | *W. cibaria* | pasture | Campo das Vertentes |
| 53 | *W. cibaria* | cow milk | Campo das Vertentes |
| 24 | *W. cibaria* | pasture | Campo das Vertentes |
| 38 | *W. cibaria* | soil | Campo das Vertentes |
| 50 | *W. cibaria* | silage | Campo das Vertentes |
| 23 | *W. cibaria* | pasture | Campo das Vertentes |
| 40 | *W. cibaria* | soil | Campo das Vertentes |
| 21 | *W. cibaria* | pasture | Campo das Vertentes |
| 30 | *W. cibaria* | pasture | Campo das Vertentes |
| 36 | *W. cibaria* | soil | Campo das Vertentes |
| 57 | *W. cibaria* | cow milk | Campo das Vertentes |
| 55 | *W. cibaria* | cow milk | Campo das Vertentes |
| 13 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 14 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 3 | *W. confusa* | utensil | Marajó Island |
| 9 | *W. paramesenteroides* | utensil | Marajó Island |
| 18 | *W. paramesenteroides* | Requeijão do Sertão | Guanambi |
| 48 | *W. cibaria* | silage | Campo das Vertentes |
| 49 | *W. cibaria* | silage | Campo das Vertentes |
| 41 | *W. cibaria* | soil | Campo das Vertentes |
| 16 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 12 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 4 | *W. confusa* | utensil | Marajó Island |
| 6 | *W. confusa* | fermented curd | Marajó Island |
| 19 | *W. paramesenteroides* | cow milk | Guanambi |
| 17 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 10 | *W. paramesenteroides* | artisanal cheese | Marajó Island |
| 8 | *W. confusa* | cow milk | Marajó Island |
| 45 | *W. cibaria* | soil | Campo das Vertentes |
| 43 | *W. cibaria* | soil | Campo das Vertentes |
| 26 | *W. cibaria* | pasture | Campo das Vertentes |
| 46 | *W. cibaria* | silage | Campo das Vertentes |
| 42 | *W. cibaria* | soil | Campo das Vertentes |
| 15 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 51 | *W. cibaria* | cow milk | Campo das Vertentes |
| 56 | *W. cibaria* | cow milk | Campo das Vertentes |
| 11 | *W. paramesenteroides* | artisanal cheese | Southern Pará |
| 5 | *W. confusa* | fermented curd | Marajó Island |
| 7 | *W. confusa* | cow milk | Marajó Island |
| 2 | *W. confusa* | utensil | Marajó Island |
| 1 | *W. confusa* | utensil | Marajó Island |

**Fig 3.** Dendrograms generated after cluster analysis of rep-PCR fingerprints of *Weissella* spp. obtained from Brazilian artisanal cheese-producing regions.

Unlike PFGE, the rep-PCR profiles were visually more diverse, which allowed the isolates to be clustered into two major groups, with similarity indexes among the included isolates ranging from 35 to 45%. Isolates that presented identical profiles according to rep-PCR analysis did not show the same SmaI restriction profiles (Figs. 2 and 3). However, as

we had observed for the PFGE results, rep-PCR allowed the isolates with identical genetic profiles from different samples to be identified. As examples, see: samples 25, 29, 35, 39, and 54, from the soil, pasture, and cow milk (*W. cibaria*); 20, 22, and 37, from soil and pasture (*W. cibaria*); 24 and 38, from pasture and soil (*W. cibaria*); 26, 43 and 45, from soil and pasture (*W. cibaria*), and 2, 5 and 7, from fermented curd, cow milk, and utensils (*W. confusa*).

### 3.2. Inhibitory activity

Most of the tested isolates demonstrated antagonism against the target strains, with a minimum level of 60% of inhibitory activity (Table 1). The pH range of the inoculated MRS after the isolates was added and incubated for 24 h is shown in Table 1. *L. monocytogenes* ATCC 15313 was susceptible to all isolates from Marajó Island and Guanambi. Only isolate 16, from Southern Pará, did not demonstrate any antagonistic activity above 60%. *S. aureus* ATCC 6538, *S.* Typhimurium ATCC 14028, and *E. coli* ATCC 11229 were greatly inhibited by almost all of the isolates from Marajó Island, Southern Pará, and by all isolates from Guanambi. Isolate 21 (*W. cibaria*) presented the highest inhibition level against *L. monocytogenes* ATCC 15313. Isolate 11 (*W. paramesenteroides*), from Southern Pará, presented the inhibitory activity against *S. aureus* ATCC 6538 and *S.* Typhimurium ATCC 14028. Despite the described inhibitory activity in the antagonism test, for the test with treated CFS (neutralized and heat treated), none isolate presented bacteriocinogenic activity against the target strains (data not shown).

### 3.3. Technological features

Among the 13 tested *Weissella* isolates, six exhibited diacetyl production (Table 2): isolate 25 (*W. cibaria*) results exhibited the most intense red color (data not shown). Four of these six isolates were obtained from cheese (Southern Pará) and two from pasture samples (Campo das Vertentes). *W. confusa* isolates demonstrated no diacetyl production.

In our research, four of the *Weissella* spp. isolates tested showed uniform and consistent clotting after milk coagulation (Table 2). Two demonstrated homogeneous coagulation (16 and 25, *W. paramesenteroides* and *W. cibaria*, respectively) while the other two isolates (17 and 22, *W. paramesenteroides* and *W. cibaria*, respectively) showed homogeneous clots, but only after 48 h of incubation. The other isolates did not coagulate milk.

Presumed extracellular proteolytic activity was observed in eleven *Weissella* isolates (Table 2); five of these isolates were obtained from artisanal cheeses (Southern Pará). EPS production was determined qualitatively and all strains proved to be negative (Table 2). Most samples showed some ability to acidify skim milk, producing a weak reduction of pH after 24 h of incubation, except for isolate 16 (*W. paramesenteroides*) which lowered milk pH by over 2.0 pH units after 12 h (Table 2). The titratable acidity results expressed as lactic acid content confirm the pH measurement results. (Table 2).

**Table 1.** Inhibitory potential of the cell-free supernatant of *Weissella* spp. isolates against target strains.

| Codes | Identification | *L. monocytogenes* ATCC 15313 | *S. aureus* ATCC 6538 | *S.* Typhimurium ATCC 14028 | *E. coli* ATCC 11229 | pH |
|---|---|---|---|---|---|---|
| 1 | *W. confusa* | 89.2 | 53.0 | 80.6 | 67.5 | 4.12 |
| 3 | *W. confusa* | 88.1 | 37.0 | 77.3 | 71.1 | 4.35 |
| 5 | *W. confusa* | 88.7 | 86.4 | 78.1 | 86.1 | 4.03 |
| 8 | *W. confusa* | 85.3 | 85.2 | 76.6 | 87.9 | 3.95 |
| 9 | *W. paramesenteroides* | 62.4 | 25.0 | 50.9 | 29.2 | 4.03 |
| 10 | *W. paramesenteroides* | 89.4 | 85.1 | 81.9 | 84.1 | 4.04 |
| 11 | *W. paramesenteroides* | 90.3 | 89.2 | 83.0 | 85.6 | 3.94 |
| 12 | *W. paramesenteroides* | 87.1 | 79.8 | 77.5 | 83.6 | 4.04 |
| 14 | *W. paramesenteroides* | 88.0 | 86.6 | 79.5 | 87.3 | 4.01 |
| 15 | *W. paramesenteroides* | 60.1 | 45.0 | 26.7 | 49.5 | 4.74 |
| 16 | *W. paramesenteroides* | 31.8 | 36.8 | 43.4 | 46.3 | 4.36 |
| 17 | *W. paramesenteroides* | 88.0 | 84.0 | 79.5 | 85.4 | 4.00 |
| 18 | *W. paramesenteroides* | 88.1 | 68.8 | 78.1 | 65.6 | 4.17 |
| 19 | *W. paramesenteroides* | 88.6 | 88.1 | 82.0 | 86.1 | 3.94 |
| 21 | *W. cibaria* | 98.5 | 87.1 | 76.8 | 82.8 | 4.79 |
| 22 | *W. cibaria* | 39.4 | 39.9 | 15.2 | 63.4 | 4.49 |
| 25 | *W. cibaria* | 44.0 | 22.2 | 49.9 | 18.6 | 4.41 |
| 30 | *W. cibaria* | 48.7 | 28.9 | 26.3 | 40.2 | 4.67 |
| 32 | *W. cibaria* | 26.6 | 37.0 | 4.2 | 21.8 | 4.71 |
| 35 | *W. cibaria* | 86.8 | 65.9 | 74.7 | 70.0 | 4.20 |
| 42 | *W. cibaria* | 89.1 | 88.5 | 80.3 | 86.2 | 3.92 |
| 49 | *W. cibaria* | 85.2 | 27.4 | 0.7 | 20.6 | 4.72 |
| 51 | *W. cibaria* | 86.7 | 63.8 | 78.4 | 74.0 | 4.01 |
| 52 | *W. cibaria* | 11.4 | 4.0 | 8.5 | 2.2 | 4.46 |
| 54 | *W. cibaria* | 78.5 | 30.5 | 70.7 | 28.0 | 4.75 |
| 56 | *W. cibaria* | 87.9 | 48.4 | 77.2 | 56.6 | 4.39 |

Percentage of inhibition is expresses as (%) = 100 – (mean $OD_{600\ nm}$ / mean $OD_{600\ nm}$ target strain) * 100

**Table 2.** Technological features of *Weissella* spp. isolates obtained from Brazilian artisanal cheese-producing regions

| Codes | Identification | Diacetyl | Coagulation | Proteolysis | EPS | Acidifying activity | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | pH | | | | % lactic acid | | | |
| | | | | | | T0 | T6 | T12 | T24 | T0 | T6 | T12 | T24 |
| 1 | *W. confusa* | - | absence of clot | + | - | 6.85 | 6.50 | 6.44 | 6.15 | 0.14 | 0.15 | 0.13 | 0.19 |
| 5 | *W. confusa* | - | absence of clot | + | - | 6.85 | 6.59 | 6.26 | 6.15 | 0.14 | 0.11 | 0.16 | 0.19 |
| 10 | *W. paramesenteroides* | - | absence of clot | + | - | 6.85 | 6.62 | 6.40 | 6.33 | 0.14 | 0.13 | 0.11 | 0.20 |
| 12 | *W. paramesenteroides* | + | absence of clot | + | - | 6.85 | 6.60 | 6.42 | 5.46 | 0.14 | 0.13 | 0.13 | 0.23 |
| 14 | *W. paramesenteroides* | + | absence of clot | + | - | 6.85 | 6.63 | 6.45 | 6.27 | 0.14 | 0.12 | 0.14 | 0.18 |
| 15 | *W. paramesenteroides* | + | absence of clot | + | - | 6.85 | 6.62 | 6.43 | 6.36 | 0.14 | 0.13 | 0.17 | 0.16 |
| 16 | *W. paramesenteroides* | + | uniform | + | - | 6.85 | 5.41 | 4.65 | 4.60 | 0.14 | 0.37 | 0.57 | 0.77 |
| 17 | *W. paramesenteroides* | - | uniform | + | - | 6.85 | 6.53 | 6.14 | 5.84 | 0.14 | 0.12 | 0.20 | 0.27 |
| 18 | *W. paramesenteroides* | - | absence of clot | + | - | 6.85 | 6.69 | 6.53 | 6.37 | 0.14 | 0.12 | 0.13 | 0.17 |
| 19 | *W. paramesenteroides* | - | absence of clot | + | - | 6.85 | 6.57 | 6.40 | 6.24 | 0.14 | 0.13 | 0.15 | 0.18 |
| 22 | *W. cibaria* | + | uniform | + | - | 6.85 | 6.30 | 5.78 | 5.35 | 0.14 | 0.17 | 0.29 | 0.38 |
| 25 | *W. cibaria* | + | uniform | - | - | 6.85 | 6.47 | 5.58 | 5.00 | 0.14 | 0.15 | 0.35 | 0.60 |
| 54 | *W. cibaria* | - | absence of clot | - | - | 6.85 | 6.77 | 6.73 | 6.66 | 0.14 | 0.12 | 0.13 | 0.12 |

Results: +, positive; −, negative

## 4. Discussion

Based on the profiles obtained by PFGE and rep-PCR (Figs. 2 and 3), the studied isolates presented high variability; the two techniques grouped the isolates differently, which is to be expected because the approaches used are totally different. Nisiotou, Dourou, Filippousi, Banilas, and Tassou (2014) have described differences found in *W. uvarum* groupings obtained using PFGE and rep-PCR in a study on grape isolates (Nemea region, Greece). rep-PCR testing presented a greater number of genetic profiles compared to PFGE (23 and 29, respectively), and the profiles had fewer similarities compared to the results obtained using PFGE. These results have also been observed by other authors who used both rep-PCR and PFGE techniques with different lactic bacteria (Cavicchioli et al., 2015; Perin & Nero, 2014). In general, clustering obtained by PFGE confirmed the rep-PCR results, where clustering tendencies between isolates species and regions have been observed (Figs. 2 and 3). However, similarity and distribution levels within the groups were not the same for the two techniques: PFGE was able to establish clearer associations between similar isolates, thus demonstrating better discriminatory properties and genetic characterizations of these isolates. As PFGE and rep-PCR require very different approaches, the first based on the digestion of entire DNA and the second based on the amplification of specific DNA regions, the profiles recorded were expected to be different (Tenover et al., 1995). Freitas et al. (2015) compared PFGE to random amplified polymorphic DNA (RAPD), another molecular technique, and concluded that PFGE provides better heterogeneity resolution between strains of the same species. Thus, in this study, the PFGE technique was chosen to screen select samples from each cluster for antimicrobial activity assays.

The three *Weissella* species studied were present in almost all types of original samples including milk, cheese, pasture, and utensils. This demonstrates the high diversity of strains found in the regions studied. Fuka et al. (2013) and others have also identified *Weissella* strains in dairy environments and isolated *W. paramesenteroides* and *W. hellenica* in Croatian raw sheep's milk cheese. Masoud et al. (2012) have also isolated the same *Weissella* species in raw milk and raw milk cheeses. The regions studied represent specific ecological niches as isolated strains from different regions did not present similar PFGE restriction profiles (100% similarity). Freitas et al. (2015) in the research on *Propionibacterium* biodiversity obtained the same results with each of the farms they studied in Campo das Vertentes, representing a different bacterial ecological niche.

Using PFGE, identical pulsotypes were found within the same geographical regions (Fig. 2). In addition to belonging to the same regional classification (Campo das Vertentes), these isolates were taken from very closely related and/or similar samples, such as isolates 20 and 21 from pasture and isolate 44, 26 and 27, from soil and pasture from the same region (Fig. 1). Martins et al. (2020) used PFGE to compare 23 isolates of *L. lactis* subsp. *lactis* isolated from the dairy environment in four different regions in Brazil and showed that isolates presented identical profiles by PFGE. Here, the isolates belonged to the same region and similar samples.

The high susceptibility levels of *L. monocytogenes* to *Weissella* supernatants (Table 1) were justified because antimicrobial activity among related microorganisms is common in Gram-positive bacteria. *E. coli* was used as a Gram-negative target microorganism and it was also shown to be highly inhibited by supernatants. Ortolani, Yamazi, Moraes, Viçosa, and Nero (2010) also reported LAB antagonist activity for foodborne pathogens *L. monocytogenes* and *S. aureus* when they studied isolates taken from raw milk and soft cheese. When Espeche, Otero, Sesma, and Nader-Macias (2009) isolated 6 strains of *W. paramesenteroides* from bovine milk samples, one sample also showed antimicrobial activity for *Streptococcus dysgalactiae* subsp. *dysgalactiae* ATCC 27957 and pathogenic *E. coli*. It should be noted that only cells were removed from *Weissella* sp. supernatants in this test, and the cells were obtained under optimum growth conditions, which does not prevent the production of more than one antimicrobial compound. LAB can produce a variety of antimicrobial compounds including hydrogen peroxide, organic acids (lactic acid, acetic acid, formic acid, for example), carbon dioxide, bacteriocins, ethanol, and diacetyl (Leroy & De Vuyst, 2004). The bacteriocin production test revealed that the isolates did not demonstrate any production capacity under the growth conditions (data not shown). However, *W. paramesenteroides* DX is able to produce the bacteriocin weissellin A when grown in M17 + 2% glucose broth at 30 °C for 60 h, according to Papagianni and Papamichael (2011). Srionnual et al. (2007) also incubated *W. cibaria* 110 in MRS broth at 30 °C and obtained bacteriocin weissellicin 110.

Decreases in pH indicate that acids were produced during the incubation of isolates in MRS. Because MRS includes dextrose and ammonium citrate in its formulation, it can be assumed that the acids produced by the strains are lactic acid and acetic acid, via glycolytic and phosphoketolase pathways, as well as acetic acid via a diacetyl/acetoin pathway (Lynch et al., 2015). These organic acids have antimicrobial capacities when the pathogenic bacteria's cytoplasmic medium experiences a decrease in pH. According to Russell (1992),

the non-dissociated form of organic acids is liposoluble and able to passively pass through cell cytoplasmic membranes. It changes the pH within cells by modifying the proton gradient and the electric charge when it interferes with the amino acid transport system and inactivates enzymes.

*Weissella* sp. is autochthonous to certain Brazilian raw milk artisanal cheeses and their production environments. The fact that they present antimicrobial activity against important foodborne pathogens indicates the microbiota may interfere with pathogens present in raw milk and inhibit them. According to Jay (1996), the effects of autochthonous microorganisms on pathogens are well established. This effect is greater in animal-derived products containing a rich autochthonous microbiota.

The six isolates capable of producing diacetyl included two *W. cibaria* and four *W. paramesenteroides* (Table 2). Studies have shown that the *W. cibaria* MG1 strain isolated from pozol can produce diacetyl via a diacetyl/acetoin pathway (L´opez-Hern´andez, Rodríguez-Alegría, L´opez-Munguía, & Wacher, 2018). Additionally, Pakdeeto, Naranong, and Tanasupawat (2003) observed diacetyl production by *W. confusa* strains under incubation conditions in modified MRS broth. Diacetyl is a volatile compound generated as a final product when citrate is converted to pyruvate. It confers a buttery aroma to fermented dairy products and it is a common LAB feature that is usually associated with *L. lactis* subsp. *lactis* bv. *diacetylactis* (Fusieger, Martins, Freitas, Nero, & Carvalho, 2020; Leroy & De Vuyst, 2004; Smit, Smit, & Engels, 2005).

Of the four isolates that were able to coagulate milk, two were *W. cibaria* (Table 2). Some studies have shown the ability of *W. cibaria* strains to ferment lactose and galactose (Bounaix et al., 2010; Lynch et al., 2014). According to Lynch et al. (2015), *W. cibaria* MG1 possesses all the necessary genes for the phosphoketolase pathway and metabolizes galactose via the Leloir pathway. Milk coagulation is important in the production of fermented products such as yogurt and cheeses. Coagulation occurs when milk pH is lowered by LAB acid production. When the caseins' isoelectric point is reached, this adds to the effects of the expelled serum to form yogurt gels and cheese curds (Robinson, 2002; Tamime & Robinson, 2007).

Presumed proteolytic activity was observed for almost all the isolates selected in our study (Table 2). Lynch et al. (2014) have also evaluated the proteolysis activity of strains of *W. cibaria* MG1 using tests in milk agar and report that this enzymatic activity is strain-specific. LAB proteolytic and lipolytic activities in cheese lead to aroma and flavor generation

and contribute to the release of bioactive peptides in milk that are beneficial to consumer health (Meisel & Bockelmann, 1999; Wouters et al., 2002).

According to Cogan et al. (1997), isolates with acidification capacities must reduce milk pH to 1.3 units after 6 h of incubation. The isolates examined in this study can be characterized as poor acid producers, except for isolate 16 (*W. paramesenteroides*), which was the only one able to reduce the pH to 5.41 after 6 h of incubation at 30 °C. It also produced a large amount of acid after 24 h of incubation (Table 2). Isolate 25 (*W. cibaria*) also produced a large amount of acid at the end of 24 h, but it required 12 h for it to reach a pH below 5.75 (Table 2).

Isolate 16 (*W. paramesenteroides*) was isolated from cheese samples (Southern Pará) which could justify its strong acidification properties given the optimal adaptation it has undergone. In addition, we can conclude that this isolate may play an important role in cheese. Not only did it show positive results for acidification and pH reduction in milk, but it also showed promising results for diacetyl production (which generates aroma), proteolytic activity (texture), and homogeneous clot formation.

## 5. Conclusion

This study shows the genetic diversity of *Weissella* strains isolated from different sources and different Brazilian regions that produce artisanal cheeses. It demonstrates the relevance of characterizing the molecular diversity of autochthonous *Weissella* in various environments. The technological characterizations helped us understand the role *Weissella* strains play in artisanal cheese microbiota and demonstrated their potential features. Further studies will be conducted to elucidate the contributing of *Weissella* species on cheese quality and sensory aspects, allowing propose their use as starter cultures in the dairy industry.

## 6. Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.111474.

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# **CHAPTER III:** *Weissella cibaria* W25: Comparative genomic analyses

**Genomic analyses of *Weissella cibaria* W25, a potential bacteriocin-producing strain isolated from pasture in Campos das Vertentes, Minas Gerais, Brazil**

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**Abstract**

*Weissella* is a genus containing Gram-positive, heterofermentative bacteria belonging to the lactic acid bacteria (LAB) group. These bacteria are endowed with promising technological and antimicrobial attributes. *Weissella cibaria* W25 was isolated from a dairy environment where raw milk cheeses are produced. Therefore, we sequenced and assembled the W25 draft genome sequence, which consists of 41 contigs totaling ~2.4Mbp, with a G+C content of 45.04%. Then we carried out comprehensive a comparative genomic analysis with *W. cibaria* 110, known to pro-duce the weissellicin 110 bacteriocin, and four other non-bacteriocin-producing *W. cibaria* strains.

**Keywords:** *Weissella*; genome sequencing; bacteriocins genes

## 1. Introduction

The study of microbial diversity in dairy and non-dairy environments plays a pivotal role in understanding the presence of these microorganisms in such ecosystems and their impact on the final product, especially when we refer to traditional and artisanal products. Each environment has unique and specific characteristics that favor and allow the development of different bacterial species [1].

Handmade cheeses and raw milk are considered potential sources of new strains of LAB [2]. The way these cheeses are made can determine the fermentation to be con-ducted by bacteria present in the grazing, animal skin, utensils, surfaces, and other places that may come into contact with the cheese during production [3]. The study of the bacterial community present in artisanal cheeses revealed the presence of species that had not yet been related to cheeses and a high diversity of lactic bacteria with differentiated technological characteristics [4]. In addition, non-dairy environments such as grass, different types of silage, and even animal skin have also been important sources of novel strains that have adapted and therefore, can provide interesting features to be explored [5].

The diversity of *Weissella* isolated from dairy and non-dairy environments is of great interest for the enrichment of knowledge of this microorganism in the final products. The genus *Weissella* is composed of bacteria classified as Gram-positive, catalase-negative, non-spore-forming, coccoid morphology, or short bacilli. They belong to the group LAB, mainly due to their production of lactic acid from the fermentation of carbohydrates [6]. The main purpose of this study is to announce and analyze the sequencing and annotation of the *Weissella cibaria* W25 genome and carry out a comprehensive comparative genomic analysis with *W. cibaria* 110, known to produce the weissellicin 110 bacteriocin and four other non-bacteriocin-producing *W. cibaria* strains.

## 2. Materials and Methods

### 2.1. Bacterial strain

The strain W25 was previously isolated from pasture sampled from a dairy farm located in the Campos da Vertentes region, in the Southeast of the Minas Gerais state, Brazil. This strain was named “isolate id 25” by Teixeira, et al. [7], identified as *Weissella cibaria* after sequencing of the gene 16S rRNA and characterized as possessing a technological potential due to the ability to coagulate milk, producing diacetyl and non-

proteolytic. Of note, the strain presented antimicrobial activity against a panel of Gram-positive and Gram-negative foodborne pathogens [7].

### 2.2. Genome sequencing and assembly

The whole genome of *W. cibaria* W25 was sequenced with Nextera technology by a whole-genome shotgun strategy using the MiSeq v3 machine and Solexa platform (Il-lumina, USA) by Neoprospecta (Florianópolis, SC, Brazil). The trimming was performed using Trimmomatic v.0.36 [8] and the Phred value > 20. The raw read files were trimmed of adapter sequences, and low-quality reads. After trimming, sequence reads were checked for quality using the fastQC v.0.11.5 [9] and then used for de novo genome assembly. Genome assembly was conducted by using MIRA Assembler v.4.9.6 [10], mode “genome, accurate”. The assembling quality was determined with QUAST v.5.0.2 [11], and ContEst16S was used to check contamination [12].

### 2.3. Genome annotation and analysis

Gene prediction and annotation were performed using the Rapid Prokaryotic Genome Annotation (PROKKA) v.1.14.5 [13], executed with default parameters and also performed by RAST automated web server [14]. To identify secondary metabolite biosynthetic gene clusters and bacteriocins, we used the antiSMASH v.6.0 [15] and the BAGEL4 webserver [16]. Moreover, the research for plasmid was evaluated by plasmidFinder [17] and the web tool PathogenFinder [18] was used to check the presence of potential virulence factors.

### 2.4. Phylogenetic analyses

The identification of the genus and species was carried out using KmerFinder [19,20] and the Type (Strains) Genome Server (TYGS) [21]. The phylogenetic trees were visualized and edited using the online tool iTol v.6 (https://doi.org/10.1093/nar/gkw290).

### 2.5. Comparative genomic

For establishing the relationship between *W. cibaria* W25 and other members of this species in the bacteriocin production context we selected four published non-bacteriocins-producing *W. cibaria* strains and *W. cibaria* 110 known to produce the weissellicin 110 bacteriocin (Table 1). The genomes obtained from the GenBank were annotated using Prokka before subjecting to analyses in order to standardize the annotations.

All the strains were submitted to the Type (Strains) Genome Server (TYGS) to confirm genus and species. Moreover, to establish the genetic similarity between all strains, analysis was done with Digital DDH (DNA–DNA hybridization) similarities based on the GGDC (Genome-to-Genome Distance Calculator) web server, version 3.0 [22]. The core genome of each group was determined with OrthoVenn2 (e-value of 10-5) [23] and the CGView Server [24] was used to comparative genome analysis using BLAST with default parameters.

### 2.6. Availability of nucleotide sequence data

This Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the accession JAFNKE000000000. The version described in this paper is version JAFNKE010000000. The raw sequencing data were submitted to Sequence Read Archive (SRA) database under accession number SRR16076638.

## 3. Results and Discussion

### 3.1. Genome sequencing, annotation, and analysis

The genome features of *W. cibaria* W25, *W. cibaria* 110, *W. cibaria* B3b, *W. cibaria* ffPR, *W. cibaria* JCM 12495 and *W. cibaria* MG1 are presented in table 1. The whole-genome sequencing of *W. cibaria* W25 resulted in maximum size of reads for the forward sequence of 305 and for the reverse sequence of 205 and with a total number of sequences of 2,906,916 bp. After genome assembly using the MIRA software, we obtained a draft genome with 41 contigs, N50 202,649 bp, and a maximum length of 331,445 bp (contigs over 500 bases).

The genome of *W. cibaria* W25 contains 2,412,435 bp, which is very similar to the genome of *W. cibaria* MG1, slightly bigger than *W. cibaria* 110, *W. cibaria* ff3PR and *W. cibaria* JCM 12495. However, the GC content is very similar between all of them varying from 44.7 to 45.1%. Genome annotation using Prokka identified a total of 2,190 coding DNA sequences (CDS) which ware more abundant than in the genome of *W. cibaria* JCM 12495 and less abundant than in other genomes. *W. cibaria* W25 presented the highest quantity of tRNA and rRNA with 11 copies of 5S ribosomal RNA (rRNA) genes, 3 copies of 16S, and one single copy of 23S rRNA genes.

**Table 1.** Comparison of the genomic feature of *W. cibaria* W25 with a bacteriocin-producing *W. cibaria* strains 110, and four other non-bacteriocin-producing strains.

| Genome feature | W25 | 110 | AB3b | ff3PR | JCM 12495$^T$ | MG1 |
|---|---|---|---|---|---|---|
| Accession [a] | JAFNKE000000000 | LRRC00000000 | JWHT00000000 | JWHV00000000 | BJEF00000000 | JWHU00000000 |
| Reference | This study | [25] | [26] | [26] | [27] | [26] |
| Contigs | 41 | 18 | 88 | 60 | 25 | 44 |
| Size (pb) | 2,412,435 | 2,347,049 | 2,465,158 | 2,357,128 | 2,323,953 | 2,436,232 |
| GC content (%)[b] | 45.04 | 44.9 | 44.7 | 44.9 | 45.1 | 44.7 |
| CDS | 2,190 | 2,209 | 2,348 | 2,228 | 2,124 | 2,284 |
| tRNA | 84 | 76 | 67 | 70 | 77 | 62 |
| rRNA | 15 | 5 | 7 | 5 | 9 | 4 |
| tmRNA | 1 | 1 | 1 | 1 | 1 | 1 |
| Repeat region | 1 | 1 | 0 | 0 | 0 | 0 |

[a] GenBank accession number; [b] using RAST program.

No plasmid gene was detected by plasmidFinder in *W. cibaria* W25 and this input organism was predicted as a non-human pathogen by PathogenFinder. These results are indicative of the safe use of this strain for future human consumption as a probiotic or as a bioprotective culture in food, for example.

According to AntiSMASH *W. cibaria* W25 possess two putative bacteriocins gene clusters, one lasso peptide (MicJ25), and one RiPP-like bacteriocin_IIc. Of, note, the Bagel4 software did not allow the identification of any bacteriocin gene. Previously, we showed that *W. cibaria* W25 has a narrow spectrum of inhibition against the most common foodborne pathogens [7] reinforcing that this strain is a suitable probiotic candidate. Li et al. (2017)[25] showed that *W. cibaria* 110 present similar results showing a large spectrum of inhibition against other LAB but the bacteriocin weissellicin 110, produced by *W. cibaria* 110, unlike most class II bacteriocins, has no inhibitory activity against *Listeria monocytogenes.*

### 3.2. Phylogenetic analyses and comparative genomic

*Weissella* phylogeny was constructed using available 16S rRNA sequences of sequenced *Weissella* species, including complete and draft genomes (Figure 1) and using the whole genome sequences (Figure 2). Figure 1 and 2 shows the formation of two major clusters, using only the 16S rRNA sequence we noticed that cluster 1 com-prised 4 strains (*W. confusa*, *W. cibaria,* and *W. muntiaci*) and cluster 2 comprises the other strains. On the other hand when we used the whole genome sequence, cluster 1 comprised *W. ceti* and *W. diestrammenae,* and cluster 2 all the other strains. Also figures 1 and 2 show that *W. cibaria*

W25 and *W. cibaria* JCM 12495 are phylogenetically closely related. These results along with the one from KmerFinder software confirmed the genus *Weissella* and the species cibaria for the strain W25 as previously announced by Teixeira et. al (2021).

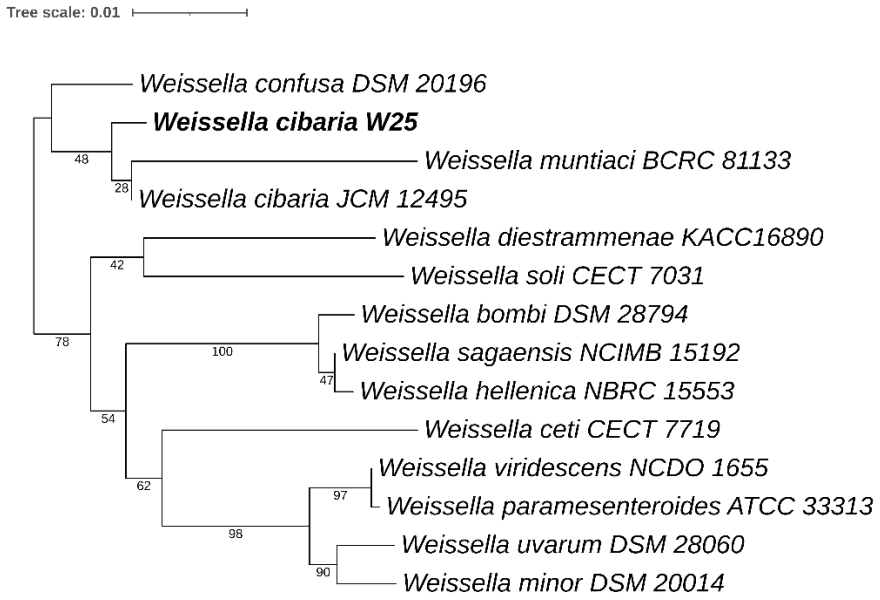


**Figure 1.** Tree inferred with FastME 2.1.6.1[28] from GBDP distances calculated from 16S rDNA gene sequences. The branch lengths are scaled in terms of GBDP distance formula $d_5$. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 67.6 %. The tree was rooted at the midpoint [29].

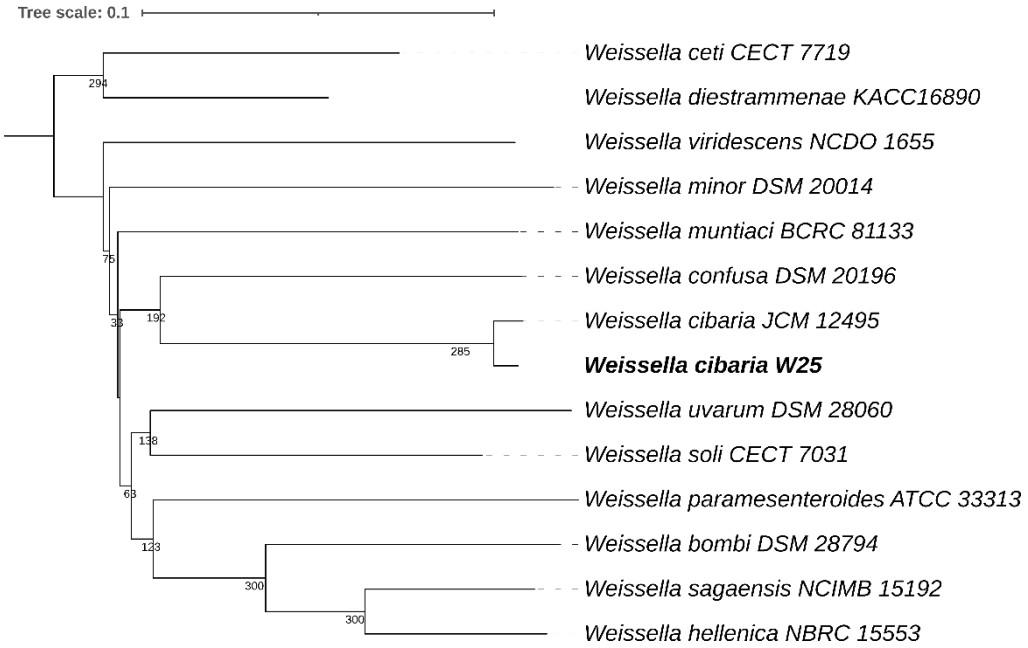


**Figure 2.** Tree inferred with FastME 2.1.6.1 [28] from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers

above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 55.9 %. The tree was rooted at the midpoint [29].

The digital DDH genomic similarity revealed that between the strains tested in this study, *W. cibaria* W25 possesses more similarity with *W. cibaria* JCM 12495 (87.10%), a non-bacteriocin producer, and less similarity with *W. cibaria* 110 (63.80%), a strain known as a bacteriocin producer.

According to the Veen diagram from OrthoVenn2, *W. cibaria* W25 shares the same quantity of protein cluster genes with *W. cibaria* 110 and *W. cibaria* JCM 12495 (Figure 3) when comparing just the three of them. When we compared with all the strains used in this study, *W. cibaria* W25 shares more unique protein cluster genes [18] with *W. cibaria* 110 (Figure 4, B) than with the others. Besides that, *W. cibaria* W25 shares 25 with all the non-bacteriocin-producing strains (Figure 4, C). There were 1,852 protein cluster genes conserved in all *W. cibaria* strains, and five of them are unique to *W. cibaria* W25 (Figure 4, A) with 10 paralogs. Among the five protein cluster coding genes OrthoVenn2 identified three of them, one related to the lipopolysaccharide biosynthetic process, one to the O antigen biosynthetic process, and one to oxidoreductase activity. Li et al. (2017)[25] also compared *W. cibaria* 110 with four other strains (*W. cibaria* MG1, *W. cibaria* AB3b, *W. cibaria* ff3PR, and *W. cibaria* KACC11862) and the comparative genomic analysis also showed the presence of unique genes that encoded the novel bacteriocin weissellicin 110 and defense system.

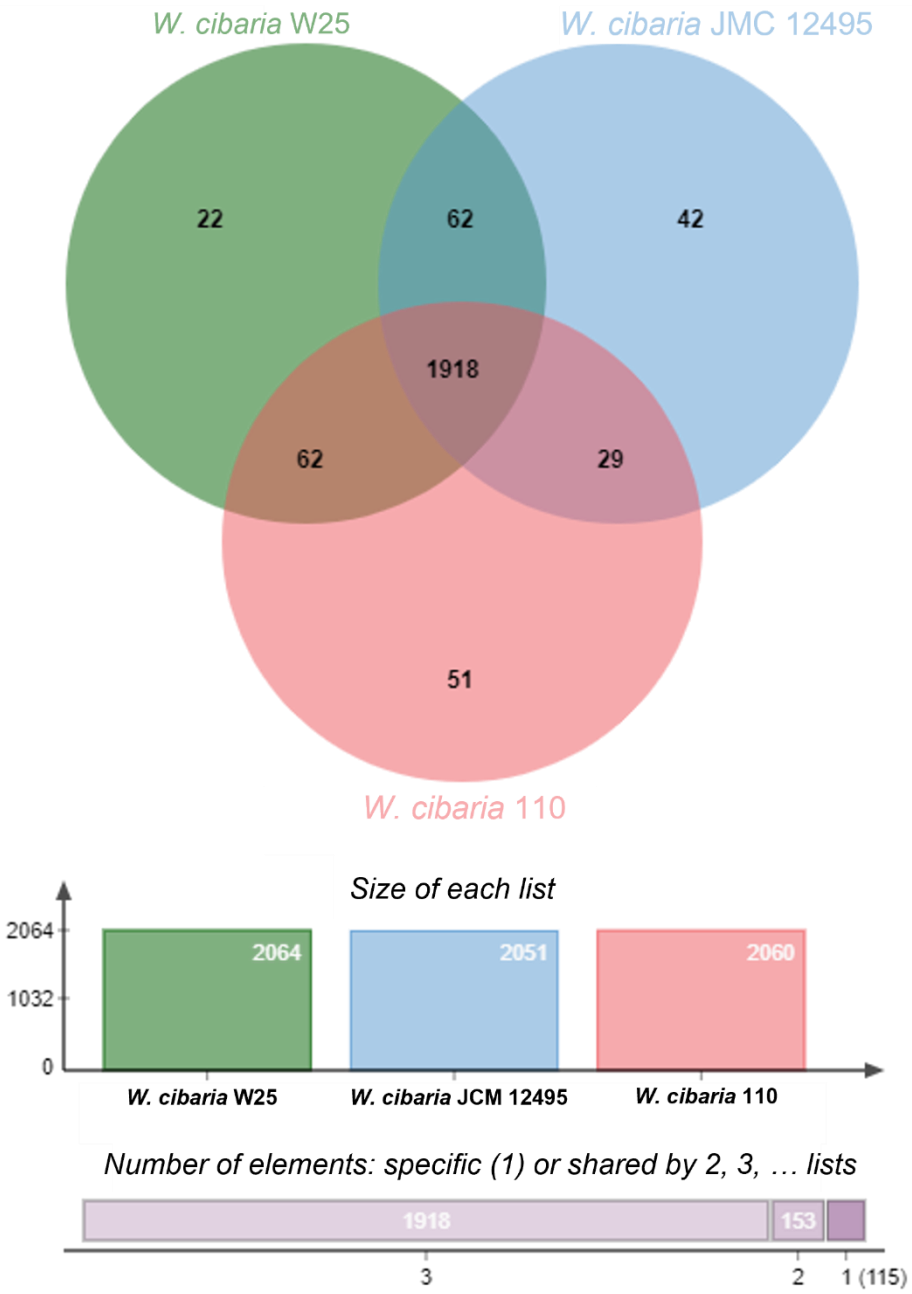


**Figure 3.** Venn diagram showing the protein-coding genes or pseudogenes in each sequenced of *W. cibaria* W25, *W. cibaria* 110, and *W. cibaria* JMC 12495. Overlapped regions represent shared proteins and the numbers in the non-overlapped regions indicate the unique protein-coding genes or pseudogenes.

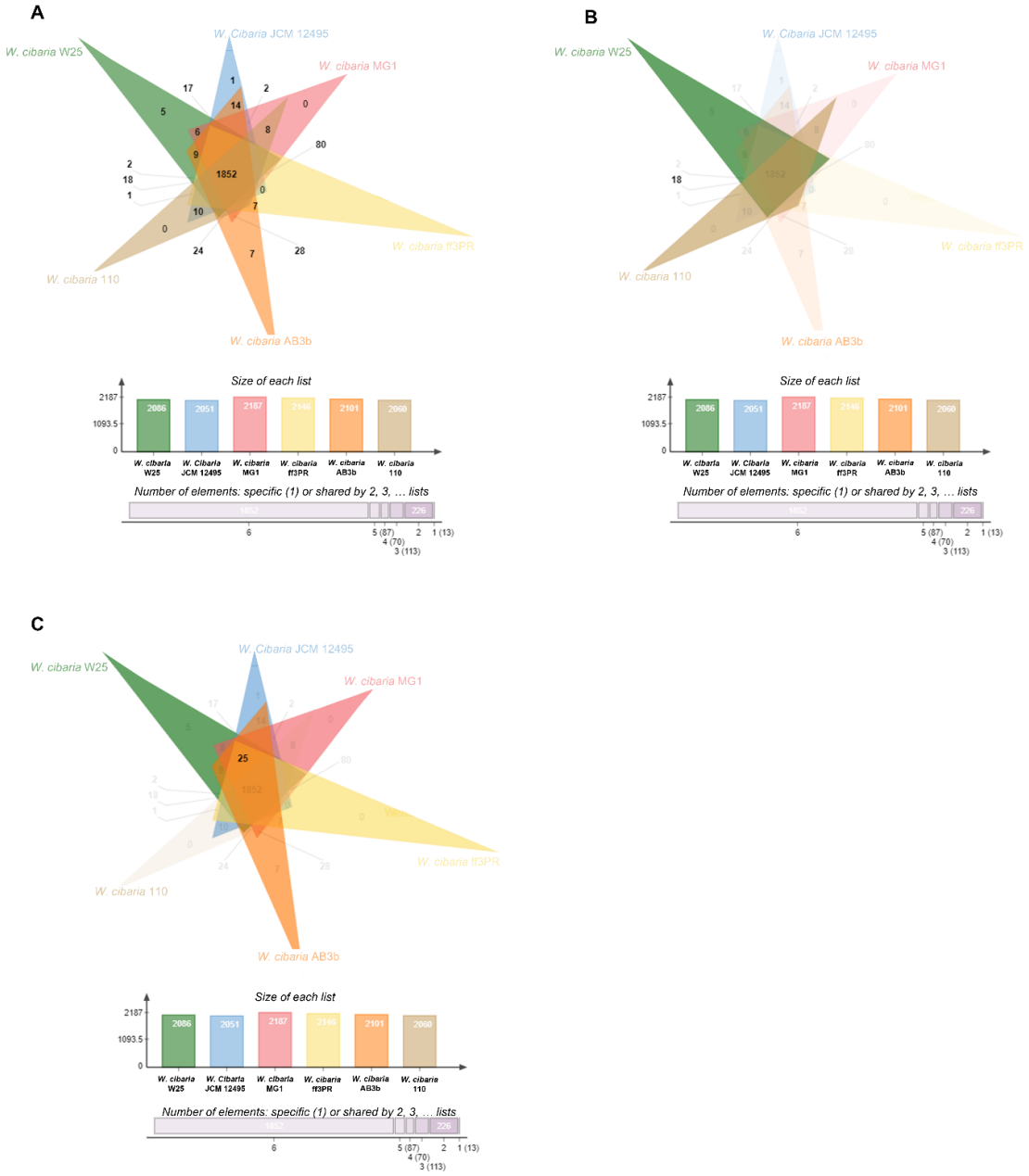


**Figure 4.** Venn diagrams showing the protein-coding genes or pseudogenes of six *W. cibaria* strains. Overlapped regions represent shared proteins and the numbers in the non-overlapped regions indicate the unique protein-coding genes or pseudogenes. A: normal Venn diagram; B: Venn diagram highlighting the cluster between *W. cibaria* W25 and *W. cibaria* 110; C: Venn diagram highlighting the clusters between *W. cibaria* W25 and the non-bacteriocin-producing strains.

We constructed a comparative genetic map using CGview server to help demonstrate the similarity between the strains (Figure 5). We used the *W. cibaria* 110 as the reference for comparison because it is a well-known bacteriocin-producing strain. Moreover, gene confirmation for weissellicin 110 was performed by the BLASTn program at the National Center for Biotechnology Information (NCBI) using the deposited sequence under accession

number LC010242 and the predicted genes of the genome from *W. cibaria* 110. We obtained results to query coverage and percent identity of 100%. Among the regions that showed to be similar between *W. cibaria* W25 and *W. cibaria* 110, we observed that the strain under study presented a similar and more complete region, when compared to the region containing weissellicin 110, than the non-bacteriocin-producing strains (Figure 5, B). This indicates that perhaps in this region of *W. cibaria* W25, there are genes for a bacteriocin, however a distinct one.

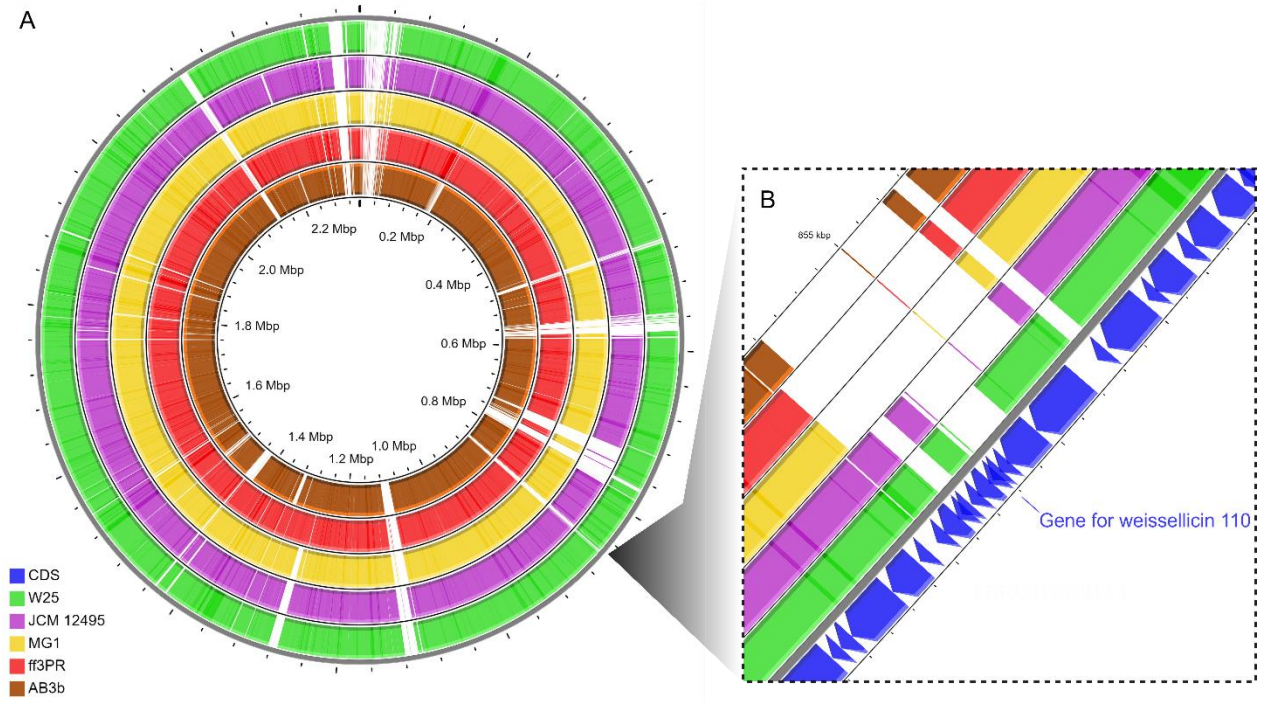


***Figure 5.*** Comparative genome map generated using CGView server, showing a full circular map obtained using BLAST (A) and an expanded view of the weissellicin 110 region (B). The rings in (A) and (B) green, purple, yellow, red, and brown indicate the *W. cibaria* strains W25, JCM 12495, MG1, ff3PR, and AB3b, subsequently, that were compared with the *W. cibaria* 110. The blue ring in (B) indicates the coding sequences (CDS) of the genome from *W. cibaria* 110.

## 4. Conclusions

According to the bioinformatics results obtained in this study, *W. cibaria* W25 has great potential to be used for human consumption once it was predicted as a non-human pathogen. Also, despite *W. cibaria* W25 showing more genomic similarity with *W. cibaria* JCM 12495 (according to DDH similarly) OrthoVenn2 showed that it has its unique protein cluster genes which can be related with the bacteriocins genes indicated by AntiSMASH confirming the possibility of producing two different bacteriocins.

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# **CHAPTER IV:** *Weissella cibaria* strains isolated from Campo das Vertentes (MG), a potential bacteriocin producers

**Genome analyses of *Weissella* strains isolated from Campos das Vertentes, Minas Gerais, Brazil revealed new bacteriocins with a large spectrum of activity**

Camila Gonçalves Teixeira, Rafaela da Silva Rodrigues, Anca Lucau-Danila, Luís Augusto Nero, Antônio Fernandes de Carvalho, Djamel Drider

**Abstract**

Lactic acid bacteria (LAB) are ubiquitous, and commonly found in different niches like gut microbiota, healthy vaginal microbiota, and naturally fermented foods, and some of LAB possess the “Generally Recognized as Safe” status. LAB produces a wide range of antimicrobial substances, including bacteriocins which are endowed with antibiotic-activities. Bacteriocins offer many advantages for biotechnological applications. Indeed, they can play a role in the medical sector as an alternative to antibiotic drugs, and microbiota editing agents, and also in the food sector, as food-additives, to fight against food spoilage agents and pathogens.

This work aimed at characterizing the *Weissella cibaria* strains isolated from Campos das Vertentes, Minas Gerais, Brazil, and their antimicrobial potential. Thus, three strains of *W. cibaria* (W21, W25, and W42) were previously selected, and the genomes of W21 and W42 were sequenced, while that of strain W25 was already sequenced (Teixeira et al., 2022). Afterwhich, we investigated the presence of genes coding for potential secondary metabolites in the genomes of the strains using the AntiSmash webtool, and located a common region coding for a putative bacteriocin identified as Bacteriocin_IIc. Then, we assessed *in vitro* this antimicrobial activity using the supernatant of these strains. According to the data, the strain W21, which has one transport-related gene missing from the bacteriocin operon compared to W25 and W42, likewise showed no differences in the *in vitro* antimicrobial test when comparing the outcomes of the three treatments (supernatant without treatment, neutralized and treated with proteinase K). Also, we compared the nucleotides and amino acids of this putative bacteriocin in the NCBI platform and there was no similarity with other so far described bacteriocin from the genus *Weissella*. In conclusion, two of the three *Weissella* strains tested in this work have the potential to produce a new bacteriocin not yet described which needs to be deeply characterized and proposed for food protection as an alternative to chemicals in foods.

**Key words**: *Weissella cibaria*, bacteriocins, antimicrobial activity

## 1. Introduction

For many years antibiotics have been used as the basis of treatment against bacterial infections and their misuse conducted to the development of antibiotic-resistant species (Blair et al., 2015). The World Health Organization (WHO) encourages new strategies and interventions to avoid the development of new multi-drug-resistant pathogens (Neill, 2016). LAB and its diverse metabolites such as organic acids, hydrogen peroxide ($H_2O_2$), and bacteriocins offer interesting prospects (Hernández-González et al., 2021).

Bacteriocins are ribosomally synthesized antimicrobial peptides/proteins produced by Gram-positive, Gram-negative, and Archaea (Hammami et al., 2010; Nes, 2011) with the aim to defeat their congeners living in the ecological niches (Soltani et al., 2021). Bacteriocins can be active against phylogenetically related or phylogenetically distant target strains, but will not be against producing strains themselves because of their developed specific immunity proteins (Drider et al., 2006). As bacteriocins are commonly produced by strains from the gut microbiota and natural food sources, in which some are “Generally Recognized as Safe” (GRAS status), interest in LAB-bacteriocins is ever-growing for the food and pharmaceutical sectors (Field et al., 2018). In addition, bacteriocins are non-toxic to cells from the Eukarya domain and can be used against many food spoilage microbes and pathogenic bacteria (Belguesmia et al., 2011; Field et al., 2018). Additionally, bacteriocins are expected to play a role in editing-microbiomes (Heilbronner et al., 2021, Drider et al., 2021) based on their mode of action, which include different natural targets (Heilbronner et al., 2021) and even liposomes (Naghmouchi et al., 2007).

Despite some LAB genera being known for their antimicrobial activity against food spoilage and pathogenic microorganisms, not all of them are effective. Some of them produce a low amount of antimicrobial compounds or have a limited spectrum of activity (Tenea et al., 2020). Therefore, the discovery of novel antimicrobial compounds endowed with broad inhibitory spectra is of interest for their potential applications as alternatives to overage antibiotics or as bio-preservative agents for the food industry. Of note, applications of LAB-bacteriocins in the food industry as bio-preservative agents are feasible and constitute an eco-friendly approach that could replace the use of chemical preservatives. The biopreservative approach is timely as it meets the consumers' expectations for natural and bio-protected foods (Venegas-Ortega et al., 2019).

Within the LAB group, strains of the *Weissella* genus are known to produce bacteriocins such as weissellicin 110, MBF, L, D, M, Y, and weissellicin A from strains of *W.*

*cibaria, W. paramesenteroides* and *W. hellenica* (Chen et al., 2014; Di Cagno et al., 2011; Kariyawasam et al., 2019; Leong et al., 2013; Masuda et al., 2012; Srionnual et al., 2007). Related to that, we isolated strains of *W. cibaria* from different artisanal cheese-producing regions in Brazil and established their technological and bacteriocinogenic traits (Teixeira et al., 2021). Most of the studied isolates demonstrated broad inhibition against several pathogens, both gram-positive and gram-negative (Teixeira et al., 2021).

This study aims to identify the antimicrobial compounds produced by *Weissella* strains isolated from Campos das Vertentes, Minas Gerais, Brazil, to schedule their future application as pathogens inhibitors in the food industry and preservation.

## 2. Material and Methods

### 2.1. Microorganisms and Culture Conditions

Three different strains of *Weissella cibaria,* previously isolated from dairy farms located in the Campos da Vertentes region, in the southeast of the Minas Gerais state, Brazil were used in this work. The strains W21 (isolate id 21) and W25 (isolate id 25) were isolated from pasture samples, whereas strain W42 (isolate id 42) was isolated from soil (Teixeira et al., 2021). These 3 strains were identified as *Weissella cibaria* after sequencing of the gene 16S rRNA and presented antimicrobial activity against a panel of Gram-positive and Gram-negative foodborne pathogens (Teixeira et al., 2021). The isolates obtained were stored at -80°C in the Man Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, England) and supplemented with 30% (v/v) glycerol (Synth, São Paulo, SP, Brazil). Before analysis, stock cultures (1% v/v) were transferred to MRS broth (Oxoid) and incubated at 30 °C overnight.

### 2.2. Genome Sequencing, Assembly, and Annotation

The genome sequence of the W25 strain was previously deposited in GenBank under accession number JAFNKE000000000 (Teixeira et al., 2022). The whole genomes of *W. cibaria* W21 and W42 were sequenced, trimmed, and assembled as recently performed for the W25 strain (Teixeira et al., 2022). Gene prediction and annotation were performed using the Rapid Prokaryotic Genome Annotation (PROKKA) v.1.14.5 (Seemann, 2014), executed with default parameters, and also performed by RAST automated web server (Aziz et al., 2008). The assembled genome was conducted by using MIRA Assembler v.4.9.6 (Chevreux et al., 1999), mode “genome, accurate”. The assembling quality was determined with

QUAST v.5.0.2 (Gurevich et al., 2013), and ContEst16S was used to check contamination (Lee et al., 2017).

### 2.3. Bacterial Species Confirmation

The identification of the genus and species was carried out using KmerFinder (Hasman et al., 2014; Larsen et al., 2014) and the Type (Strains) Genome Server (TYGS) (Meier-kolthoff & Göker, 2019). The phylogenetic tree was visualized and edited using the online tool iTol v.6 (Letunic & Bork, 2016).

Genus and species were also confirmed by Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry on an Autoflex SpeedTM (Bruker Daltonics, Bremen, Germany) with flex control software (Bruker Daltonics).

Protein extraction for bacterial identification was made according to Bruker's recommendation after 24 h of culture in MRS media. Briefly, bacterial pellets were washed with 70% ethanol solution, centrifuged to remove all added ethanol, and incubated for 5 min at room temperature with 50 µL of 70% formic acid, and another 5 min with an equal volume of 100% acetonitrile. After a quick step of centrifugation at maximum speed for 2 minutes, 1 µL of supernatant was spotted 3 times on a polished steel MALDI target plate (Bruker Daltonics) and dried at room temperature. Each spot was overlayed with 1 µL of matrix solution [10 mg/ml of HCCA dissolved in ACN/water/TFA (50/47.5/2.5; v/v/v)] and dried again (Heuson et al., 2019; Nacef et al., 2017; Zidour et al., 2017). MALDI-TOF mass spectrometer calibration was performed using the bacterial standard test recommended by Bruker. MALDI-MS profiles were acquired in positive linear mode across the m/z range of 2–20 kDa using the manufacturer's automatic method MBT_FC.par (Bruker Daltonics).

### 2.4. Antimicrobial Potential

To identify secondary metabolite biosynthetic gene clusters and bacteriocins, we used the antiSMASH v.6.0 (Blin et al., 2021) and the BAGEL4 web server (Heel et al., 2018). The bacteriocin genes detected were blasted on the NCBI platform and the *in silico* prediction of promoters (-10 and -35) and terminator region for the bacteriocin coding gene was performed manually and using Arnold Finding Terminator (Gautheret et al., 2001), respectively. The putative promoter region of each gene was suggested based on the consensus sequences for the -10 box (TATAAT) and -35 box (TTGACA) and considering their position upstream of the start codon.

We tested the antimicrobial activity against *Escherichia coli* ATCC 8739, *Kocuria rhizophila* CIP 5345, *Listeria innocua* CIP 80.11, and *Salmonella enterica* using the microdilution assays according to Teixeira et al. (Teixeira et al., 2021) and the spot on the lawn assay according to Moraes et al. (Moraes et al., 2010). *Weissella* spp. strains were cultured in MRS broth (Oxoid) at 30°C for 24 h and a cell-free supernatant (CFS) was obtained by centrifugation (10,000 × *g* for 10 min at 4 ºC) and filtration (Ø = 0.45 µm). Target strains were cultured in a brain heart infusion (BHI, Oxoid) at 37 °C for 24 h. For the microdilution assay aliquots of the supernatant (100 µL, at a concentration of about $10^4$ CFU/mL) were transferred to 96-well microtiter plates. Then, 50 µL aliquots of the selected *Weissella* spp. strains' CFS were added to each well. The microplates were incubated at 37 ºC for 24 h in a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cultures' optical densities (OD) were measured every 30 min (λ = 600 nm). For the spot-on-the-lawn assay, the supernatant was ten-fold concentrated and spotted on BHI agar plate. After absorption at room temperature (10-15 min), the agar was covered with an overlayer of semi-solid BHI (8 ml) inoculated with $10^5$ CFU/mL of the target and incubated at 37 °C for 24 h. The absence of an inhibition zone in the proximity of the well indicates no antimicrobial activity of the supernatant.

After confirming the antimicrobial activity against *E. coli*, *K. rhizophila*, *L. innocua*, *S. enterica,* and *S. aureus*, the supernatant of the *Weissella* strains were treated with proteinase K in order to confirm the proteinaceous nature of the inhibitory substances. On the other hand, the pH of the culture supernatant was adjusted/neutralized to discard the effect of any inhibition linked to acid production. Of note, after these treatments, the antibacterial activity was confirmed against the aforementioned target organisms with both assays as described above.

## 3. Results and Discussion

### 3.1. Genome Sequencing, Assembly, and Annotation

The information obtained after genome sequencing, assembly and annotation is presented in table 1.

**Table 1.** Genome assembly and annotation statistics for W. cibaria W21, W25, and W42 strains.

| Parameters | Strains | | |
|---|---|---|---|
| | **W25[1]** | **W21** | **W42** |
| **Contigs** | 41 | 50 | 42 |
| **Total length (bp)** | 2,412,435 | 2,461,013 | 2,455,505 |
| **Largest contig** | 331,445 | 290,433 | 290,388 |
| **N50** | **202,649** | 141,851 | 159,842 |
| **N [a]** | 40 | 434 | 424 |
| **G+C content (%)** | 45.04 | 44.96 | 44.95 |
| **CDS** | 2,190 | 2,244 | 2,237 |
| **rRNA** | 15 | 19 | 18 |
| **tRNA** | 84 | 87 | 86 |
| **tmRNA** | 1 | 1 | 1 |
| **Repeat region** | 1 | 1 | 1 |

[1] Results according to Teixeira et al. [19]; [a] The total number of uncalled bases in the assembly.

A slight difference between the three genomes regarding the number of contigs and the size of the genomes (Table 1). According to Björkroth et al. (Björkroth et al., 2002), the G+C content in *W. cibaria* species should be 44 to 45 mol% DNA base ratio, which was what we observed. We can also see that the quantities of rRNA and tRNA genes are comparable between the same species but slightly different due to the fact to be different strains from the same species.

### 3.2. Bacterial Species Confirmation

The identification of the genus and species using KmerFinder (Hasman et al., 2014; Larsen et al., 2014) and the MALDI-TOF are in agreement with the molecular identification based on the 16S rRNA/DNA sequencing realized by Teixeira et al. (Teixeira et al., 2021). The MALDI-TOF analysis is utilized for the identification and confirmation of the LAB bacteria genus and species based on its recognized robustness and appropriateness for LAB (Abdelkader et al., 2021; Nacef et al., 2017) including those belonging to the *Weissella*

genus (Abdelkader et al., 2021; Kim et al., 2021; Ogunremi et al., 2022). Besides that, it has been proven to be a fast method of identification of bacteria at the species and genus level and can be utilized for routine analysis due to its effectiveness (Nacef et al., 2017; Zidour et al., 2017). This technique has been already used to identify *Weissella* genus and species (Abdelkader et al., 2021; Kim et al., 2021; Ogunremi et al., 2022).

The results from the genetic relatedness of *W. cibaria* W21, W25, and W42, constructed using their whole-genomes and those from other *Weissella,* are expressed in the phylogenetic tree in figure 1.

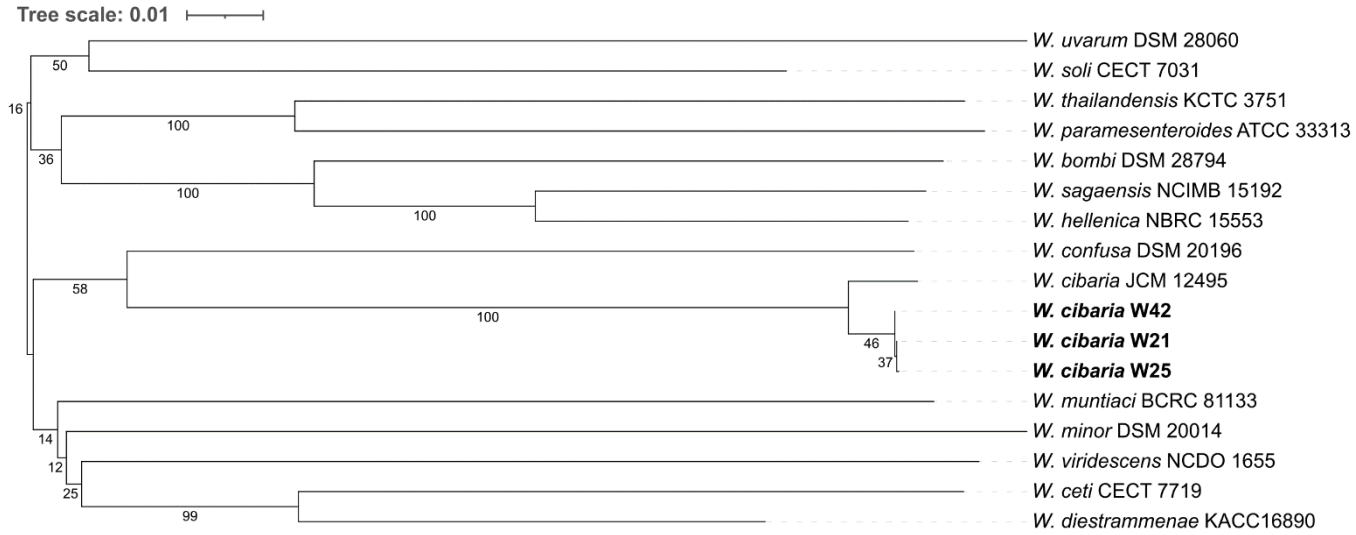


**Figure 1**. Tree inferred with FastME 2.1.6.1 (Lefort et al., 2015) from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula *d5*. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with average branch support of 56.6 %. The tree was rooted at the midpoint (Farris, 1972).

Figure 1 besides confirming the genus and species of the three sequenced strains also confirmed the similarity of the results from table 1 grouping the three strains in the same cluster with a high percentage of similarity.

### 3.3. Antimicrobial potential

In the previous report [15], we mentioned the presence of two putative bacteriocins genes cluster (Teixeira et al., 2022), one lasso peptide (MicJ25), and one RiPP-like bacteriocin_IIc. According to the analysis for *W. cibaria* W25, AntiSMASH (Blin et al., 2021) detected three regions in its genome, C6, C18, and C75.

The C6 region contains 2 core biosynthetic genes, one additional biosynthetic gene, one transport-related gene, and nine putative genes. The two core biosynthetic genes are a

possible bacteriocin classified as Bacteriocin_IIc (Figure 2) and a possible protein related to the ABC transport. The additional biosynthetic gene was identified as a phenylalanine-specific permease and the transport-related gene was identified as a possible ABC transporter ATP-binding protein.

In the C18 region, AntiSMASH (Blin et al., 2021) detected one core biosynthetic gene named biosynthetic (rule-based-clusters) hydroxymethylglutaryl-CoA synthase, and one additional biosynthetic gene identified as biosynthetic-additional (rule-based-clusters) Flavoprotein, one transport-related gene identified as transport SMCOG1184: major facilitator transporter and 29 putative genes.

Finally, in the C75 there was one core biosynthetic gene named biosynthetic (rule-based clusters) lasso peptide: micJ25 and three putative genes.

In the genome of W21, we detected two regions C1 and C9 with two and one core biosynthetic genes, respectively. In the C1 region, we found, as in the W25, a possible bacteriocin gene named Bateriocin_IIc. The difference between this region compared to the C6 in the genome of the W25 strain is that there are all the same genes except for the transport-related gene, as we found in W25. Region C9 of W21 has the same genes as region C18 in the W25 genome strain.

In the genome of strain W42, AntiSMASH (Blin et al., 2021) detected two different regions and one possible bacteriocin. Region C3 has the same genes as the C6 region in the W25 genome strain. Also, the C2 region has the same genes as the region C18 in W25. The bacteriocin_IIc detected by AntiSMASH in the region C6 of *W. cibaria* W25 is represented in figure 2.

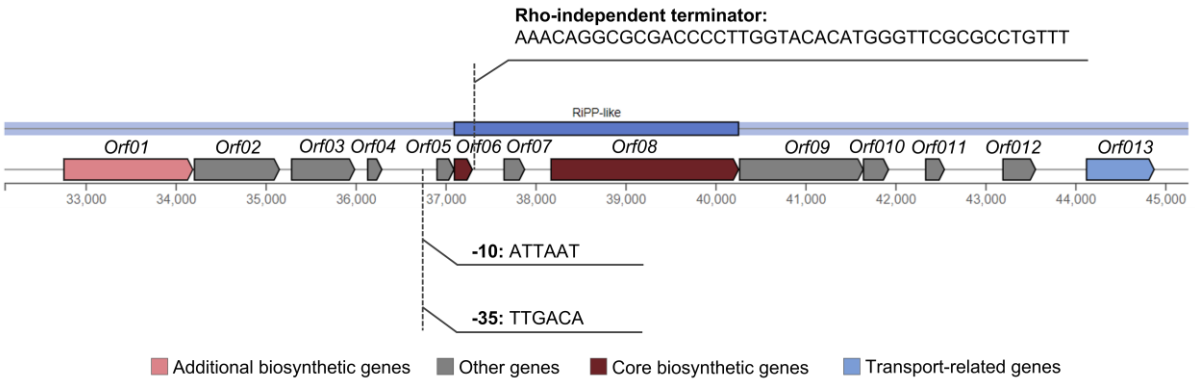


**Figure 2.** In silico prediction of promoters (-10 and -35) and terminator region for the bacteriocin coding gene. Figure adapted from AntiSMASH results.

The gene for the bacteriocin_IIc was identified in all three *Weissella* strains, and the region containing the gene has also other genes such as the ABC transport protein-related

gene and other Orfs non-identified by AntiSMASH. However, the strain W21 is missing one transport-related gene. When the sequence of amino acids of this putative bacteriocin was blasted in the NCBI platform it shows a 98.48% of identity with Blp family class II bacteriocin from *W. cibaria*.

The putative sequences of the promoter region for the bacteriocin_IIc gene are depicted in Figure 2. The results for the terminator region indicate that it is a Rho-independent terminator which is based on a palindrome sequence downstream from the target gene. The distance between the Orf05 and Orf06 is very short, (3 nucleotides) and therefore we could not identify any putative promoter sequences for gene Orf06 and terminator sequences for gene Orf05 which means that these Orfs could be co-transcribed.

There are already some well-known bacteriocins from the *Weissella* genus such as Weissellicin 110, MBF, L, D, M, Y, and Weissellin A from strains of *W. cibaria, W. paramesenteroides,* and *W. hellenica* (Chen et al., 2014; Di Cagno et al., 2011; Kariyawasam et al., 2019; Leong et al., 2013; Masuda et al., 2012; Srionnual et al., 2007). The Weissellicin 110 is the most characterized bacteriocin from the specie *W. cibaria*, despite of that no other bacteriocin was well-study from the strain *W. cibaria* and the identified sequence by AntiSMASH didn't reveal any similarity with the studied bacteriocins from *Weissella* genus.

The *in vitro* evaluation of antimicrobial activity against *E. coli*, *K. rhizophila*, *L. innocua,* and *S. enterica* is presented in Figure 3 for the microdilution assay. The results of the spot-on-the-lawn assay show that all the supernatant previous to the treatment presented a clear zone around the spot, but in the test with the treated supernatant, no clear zone was detected.

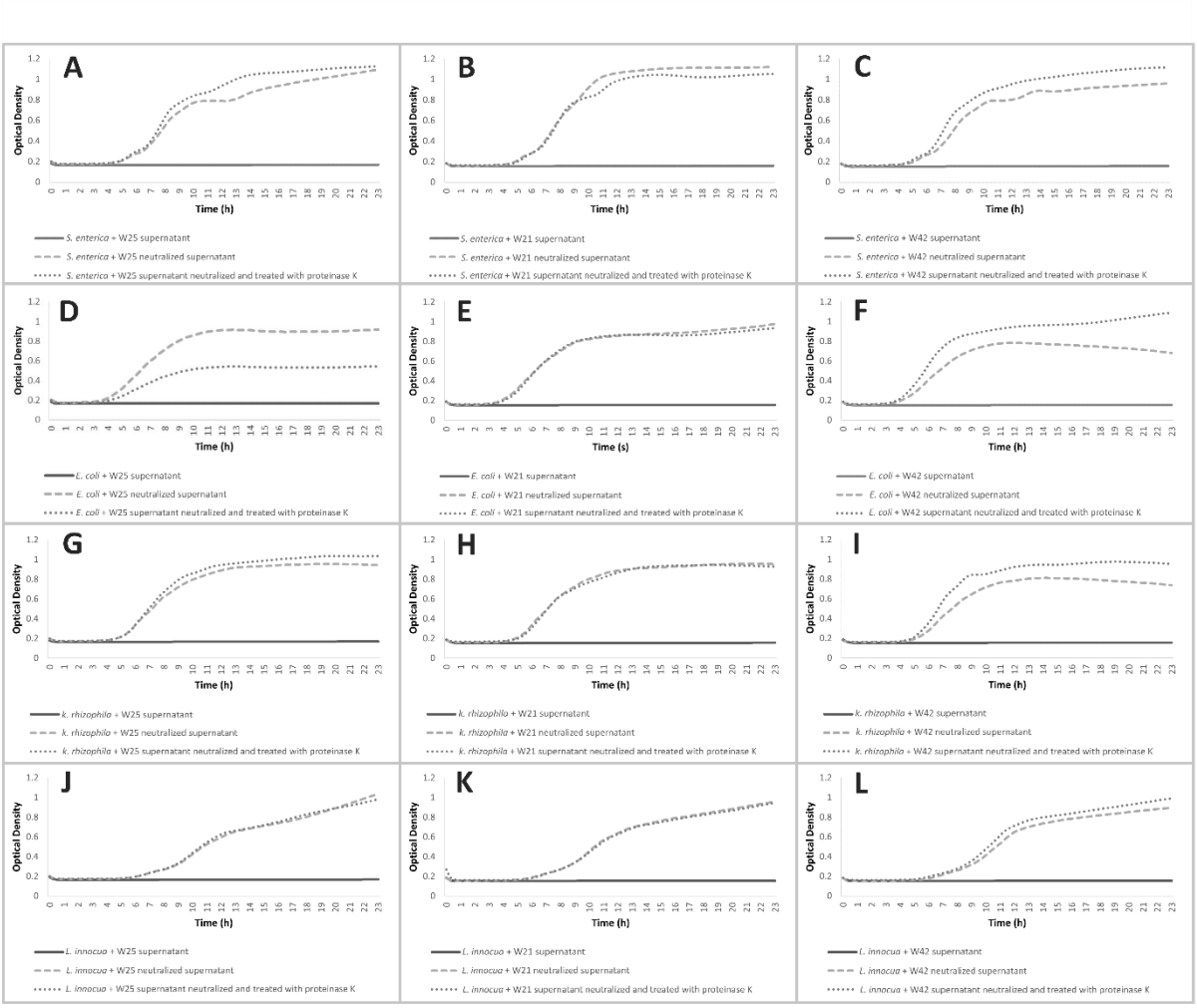


**Figure 3.** Antimicrobial activity of *Weissella* strains supernatants (Non-treated, Neutralized, and Neutralized, and treated with proteinase K) against the targets *E. coli, K. rhizophila, L. innocua*, and *S. enterica*. The analysis was in duplicate.

The results showed that when we used the supernatant without treatment there was no growth of any target, but when the supernatant is neutralized or neutralized and treated with proteinase K there was a huge decrease in the antimicrobial activity. However, as we can see in some growth curves from figure 3 (A, C, D, F, and I) after neutralization of the supernatant there was a remaining antimicrobial activity and this one was lost after the treatment with proteinase K. This is an indication that the major compound responsible for antimicrobial activity can be some organic acids produced by the tested *Weissella* strains or a combination between produced compounds. Several compounds produced by *Weissella* strains has antimicrobial activity such as organic acids, hydrogen peroxide, bacteriocins, and other (Cheng et al., 2016; Fhoula et al., 2022; Yeu et al., 2021; Yu et al., 2019). Also, the synergism between two or more compounds, such as lactic acid and acid citric with bacteriocin, can increase the antimicrobial activity of the supernatant (Soltani et al., 2022).

Teixeira et al. (Teixeira et al., 2021) also tested the W25 and W21 strains against different targets such as *L. monocytogenes* ATCC 6538, *S.* Typhimurium ATCC 14028, *S. aureus* ATCC 6538, and *E. coli* ATCC 1122. The authors observed a higher level of inhibition in the supernatant of W21 compared to W25 and also W21 presented the highest inhibition level against *L. monocytogenes* ATCC 15313. However, in the present work comparing the same strains we can see that after the treatment with proteinase K, there was no difference in the antimicrobial activity which means that the inhibitory activity from W21 is not related to the production of bacteriocins. This can be confirmed by the AntiSMASH results, once in this genome, the region that has the lasso peptide has no transport-related gene as in the genome of W25 and W42. According to AntiSMASH results, *W. cibaria* W25 and W42 possess one putative lasso peptide and both of them have the same genes in the region containing this bacteriocin. Also, these two strains are the ones in the antimicrobial test that maintained some antimicrobial activity after neutralizing and lost it after being treated with proteinase K.

## 4. Conclusions

The three *W. cibaria* strains studied in this work present high antimicrobial inhibitory activity of the non-treated supernatant against all the target strains tested. Besides that, the genome of all of them presents the putative gene for bacteriocin identified as bacteriocin_IIc by the AntiSMASH webserver. However, the strain *W. cibaria* W21 did not present the transport-related gene presented in the others two. This fact was confirmed by the *in vitro* test with the supernatant neutralized and with the supernatant neutralized treated with proteinase K. Once the supernatant was neutralized it lost antimicrobial activity, however when the supernatant of W25 and W42 was treated with proteinase K, the remaining antimicrobial activity was lost and for W21 there was no difference between the tested supernatants against all targets. This is an indication of the production of bacteriocin. Moreover, the bacteriocin identified has no similarity with the well-known bacteriocin produced by *W. cibaria* nor with the other strains from this genus being a potential new bacteriocin for the food and pharmaceutical industries.

## 5. Data Availability

Draft genome sequence of the *W. cibaria* strains W21 and W42 was submitted to NCBI under GenBank accession numbers JALRNM000000000 and JALRNN000000000, respectively.

## Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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**CHAPTER V:** *Weissella cibaria* strains isolated from Campo das Vertentes (MG), biosecurity and probiotics analyses

**Assessment of biosecurity traits and *in situ* antibacterial activities of *Weissella cibaria* strains isolated from Campos das Vertentes, Minas Gerais, Brazil focused for a potential probiotic and food application**

Camila Gonçalves Teixeira, Yanath Belguesmia, Rafaela da Silva Rodrigues, Anca Lucau-Danila, Luís Augusto Nero, Antônio Fernandes de Carvalho, Djamel Drider

**Abstract**

The antagonistic *Weissella cibaria* strains W21, W25 and W42 are characterized in this study for additional beneficial properties. These strains display therefore high scores of adhesion to intestinal cell-lines and capacities to exclude in situ pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA S1) and *Escherichia coli* 184, which is resistant to colistin. Further, *W. cibaria* W21, W25 and W42 were hemolysis-negative, non-cytotoxic towards a mix of eukaryotic Caco2 and HT-29 cell-lines, do not induce inflammation on the promonocytic human cell line U937, but were unable to survive conditions mimicking the human gastrointestinal tract. Their biosafety aspect was addressed in silico and experimentally by studying their blood-hemolysis and susceptibility to antibiotics. To sum up, *W. cibaria* W21, W25 and W42 have no acquired related antimicrobial resistance genes and mobile genetic elements (MGE) in their genomes. Nevertheless, their intrinsic resistance to vancomycin was established in vitro and confirmed with in silico analyses. Their difficulties to face the gastrointestinal conditions could be overcome by using appropriate delivery systems. Taking all these data together, we claim that *W. cibaria* W21, W25 and W42 are coming as excellent strains candidates with wide a range of industrial applications and bio-functional properties.

**Keywords.** *Weissella cibaria*, safety, antibacterial activity, cytotoxicity, probiotics, gut

## 1. Introduction

Brazil is a country, in which several regions produce their own artisanal cheeses, offering, therefore, access to rich bacterial microbiota and beneficial microorganisms such as lactic acid bacteria (LAB), that could be employed in the food and pharmaceutical sectors [1]. Related to that, handmade cheeses and raw milk are endless sources of novel LAB strains [2], and each handmade cheese has its specific microbiota. Environmental microorganisms can also reach the product during the manufacturing process, influence the fermentation process and consequently impact the organoleptic and aromatic qualities of the final product [3]. LAB assigned to the *Weissella* genus are Gram-positive bacteria, catalase-negative, asporogenous, coccoid morphology, or short bacilli with heterofermentative metabolism and CO2 production from carbohydrate uptake, making them suitable for use in fermentation processes. *Weissella*, as well as Fructilactobacillus, Convivina, Leuconostoc, and Oenococcus, are known as Fructophilic LAB because they usually metabolize fructose instead glucose under certain conditions. *Weissella* spp. inhabit a wide range of ecological niches including plants, vegetables, soil, water, and fermented foods, where they assume a probiotic role [4]. *Weissella* species can also be found in habitats associated with the human or animal body, e.g., the gastrointestinal tract, vaginal microbiota, or human breast milk [5, 6].

*Weissella* strains endowed with antagonistic activities against different foodborne pathogens have been isolated from different Brazilian regions producing artisanal cheeses [7]. Of note, the *Weissella* genus has emerged in 1993, after reconsidering taxonomically atypical *Leuconostoc* [4]. Phylogenetically, the genus *Weissella* belongs to the *Leuconostocaceae* family, order *Lactobacillales*, class *Bacilli, phylum Bacillota* and domain *Bacteria.* Currently, the genus *Weissella* contains 35 species (www.bacterio.net). *Weissella* species are facultatively anaerobic, obligately fermentative, and non-motile bacteria, except *W. beninsensis* which is reported as being motile [8]. The accurate identification of *Weissella* strains is to be performed by sensitive methods like 16SrRNA gene sequencing or Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS), rather than traditional phenotypic methods, which lead to their misidentification [5]. *Weissella* is enormously reported for diversity and high production of exopolysaccharides with potential for industrial applications and bio-functional properties [9, 10].

The ability of *Weissella* and overall LAB to produce antimicrobial compounds retain very strong interest because of potential applications in the food sector. Indeed, the last

decade has seen a new challenge for the food industry, which consists to identify natural preservative compounds that will augment food safety and security after their incorporation into foods [11]. Foods free of chemicals are new demands of consumers and not necessarily of consumers of high living standards. Thus, alternatives based on the incorporation of beneficial microorganisms or their metabolites in the food matrix might be the next solution.

The use of a novel strain as an ingredient either for human or animal feed requires one to meet strict criteria related to safety by discarding any adverse effect like hemolysis, carriage of genes coding for antibiotic resistance, or pro-inflammation reaction [12, 13].

To discover novel lactic acid bacteria, we aim in this work to characterize *W. cibaria* W21, W25, and W42, isolated in different Brazilian farms, in Campos das Vertentes (at Minas Gerais state), known for producing artisanal cheeses with raw milk, and address their probiotic profiles.

## 2. Material and Methods

### 2.1. Safety of *Weissella cibaria* W21, W25, and W42 strains

#### *2.1.1. Bioinformatics analyses of Weissella's genomes*

To locate genes coding for the well-known virulence factors in the genomes of *W. cibaria* W21, W25, and W42 strains, we used web tools recommended by the European Food Safety Authority. The search for MGE-like plasmids was done by employing plasmidFinder [14], while the PathogenFinder web tool [15] enabled their profile as potential human pathogens. Genes coding for the virulence factors were investigated by using the "*Virulence Factors Database*" (VFDB) (http://www.mgc.ac.cn/VFs/main.htm) [16], while the search for genes coding for antibiotics resistance was realized by using the RGI (Resistance Gene Identifier) tool and the "*Comprehensive Antibiotic Resistance Database*" (CARD) (https://card.mcmaster.ca/) [17]. To this end, we import the contigs selecting the parameters "perfect and strict hits only" and "high-quality coverage". Finally, the ResFinder 4.1 [18] server (https://cge.cbs.dtu.dk/services/ResFinder/) was used to identify the acquired antimicrobial resistance genes with a selected % ID threshold of 90 % and the selected minimum length of 60%.

For the detection and annotation of prophage sequences within bacterial genomes, the PHASTER [19] (PHAge Search Tool Enhanced Release) (http://phaster.ca) tool was used. The CRISPRCasFinder [20] (https: //crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index) was also used to detect the CRISPR and Cas genes by

importing the contig FASTA sequences and selecting “Perform cas detection” and leaving the rest of the parameters by default.

*2.1.2. Hemolysis and antibiotic susceptibility*

*In vitro* tests enabling the establishment of hemolytic activity and antibiotics susceptibility of these strains were performed according to protocols reported by Colombo et al. [21]. To evaluate, the hemolytic activity, these strains were cultured on Columbia Blood Agar Settle plate (Merck KGaA, Darmstadt, Germany). After 24h at 37°C, the plates were analyzed for microbial hemolytic activity, considering a total or β-hemolysis as clear halos around the colonies, partial or α-hemolysis as greenish halos around the colonies, and γ-hemolysis as the absence of hemolysis [21].

Susceptibility to antibiotics was determined using the disk diffusion assay (Oxoid), and according to the protocol reported by Colombo et al. [21]. Each strain was diluted in a solution of 0.85 % NaCl (w/v) until obtained turbidity equivalent to 0.5 McFarland standard and then was swabbed onto the surface of an MRS agar plate (where the antibiotic disks were placed). These antibiotic disks contained imipenem (carbapenem), vancomycin (glycopeptide), clindamycin (lincosamide), erythromycin (macrolide), ampicillin (penicillin), ciprofloxacin (fluoroquinolone) and amoxicillin + clavulanic acid]. The plates were incubated at 37°C for 18h. After this, the diameter of the inhibition was measured and the resistance profile was determined according to CLSI breakpoints [22].

**2.2. Cytotoxicity and Inflammatory activity of *Weissella* strains in human cell**

*2.2.1. Cytotoxicity assay*

For the cytotoxicity test, we utilized a mix of human cell lines composed of 80 % of human colon adenocarcinoma Caco2 and 20 % of HT-29. The preparation of the mix of human cell culture was realized according to Pouille et al. [23]. The mix of Caco2 cell and HT-29 line were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific), supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS) (Biowest, Nuaillé, France), 100 units/mL penicillin, 100 µg/mL streptomycin (Gibco, Life Technologies, Grand Island, NY, USA) and 10 mM nonessential amino acids (Gibco, Life Technologies, Paisley, UK). Cells were cultured under standard cell culture conditions (37°C and 5 % $CO_2$). The culture medium was changed regularly and when the cells reached sub-

confluence (80– 90 %), they were sub-passaged. The cells were cultured at 1.5 × $10^4$ cells/mL in 96-well plates for 24 h and then exposed to the 3 different *Weissella* strains.

The preparation of the *Weissella* cultures to contact the mix of human cell cultures was realized as described below. One percent of frozen *Weissella* strains were propagated in MRS broth (Oxoid) for 24 h, centrifugated at 10,000 rpm for 5 min and the supernatant was removed. The obtained pellet was washed with 5 mL of phosphate buffer saline (PBS, pH 7.4), resuspended in 2 mL of DMEM without antibiotics and fetal bovine serum, and diluted until a final concentration of $10^7$ CFU/mL. The suspension of cells was incubated at 37°C until contact with the mix of human cells. For the contact, the mix of human cell culture fixed in the bottom of the 96 wells plate was washed with DMEM and the suspensions of *Weissella* strains were added in a proportion of 1:1 and 1:10 (MOI). After the plate was incubated for 24 h at 37°C.

Cell viability of Caco2 and HT-29 after 24 h of incubation with *Weissella* strains was measured using a cell counting kit-8 assay (CCK-8, Dojindo Molecular Technologies, Inc. Japan). After incubation, the 96-well plate was washed with DMEM with antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). After the washing procedure and removing all *Weissella* strains cells, 150 µL of CCK-8 solution (5 %) was added to each well of the 96-well plate, followed by incubation for 1.5 h at 37 °C. Then, 10 mL of stop solution provided in the CCK-8 kit was added to each well. The absorbance of the mixture was measured at 450 nm using a microplate reader (Synergy H1, BioTek, USA). Cell viability was calculated based on the relative absorbance compared with the control group.

### *2.2.2. Inflammatory effects of Weissella strains*

To investigate the inflammatory and anti-inflammatory effects of *Weissella* strains, we used the promonocytic human cell line U937 according to the protocol described by Pouille et al. [23]. The cell line U937 was cultured in Roswell Park Memorial Institute 1640 Medium (RPMI, Gibco, Thermo Fisher Scientific) supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine in a humidified 5 % $CO_2$ atmosphere at 37 °C. The culture medium was replaced regularly and when the cells reached sub-confluence (80– 90 %), they were sub-passaged. For the differentiation of the cell line in macrophages, U937 cells were seeded at approximately 1 × $10^5$ cells/well in 96-well plates with 60 ng/mL of phorbol-12-myristate-13-acetate (PMA, Thermo Fisher Scientific) for 48 h. The adherent cells were washed with PBS and incubated for 2 h with LPS (10 µg/mL; LPS from *E. coli* O26:B6, Millipore Corporation) and *Weissella*

strains. Cell supernatants were collected on ice and centrifuged (1,500 rpm 5 min) to eliminate cell debris. The supernatants were aliquoted and stored at -80°C until further analysis. *Weissella* strains samples were prepared as described above, resuspended in not supplemented RPMI, and added to each well in 1:10 MOI (multiplicity of infection). The inflammatory control is U937 differentiated with LPS (50 µg/mL) and the anti-inflammatory control is U937 differentiated with LPS (50 µg/mL) and dexamethasone (20 µM).

Multiple cytokines in culture supernatants of the U937 cell line were detected with a customized Milliplex Map kit (Human High Sensitivity T Cell Magnetic Bead Panel, Millipore, Billerica, MA), following the manufacturer's instructions. Briefly, antibody-immobilized beads for detection of interleukin -1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, tumor necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-γ (IFNγ) were incubated with 5-times-diluted culture supernatants of U937. The beads were subsequently treated with detection antibodies as well as PE-conjugated streptavidin and the quantification was carried out using the Luminex® 100/200 (Luminex Corporation, Austin, TX, USA) system and the Luminex xPONENT® for LX100/200 software. The cytokine IL-8 was quantified using a human IL-8/CXCL8 Quantikine® ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). Medium samples were diluted 100-fold according to the kit recommendations.

### 2.3. Survival of *Weissella cibaria* strains under conditions mimicking a human gastric digestion

The survival of *Weissella* strains in simulated human gastric and intestinal juices was assessed as described by Fonseca et al. [24] with modifications. The composition of the simulated gastric juice was pepsin (2 g/L) (Sigma Aldrich, St Louis, MO, USA) and pH 3.5 PBS, adjusted with HCl 0.5 M. Each isolate was grown at 37 °C overnight in sterile MRS broth (Oxoid) and inoculated in the solution until the final concentration of ~ $1 \times 10^{10}$ CFU/mL and followed by incubation at 37°C for 90 min with constant shaking at 160 rpm. After incubation, the samples were taken and serially diluted, then plated on MRS agar plates and incubated at 37°C for 24 h. Then, the simulated duodenal juice was prepared by adding pancreatin (Dinâmica, Brazil) and bile salts solutions to obtain final concentrations of 0.2 % and 0.5 % (w/v), respectively, and the pH was then adjusted to 7.0 by adding 1 M sodium hydroxide (NaOH). After mixing, samples were incubated at 37 °C for 180 min with agitation (160 rpm), and then viable cell counts were determined. All sample counts were determined by plating on MRS (Oxoid) agar. The experiments were repeated three times and performed

in triplicate. Results were expressed as mean log colony-forming units per mL (CFU/mL).

The rate of survival was calculated as the percentage of *Weissella* colonies grown on MRS agar relative to the initial bacterial concentration:

$$Survival\ rate\ (\%) = \frac{\log CFUN1}{\log CFUN0} \times 100$$

Where N1 is the viable count of isolates after incubation and N0 is the initial viable count.

### 2.4. Adhesion of *Weissella cibaria* strains to the human Caco-2 cell line

The adhesion capacity test for the three selected strains to the human colon adenocarcinoma cell lines (Caco-2) was performed according to Fonseca et al. [24] with slight modifications. The Caco-2 cells were sub-cultured ($2 \times 10^5$ cells/mL) in 24-well tissue culture plates (Sarstedt, Germany) and grown at 37 °C in a humidified atmosphere of 5 % $CO_2$ for 21 days to promote differentiation in cell media. The culture medium was changed on alternate days. For the adhesion assay, bacteria were cultured in MRS broth overnight at 32°C, and after washing twice with PBS, the cultures were resuspended in the DMEM at a concentration of approximately $10^7$ CFU/mL. After the DMEM of the 24-well tissue culture plates was removed, the culture cells were washed with the same media. Then 400 µL of DMEM and 100 µL of *Weissella* strains were added to each well. The plate was incubated at 37°C overnight in a 5 % $CO_2$ atmosphere. Subsequently, the cells were washed three times with 1 mL of PBS to remove non-adherent bacteria cells and then lysed with 300 µL of trypsin. After 15 min of incubation at 37°C, the solution with released bacteria cells was transferred to 1.5 mL Eppendorf and the bacteria cells were centrifugated (9,000 × g/ 5 min), the supernatant was removed and the pellet was resuspended in 500 µL of PBS. After recovering the bacteria cells were serially diluted and plated on MRS agar. The plates were incubated at 32°C for 24 h. Then, enumerations of *W. cibaria* strains colonies were performed. Experiments were performed in duplicate and repeated three times.

### 2.5. In situ inhibition of pathogenic bacteria

For this analysis, Caco-2 cell line cultures were cultivated and *Weissella* strains were propagated as above described. Before the experiment, the DMEM of the 24-well tissue culture plates was removed and the culture cells were washed with the same media. For the pathogen inhibition test, the exclusion assay was performed according to the protocol described by Fonseca et al. [24] with modifications. The pathogens used in this experiment

were *Escherichia coli* 184 and *Staphylococcus aureus* MRSA SA1, which were propagated separately in BHI medium (Oxoid) at 37°C for 24h.

Caco-2 cells were first preincubated with 100 µL of *Weissella* strains ($10^7$ CFU/mL) for 2h at 37°C in a 5 % $CO_2$ atmosphere and then 100 µL of *E. coli* ($10^7$ CFU/mL) or *S. aureus* ($10^7$ CFU/mL) was added to each well. The plates were incubated at 37 °C overnight in a 5 % $CO_2$ atmosphere.

After the incubation period, the cells were washed three times with 1 mL of PBS to remove non-adherent bacteria cells and then lysed with 300 µL of trypsin. After 15 min of incubation at 37°C, the solution with released bacteria cells was transferred to 1.5 mL Eppendorf and the bacteria cells were centrifugated (9,000 × g/ 5 min), the supernatant was removed and the pellet was resuspended in 500 µL of PBS. After recovering, the bacteria cells were serially diluted and plated. *E. coli* was plated in Eosin Methylene Blue agar (EMB) and *S. aureus* in BHI supplemented with the antibiotic erythromycin (100 µL/mL) that prevented the growth of *Weissella* strains. The plates were incubated at 37 °C for 24 h. After this time, enumerations of pathogen colonies were performed. Experiments were performed in duplicate and repeated three times.

## 3. Results and Discussion

This study aimed at evaluating different criteria allowing *W. cibaria* W21, W25, and W42 strains to come as excellent probiotic candidates. First, the safety was assessed based on their phenotypic (antibiotic resistance, hemolysis) and genotypic analyses (virulence genes including antibiotic resistance genes). Then, these strains have been confronted with the harsh conditions of the gastrointestinal tract.

### 3.1. *Weissella cibaria* W21, W25 and W42 strain are safe

Strains belonging to the *Weissella* genus contain traits in their genome that confer versatility. In particular, *Weissella cibaria* encodes several beneficial genes that are useful in biotechnological applications. Based on the ResFinder data, no acquired antimicrobial resistance genes were found in the genomes of the 3 tested *Weissella* strains. Moreover, the research for plasmid evaluated by plasmidFinder [14] concluded the absence of MGE, delineating the stability of the genomes.

According to the findings of CRISPRCasFinder [20] (**Table 1**), each of the three *Weissella* strains' genomes harbors a single CRISPR array, with evidence level 4 indicating that these arrays may be legitimate [20].

**Table 1.** CRISPR and *Cas* genes founded in genomes of the 3 *Weissella cibaria* strains isolated from Campos das Vertentes, Minas Gerais, Brazil.

| CRISPR region | | | | | | | CAS protein | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | **Region** | **Start** | **End** | **DR consensus** | **DR Length** | **Spacers** | **Gene name** | **Start** | **End** | **Orientation** |
| W25 | C4 | 12728 | 13621 | GTTTTAGTGTCATGTTGAATAGAATGCTTCTCAAAC | 36 | 13 | Type: CAS-TypeIIA Start: 6,032; End: 12,578 | | | |
| | | | | | | | cas9_TypeII | 6,032 | 10,498 | + |
| | | | | | | | cas1_TypeII | 10,710 | 11,582 | + |
| | | | | | | | cas2_TypeI-II-III | 11,579 | 11,914 | + |
| | | | | | | | csn2_TypeIIA | 11,907 | 12,578 | + |
| W42 | C22 | 95084 | 95977 | GTTTGAGAAGCATTCTATTCAACATGACACTAAAAC | 36 | 13 | Type: CAS-TypeIIA Start: 96,127; End: 102,673 | | | |
| | | | | | | | csn2_TypeIIA | 96,127 | 96,798 | - |
| | | | | | | | cas2_TypeI-II-III | 96,791 | 97,126 | - |
| | | | | | | | cas1_TypeII | 97,123 | 97,995 | - |
| | | | | | | | cas9_TypeII | 98,207 | 102,673 | - |
| W21 | C25 | 12686 | 13579 | GTTTTAGTGTCATGTTGAATAGAATGCTTCTCAAAC | 36 | 13 | Type: CAS-TypeIIA Start: 11,865; End: 12,536 | | | |
| | | | | | | | csn2_TypeIIA | 11,865 | 12,536 | + |
| | | | | | | | Type: CAS-TypeIIU Start: 5,990; End: 12,536 | | | |
| | | | | | | | cas9_TypeII | 5,990 | 10,456 | + |
| | | | | | | | cas1_TypeII | 10,668 | 11,540 | + |
| | | | | | | | csn2_TypeIIA | 11,865 | 12,536 | + |

The CRISPR-associated protein Csn2_TypeIIA is a mandatory protein of the Type IIA system and the gene is present in the three sequenced genomes. According to Roberts

and Barrangou [25], the Type II systems are rare in nature compared to types I and III, which are also more well-characterized [25]. The CRISPR-Cas system is a defense mechanism used by bacteria to protect themselves against infections caused by foreign genetic elements. Thus, strains endowed with the CRISPR-Cas system could acquire fewer sequences issued from other bacteria preventing therefore horizontal gene transfer and leading to a low level of drug resistance [26]. Related to that, the CARD analysis only located genes involved in the resistance to vancomycin, (*vanY* gene in the *vanB* cluster and *vanT* gene in the *vanG* cluster) for all 3 tested strains (**Table 2**), and this resistance was confirmed experimentally in *vitro* (**Table 3**), demonstrating the convergence of these two methods.

**Table 2.** Antibiotic resistance genes identified in *Weissella cibaria* strains using CARD (Comprehensive Antibiotic Resistance Database) webserver.

| Strain | RGI Criteria | ARO Term | Detection Criteria | AMR Gene Family | Drug Class | Resistance Mechanism | % Identity of Matching Region | % Length of Reference Sequence |
|---|---|---|---|---|---|---|---|---|
| W21<br>W25<br>W42 | Strict | vanY gene in vanB cluster | Protein homolog model | vanY, Glycopeptide resistance gene cluster | Glycopeptide antibiotic | Antibiotic Target alteration | 34.95 | 97.39 |
| | Strict | vanT gene in the vanG cluster | protein homolog model | Glycopeptide resistance gene cluster, Van T | Glycopeptide antibiotic | Antibiotic target alteration | 33.33 | 69.38 |

**Table 3.** Results from *in vitro* antibiotic resistance test.

| Code | Antibiotics spectrum (mm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Ampicillin | Amoxicillin + clavulanic acid | Imipenem | ciprofloxacin | Vancomycin | Erythromycin | Clindamycin |
| W21 | 29 | 29 | 35 | 24 | 0 | 24 | 24 |
| W25 | 31 | 31 | 33 | 20 | 0 | 27 | 26 |
| W42 | 28 | 32 | 32 | 22 | 0 | 27 | 26 |

Results are expressed in mm of inhibition halo from antibiotics.
Interpretation according to CLSI breakpoints [18].

In a direct line, [27] reported that 60% of *Weissella* species are resistant to several antibiotics [27]. Recently, Tenea and Hurtado found putative resistance genes in the genome of *W. cibaria* UTNGt21O [28]. Overall, the CARD and the *in vitro* antibiotic tests data obtained here corroborate the low level of resistance, once the three tested strains presented resistance to vancomycin, which is intrinsic for this genus [10]. Indeed, *Weissella* is known to be intrinsically resistant to vancomycin and has a high minimum inhibitory concentration (MIC) of ≥ 256µg/ml. However, antimicrobial susceptibilities of other species

like *W. confusa* are not fully understood. Currently, there are no standard methods and interpretation criteria for antimicrobial susceptibilities established for *Weissella* spp. by the Clinical and Laboratory Standards Institute (CLSI). Intrinsic resistance to antibiotics is not redhibitory or a break [12] in the procedure of the selection of these strains for probiotic applications. The transferability of antibiotic resistance (AR) genes and plasmids present in bacteria is associated with human health. Moreover, the number of spacers in the CRISPR-Cas system represents the ability to eliminate heterologous genes, such as prophage. Thus, the more spacer sequences are present, the more the strain has a limited capacity to acquire foreign sequences like prophages [26]. Here, we only could locate using PHASTER two prophages (1 entire and 1 incomplete) in the genomes of the three strains (**Table 4**).

**Table 4.** Prophage regions identified in *Weissella cibaria* strains genome using the PHASTER webserver.

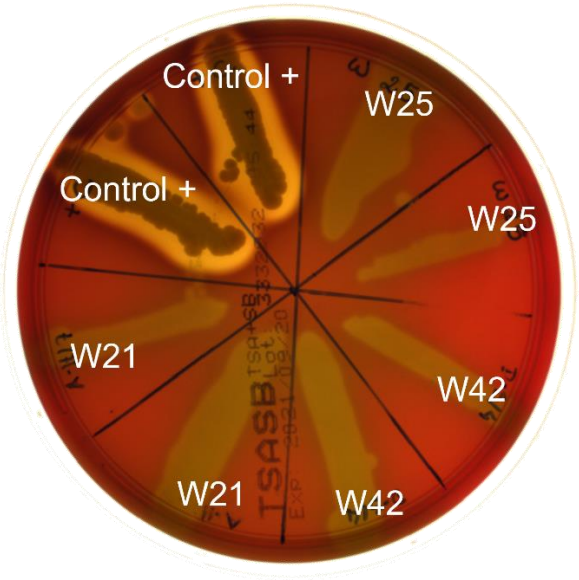
| Strain | Region | Length (kb) | Completeness* | Score | GC % | Most Common Phage |
|---|---|---|---|---|---|---|
| W21 | 1 | 17.9 | Incomplete | 20 | 41.52 | PHAGE_Lactoc_PLgT_1_NC_031016(3) |
| | 2 | 32 | Intact | 110 | 42.64 | PHAGE_Entero_phiFL1A_NC_013646(14) |
| W42 | 1 | 17.9 | Incomplete | 20 | 41.51 | PHAGE_Lactoc_PLgT_1_NC_031016(3) |
| | 2 | 35.8 | Intact | 110 | 42.30 | PHAGE_Entero_phiFL1A_NC_013646(14) |
| W25 | 1 | 17.9 | Incomplete | 20 | 41.54 | PHAGE_Lactoc_PLgT_1_NC_031016(3) |
| | 2 | 35.8 | Intact | 110 | 42.30 | PHAGE_Entero_phiFL1A_NC_013646(14) |

*Intact (score > 90); Questionable (score 70-90); Incomplete (score < 70)

Dong et al. [26] demonstrated that the correlation between spacers and prophages is negative [26]. Prophages are MGE which is considered the primary factor in genetic diversity enabling niche adaptation in bacteria [29]. As above-mentioned, two prophages were located in the genomes of the three strains *W. cibaria* W21, W25, and W42, one of them was intact, whereas the second was incomplete in each of the tested strains. The length of the intact prophage varies from 32 to 35.8 Kb with the most common phage: PHAGE_Entero_phiFL1A_NC_013646(14). The presence of intact regions could be a selective advantage and help bacteria against other prophage infections [30, 31].

Several genes were predicted as putative virulence factors by VFDB (data not shown). However, no one of the well-known virulence genes *gelE* (gelatinase), *hyl* (hyaluronidase), *asa1* (aggregation substance), *esp* (enterococcal surface protein), *cylA* (cytolysin), *efaA* (endocarditis antigen), and *ace* (adhesion of collagen), were detected in the genomes of *W. cibaria* W21, W25, and W42 strains. Besides, most of the genes detected by the VFDB webserver are related to other cellular functions such as genes for putative enolase (*eno*) phosphopyruvate hydratase. Enolase is a key glycolytic enzyme in the cytoplasm of prokaryotic essential for the degradation of carbohydrates via glycolysis [32].

Some researchers reported the presence of some virulence-encoded genes such as collagen adhesin proteins and genes associated with toxin production systems (including botulinum neurotoxin homolog) in some *Weissella* species like *W. cebi*, *W. confusa* [33] and *W. oryzae* [34], respectively. None of these genes were found in our *W. cibaria* strains tested. Also, Tenea and Hurtado [28] report two genes (*hlyD*, *hlyIII*) in some strains of *W. cibaria* [28], however in our study no genes involved in hemolysis activity were observed in the genomes of the *W. cibaria* strains W21, W25, and W42; and this was phenotypically confirmed by assessing this activity on the blood-agar medium (**Figure 1**). Therefore, slight hemolysis that could be graded as an α-hemolysis was detected.




Control +: *S. aureus* ATCC 29213

**Figure 1.** Hemolytic activity of *Weissella* strains W21, W25, and W42.

Despite several virulence factors detected by the VFDB web server, the results indicate that no one of the known virulence factors was present among them and the analysis of PathogenFinder [15] indicates that the three input organisms were predicted as a non-human pathogen. Therefore, these three strains of *W. cibaria* can be considered safe for use as probiotics. Of note, to evaluate the safety of probiotics, some guidelines consider several factors in advance, like excessive immune stimulation in sensitive individuals, systemic infection, gene transfer, or deleterious metabolic effects [35]. To the best of our knowledge, *W. cibaria* has not current use as a probiotic ingredient, although this species has been frequently isolated from fermented foods and human feces and is well-known for its beneficial effects.

### *3.2. Weissella cibaria* W21, W25, and W42 strains are not cytotoxic toward Eukaryotic cells and do not induce inflammatory effects

For the cytotoxicity test, we used a mix of human cell cultures with 80 % of Caco2 and 20 % of HT-29 5 (**Figure 2A**) and with the human U937 cells (**Figure 2B**).

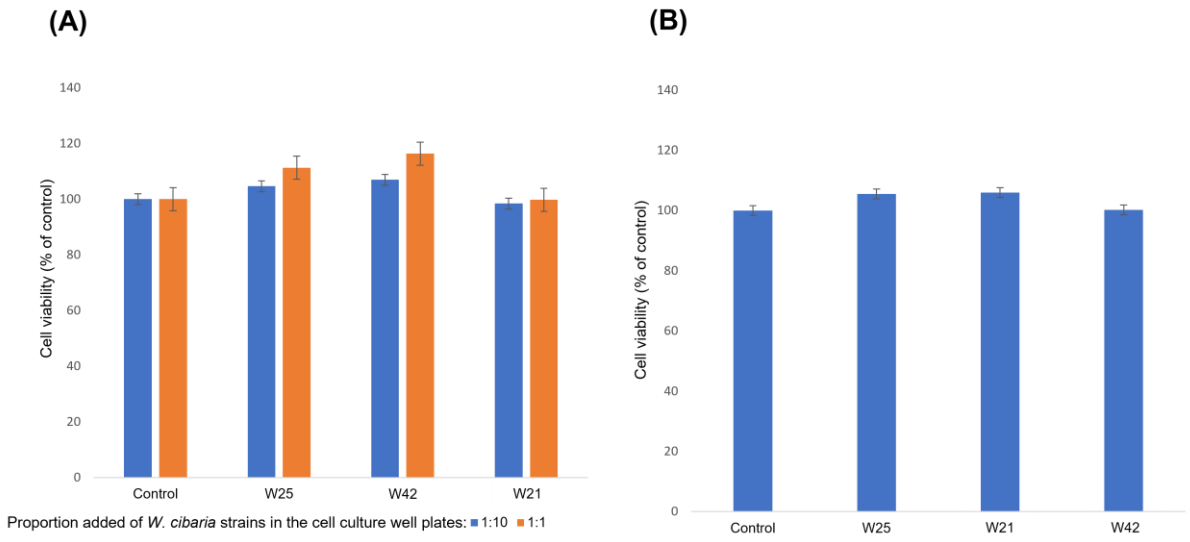



**Figure 2.** Effect of *W. cibaria* strains on cytotoxicity of 80 % of Caco2 + 20 % of HT-29 cells (A); and U937 cells (B).

It is noteworthy that no cytotoxicity was found against these human lines, matching with other studies that underpinned the safety of *W. cibaria* strains on RAW 264.7 macrophage cell line and human mouth epithelial cell line KCLB 10017 [36, 37]. Interestingly, we assessed the inflammatory and anti-inflammatory capacity of *Weissella* strains against the human U937 cells and these strains showed neither inflammatory nor

anti-inflammatory responses. Taken all together, these data strengthen the safety aspects of these strains and promote their potential in the intestinal health of humans and animals. Similarly, Kyung-Hyo Do et al. [38] showed significant therapeutic efficacy of *W. cibaria* CMU and *W. cibaria* CMS1 in improving lung inflammation exacerbated by DEPM administration in the murine model [38].

### 3.3. Survival of *W. cibaria* strains in the simulated gastro-intestinal tract

We noted during the assessment of the survivability of *Weissella* strains in the gastrointestinal tract, a small loss of the viability of the cells after 1.5 h in conditions mimicking the stomach compartment, and also no survivability was observed upon 3 h of incubation in conditions simulating those of the duodenal compartment (**Table 5**).

**Table 5.** Survival of *Weissella cibaria* strains under conditions simulating gastric digestion.

| **Strains** | **Time** | | | | | | **Survival rate (%) of time 1.5h** |
|---|---|---|---|---|---|---|---|
| | **0h** | | **1.5h** | | **4.5h** | | |
| | **pH** | **log10 (CFU/mL)** | **pH** | **log10 (CFU/mL)** | **pH** | **log10 (CFU/mL)** | |
| **W25** | 3.5 | 10.04 | 3.5 | 9.68 | 7 | ND | 96.4 |
| **W42** | 3.5 | 9.80 | 3.5 | 9.37 | 7 | ND | 95.6 |
| **W21** | 3.5 | 9.80 | 3.5 | 9.60 | 7 | ND | 98.0 |

ND: *not detected by technique limit*

The ability of bacteria to survive under gastric digestion conditions is considered a key criterion for probiotic selection [39]. To overcome this drawback, some strategies of delivery are being researched such as encapsulation, which could help the passage through the gastrointestinal tract. Encapsulation acts by protecting bacteria from acidic stress and rendering their delivery process directly in the intestine where they can act as probiotics and provide health incentives to the host [40, 41]. A recent study showed that the HigBA TA system of *W. cibaria* 018 responded to the bile salt stress, but not to acid stress, which might offer novel perspectives to understand the tolerant mechanism of probiotics in the GIT environment [42]. Assessing gastrointestinal tract survival by the agar enumeration does not take into account the viable but nonculturable cells (VBNC) [43]. Previous studies used the cytometry method, which includes both viable bacteria and VBNC and as expected, the

survival percentages obtained by this method were significantly higher in comparison to the data obtained after their growth and enumeration by agar plating method [39].

### 3.4. *Weissella cibaria* W21, W25 and W42 strains adhere to Eukaryotic cells and exclude pathogens

The adhesion of *W. cibaria* strains to the Caco-2 cell lines was almost 100 % effective for W21 and W25 strains (**Table 6**). The initial inoculum was 7-8 log CFU/mL and all the *W. cibaria* strains W21, W25, and W42 tested strains were able to adhere in the Caco-2 cell line, with lower adhesion proportion for W42.

**Table 6.** Adhesion of *Weissella cibaria* strains to Caco-2 cells and exclusion of pathogenic bacteria by *W. cibaria* strains.

| | **Adhesion** | **Exclusion** | |
|---|---|---|---|
| | **log10(CFU/mL)** | **log10(CFU/mL)** | |
| **Strains** | | ***S. aureus* MRSA SA1** | ***E. coli* 184** |
| **Control** | | 6.53228176 | 6.46542394 |
| **W25** | 7.335792102 | 6.6515239 | 6.49991994 |
| **W42** | 6.33243846 | 6.85370046 | 6.42275553 |
| **W21** | 7.33243846 | ND | 6.44388297 |

ND: *not detected by technique limit*

The adhesion to the intestinal cell leads to a hurdle against enteropathogenic bacteria, avoiding their adhesion to the intestinal cells and competing with them for nutrients [44, 45]. Remarkably, the data from the exclusion assays revealed that only *W. cibaria* W21 was able to impede completely the adhesion of *S. aureus* MRSA S1 to the Caco-2 cells (**Table 6**), suggesting intraspecific differences between these strains. In the present work, only two pathogenic bacteria were tested *in situ*: *S. aureus* MRSA S1 and *E. coli* 184, but these strains have a broad spectrum as they are active against *L. monocytogenes* and *Salmonella enterica* [7]. Impeding pathogens to adhere to human cells may result from different mechanisms, including the production of antimicrobial substances by LAB, competition for binding sites, and further criteria [46]. Some pathogens have different levels of adhesion and invasion on the eukaryotic cells, and notably, some pathogens are more or less stable in mucus-producing intestinal cell models (HT-29-MTX) compared to non-mucus-producing cells (Caco-2) [47]. Such ability of resilience is directly related to the expression of specific proteins, pili, fimbriae, and flagella [48]. The *W. cibaria* strains like strain BYL4.2

are also able to inhibit fungi like *Penicillium chrysogenum* via the production of organic acids [49]. This antifungal activity is relevant in the food sector, as fungi can cause spoilage and deterioration of a variety of food products like dairy products, cereals, fruits and vegetables, and grains, decreasing their quality of food and shortening the shelf life, thereby causing considerable economic losses [50]. In the pharmaceutical sector, advantages are focused on food-derived chemopreventive and anti-tumor agents or formulations with fewer side effects than chemotherapy, and food-products like kimchi containing *W. cibaria* strains are under consideration for this purpose [51]. It should be noted that a strain of *W. cibaria* (CMS51) has been commercially used in Korea as an oral care probiotic for several years for its antiviral properties [52].

## 4. Conclusion

De novo sequencing in conjunction with the functional properties enabled us to provide insightful information on *Weissella* species. Indeed, *W. cibaria* W21, W25, and W42 strains are reported as safe according to the bioinformatic analyses, as these strains do not contain acquired antimicrobial resistance genes and MGE in their genomes, which could discard horizontal gene transfer. These strains harbor, nonetheless, in their genomes a CRISPR-Cas system which constitutes a defense system against infections caused by heterologous genes. *W. cibaria* W21, W25, and W42 do not contain any antibiotic resistance gene, except the vancomycin resistance gene; a phenotype confirmed experimentally. Such resistance is considered intrinsic for the *Weissella* genus and other LAB with GRAS status. Only one intact prophage was detected in the genome of these strains, which can also help the bacteria against other prophage infections. Finally, no one of the known virulence factors were detected in these genomes and no *in vitro* hemolytic activity was evidenced. Further, no cytotoxicity and no inflammatory effects were detected for *W. cibaria* W21, W25, and W42 strains tested in this study.

Regarding the probiotic traits of the *W. cibaria* W21, this strain shows higher potential as it demonstrates a high exclusion ability of *S. aureus* MRSA S1 in the intestinal cells. However, none of the three strains survived through the simulated gastrointestinal tract. To overcome this further studies regarding protective strategies to help the passage of these strains to the gastric system are needed and more studies with other enteropathogenic strains should be explored.

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**Conflict of Interest:** The authors declare that they have no competing interest.

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# GENERAL CONCLUSION

Microorganisms of the genus *Weissella* have a wide range of applications in food products, since they are capable of producing a wide variety of compounds. However, few studies have been performed yet and the industrial application of *Weissella* is not yet a reality.

In addition, this work allowed us to understand the diversity of *Weissella* species found in the different regions producing artisanal cheeses in Brazil, comparing two molecular techniques used for clustering analyzes. It also allowed the understanding of how the isolated strains are distributed in the environments that produce artisanal cheese, besides helping to understand the role they play in the artisanal cheese itself. It also showed that some of them have a favorable technological potential for future uses in the food industry as starter cultures and for the study of cheese defect prevention.

Besides that our work culminated in a possible new bacteriocin from the *Weissella* genus leaving the perspective of further investigation on the producing of this bacteriocin. Also with this work we have a panorama of the possibility of using different strains of *Weissella* for human or animal consumption and as food ingredients. We also notice the relative importance of studies technologies to help the use of this strains as probiotics.

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**O gênero *Weissella* na indústria de alimentos: Uma revisão**

**El género *Weissella* en la industria alimentaria: Una revisión**

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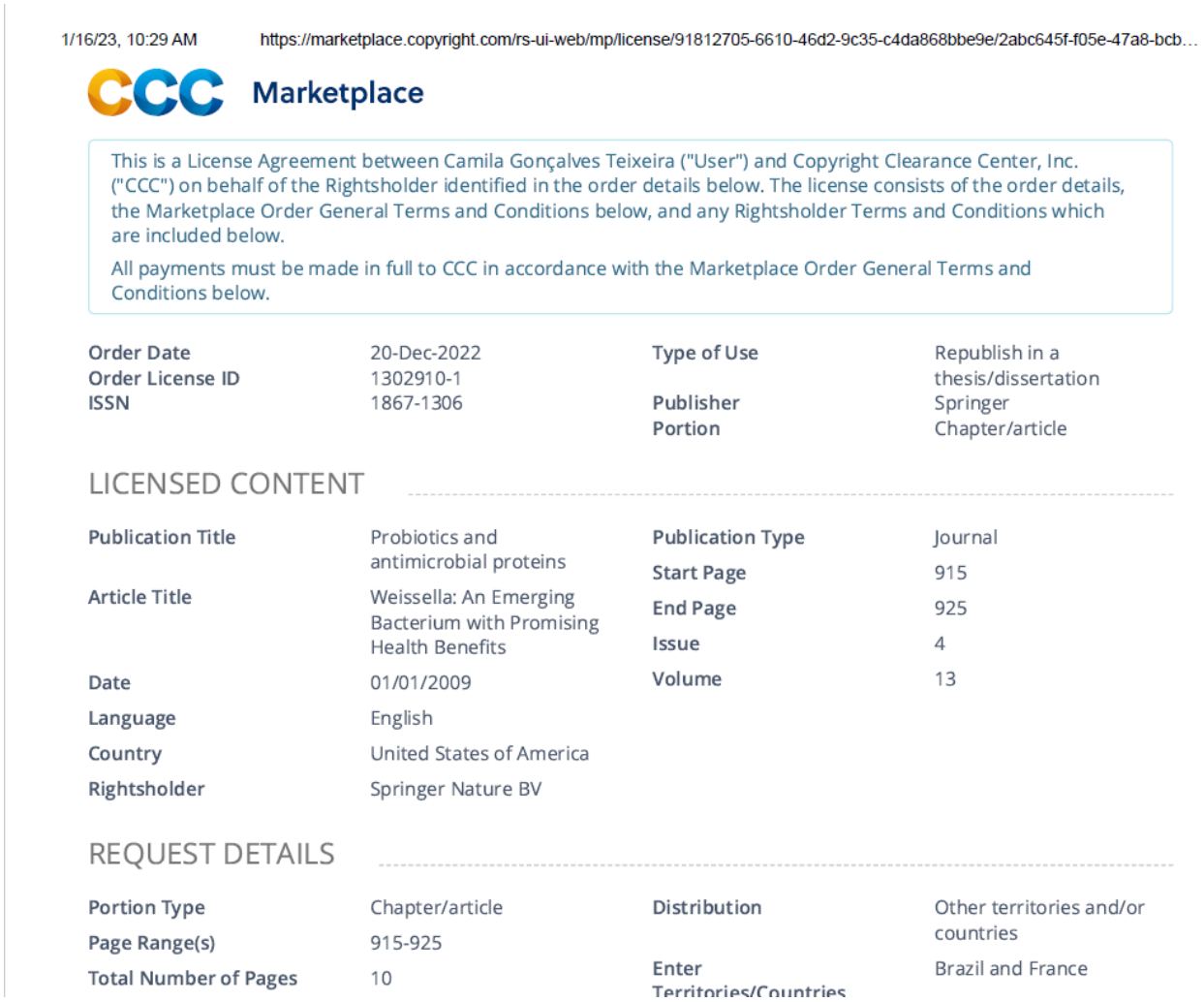

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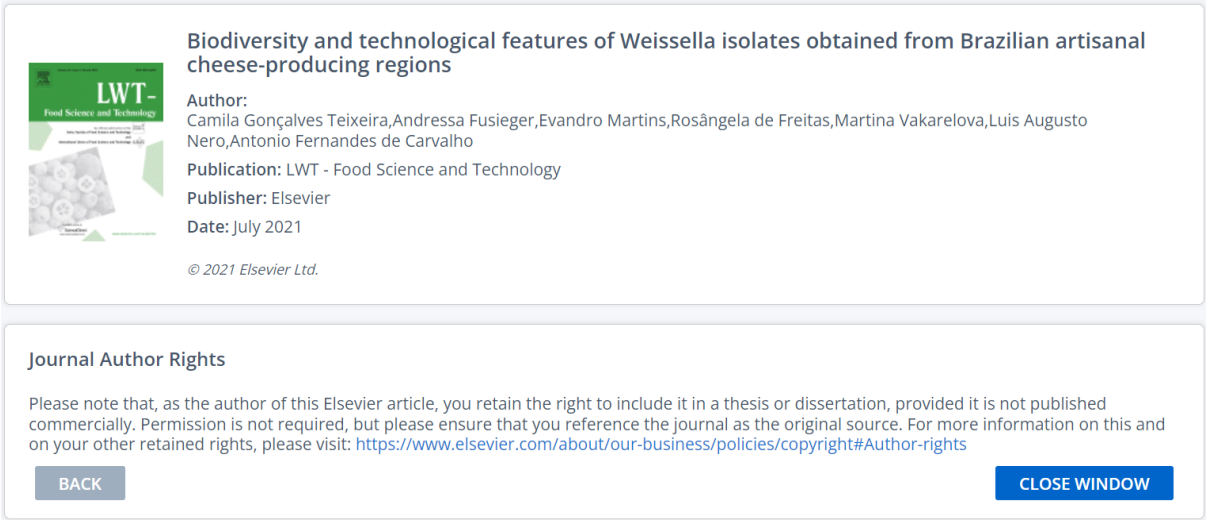

Biodiversity and technological features of Weissella isolates obtained from Brazilian artisanal cheese-producing regions

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# Genomic Analyses of *Weissella cibaria* W25, a Potential Bacteriocin-Producing Strain Isolated from Pasture in Campos das Vertentes, Minas Gerais, Brazil

Camila Gonçalves Teixeira [1,2], Rafaela da Silva Rodrigues [1,3], Ricardo Seiti Yamatogi [3], Anca Lucau-Danila [2], Djamel Drider [2], Luís Augusto Nero [3,*] and Antônio Fernandes de Carvalho [1,*]

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