CAMILA GONÇALVES TEIXEIRA

BIODIVERSITY AND TECHNOLOGICAL POTENTIAL OF THE Weissella STRAINS ISOLATED FROM DIFFERENT REGIONS PRODUCING ARTISANAL CHEESES IN BRAZIL

Dissertation submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of Magister Scientiae.

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“Ninguém é suficientemente perfeito, que não possa aprender com o outro e, ninguém é totalmente estruído de valores que não possa ensinar algo ao seu irmão. ”

(São Francisco de Assis)

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TEIXEIRA, Camila Gonçalves, M.Sc., Universidade Federal de Viçosa, julho de 2018. **Biodiversity and technological potential of the Weissella strains isolated from different regions producing artisanal cheeses in Brazil.** Orientador: Antônio Fernandes de Carvalho.

O gênero Weissella é composto por bactérias pertencentes ao grupo conhecido como bactérias ácido láticas (BAL), portanto é capaz de produzir ácido lático através da fermentação de carboidratos. Bactérias desse gênero são autóctones tanto de ambientes lácteos como não lácteos e muitas estirpes possuem capacidade de produzir vários compostos de interesse para a indústria de alimentos, como exopolissacarídeos, bacteriocinas e peróxido de hidrogênio, por exemplo. Este trabalho teve início com o estudo da biodiversidade das estirpes de Weissella isoladas nas regiões produtoras de queijos artesanais no Brasil. Para isso foram realizadas as análises de PFGE e Rep-PCR que permitiu agrupar as estirpes estudadas. Com essas análises observou-se que cada região estudade 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 estirpes de mesma espécie. Após selecionar os representantes de cada grupo analizou-se o potencial antimicrobiano dessas estirpes através das análises de antagonismo e produção de bacteriocinas contra os patógenos listeria monocytogenes ATCC 15313, Staphylococcus aureus subsp. Aureus ATCC 6538, Salmonella entérica ATCC 14028 e Escherichia coli ATCC 11229. Os resultados obtidos mostraram que a maioria dos isolados testados apresentou boa inibição dos patógenos havendo porcentagem de inibição maior que 60% no teste de antagonismo e com diminuição do pH do meio após 24 h de incubação devido a produção de ácido. No entanto não houve inibição considerável dos patógenos quando realizou-se o teste de produção de bacteriocinas. Após o estudo relacionado ao potencial antimicrobiano das estirpes de Weissella, avaliou-se o potencial tecnológico dessas culturas visando caracterizar fenotipicamente e direcionar seu potencial de aplicação na indutria de produtos lácteos. Para tanto treze estirpes foram selecionadas a um nível de similaridade de 72% no PFGE e realizou-se testes em leite desnatado reconstituído 10% (m/v) para determinar a capacidade de coagulação, produção de diacetil, produção de exopolissacarídeo, capacidade de acidificação, atividade proteolítica extracelular e características do coágulo formado. Como resultado algumas estirpes apresentaram resultados positivos para a maioria dos testes realizados, exceto no teste de produção de exopolissacarídeo havendo destaque para a estirpe de número 16 isolada de queijo da região do Sul do Pará, cujos resultados apresentaram boa capacidade de acidificação e decréscimo do pH do leite, e resultados positivos para os testes de produção de diacetil, atividade proteolítica e formação de coágulo homogêneo. Como conclusão, este trabalho permitiu compreender a diversidade das espécies de Weissella encontradas nas diferentes regiões produtoras de queijos artesanais no Brasil e como elas estão distribuídas nesses ambientes, além de ajudar a entender o papel que elas exercem nos próprios queijos artesanais. Mostrou também que algumas delas possuem um potencial tecnológico favorável para futuros usos na indústria de alimentos como culturas iniciadoras e para o estudo da prevenção de defeitos em queijos.

ABSTRACT

TEIXEIRA, Camila Gonçalves, M.Sc., Universidade Federal de Viçosa, July, 2018. **Biodiversity and technological potential of the Weissella strains isolated from different regions producing artisanal cheeses in Brazil.** Adviser: Antônio Fernandes de Carvalho.

The genus Weissella is composed of bacteria belonging to the group known as lactic acid bacteria (LAB), therefore it is capable of producing lactic acid through the fermentation of carbohydrates. Bacteria of this genus 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, such as exopolysaccharides, bacteriocins and hydrogen peroxide, for example. This work began with the study of the biodiversity of Weissella strains isolated in the regions producing artisanal cheeses in Brazil. For this, the analyzes of PFGE and Rep-PCR were carried out, which allowed to group the studied strains. With these analyzes it was observed that each study region represents a specific ecological niche and in addition it was also observed that the PFGE technique makes it possible to obtain a better result of heterogeneity between the strains of the same species. After selecting the representatives of each group the antimicrobial potential of these strains was analyzed through antagonistic analyzes and bacteriocin production against the pathogens Listeria monocytogenes ATCC 15313, Staphylococcus aureus subsp. aureus ATCC 6538, Salmonella enterica ATCC 14028 and Escherichia coli ATCC 11229. The results obtained showed that most of the isolates tested showed good inhibition of the pathogens, with inhibition percentage higher than 60% in the antagonism test and with a decrease in the pH of the medium after 24 h of incubation due to acid production. However, there was no significant inhibition of pathogens when the bacteriocin production test was performed. After the study related to the antimicrobial potential of the strains of Weissella, the technological potential of these cultures was evaluated aiming to characterize phenotypically and to direct its potential of application in the dairy industry. Thirteen strains were selected at a similarity level of 72% in PFGE and 10% (w/v) reconstituted skim milk were tested to determine coagulation capacity, diacetyl production, exopolysaccharide production, acidification capacity, extracellular proteolytic activity and characteristics of the clot formed. As a result,

some strains showed positive results for most of the tests performed, except for the exopolysaccharide production, highlighting the strain number 16 isolated from cheese of the southern of Pará, showing good acidification capacity and pH decrease of milk, and positive results for the tests of diacetyl production, proteolytic activity and homogeneous clot formation. As a conclusion, this work allowed us to understand the diversity of Weissella species found in the different regions producing artisanal cheeses in Brazil and how they are distributed in these environments, besides helping to understand the role of these strains in artisanal cheeses. 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.

GENERAL INTRODUCTION

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 as 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 are able to phylogenetically relate the strains. In addition, phenotypic characterization is necessary to understand the characteristics and potential of each of them.

This work consists of two chapters, the first being a review article on the importance of the genus Weissella and its potential use for the food industry. Chapter 2 deals with the genetic diversity of Weissella strains isolated from different environments producing artisanal cheese in Brazil. Also in Chapter 2 is the technological characterization.

The study of diversity led to the formation of phylogenetically similar groups, leading to the selection of representatives from each group to perform the technological tests. Phenotypic analyzes were carried out to characterize fermentation, diacetyl and exopolysaccharide production, proteolysis capacity and antagonistic capacity and production of bacteriocins. This characterization aimed to verify if any of these isolates have potential to be used in the industry.

The objective of this study was to understand the microbial diversity in different environments (dairy and non-dairy) seeking the application of strains with potential to be used in the food industry aiming at new flavor profiles and new applications for the development and improvement of fermented industrialized foods.

CHAPTER 1: THE WEISSELLA GENUS IN THE FOOD INDUSTRY

The Weissella genus in the food industry: a review

INTRODUCTION

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 due its production of lactic acid from the fermentation of carbohydrates (COLLINS et al., 1993).

Weissella species are distributed in different habitats, such as soils, milking machines, sugar cane and some strain with interesting technological features can be isolated from fermented foods, such as cheeses made from raw milk, fermented vegetables and fermented milk (AYENI et al., 2011; MASOUD et al., 2012; JANS et al., 2012; MATHARA et al., 2004; CHAO et al., 2008, 2009; LEE et al., 2005; WOUTERS et al., 2013). From the point of view of food technology, some strains had potential in production of exopolysaccharides, non-digestible oligosaccharides, beyond its probiotic potential (SRIONNUAL et al., 2007; CHEN et al., 2014b; BARUAH et al.,2016; ZANNINI et al., 2013; ELAVARASI et al., 2014). Then, the bacterias belonging to the genus Weissella may have great technological importance being also involved in the control of foodborne diseases due its production of bacteriocins and hydrogen peroxide.

THE HISTORY OF THE GENUS WEISSELLA

The genus Weissella was proposed by Collins et al. (1993), 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. (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 16Sr DNA 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. 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. To the new species was proposed the name Weissella hellenica.

Bacteria belonging to Weissella genus are hardly differentiated from Leuconostoc strains and heterofermentative Lactobacillus by phenotypic characteristics; thus, the description was only possible through molecular taxonomic analyzes (FUSCO et al., 2015). Collins et al. (1993) proposed the reclassification of Leuconostoc paramesenteroides, Lactobacillus confusus, Lactobacillus halotolerans, Lactobacillus kandleri, Lactobacillus minor, and Lactobacillus viridescens to Weissella paramesenteroides, Weissella confusa, Weissella halotolerans, Weissella kandleri, Weissella minor and Weissella viridescens, respectively. Subsequently, other studies have identified new species, and currently 19 species are validated (Table 1).

Table 1. Species belonging to the genus Weissella.

Strain	Reference
W. viridescens	(NIVEN; EVANS, 1957)
W. paramesenteroides	(GARVIE, 1967)
W. confusa	(Holzapfel e Kandler, 1969 apud COLLINS et al., 1993)
W. kandleri	(HOLZAPFEL; WYK, 1982)
W. halotolerans	(KANDLER; SCHILLINGER; WEISS, 1983)
W. minor	(KANDLER; SCHILLINGER; WEISS, 1983)
W. hellenica	(COLLINS et al., 1993)
W. thailandensis	(TANASUPAWAT et al., 2000)
W. soli	(MAGNUSSON et al., 2002)
W. cibaria	(BJÖRKROTH et al., 2002)
W. koreensis	(LEE et al., 2002)
W. ghanensis	(DE BRUYNE et al., 2008)
W. beninenses	(PADONOU et al., 2010)
W. fabaria	(DE BRUYNE et al., 2010)
W. ceti	(VELA et al., 2011)
W. fabalis	(SNAUWAERT et al., 2013)
W. oryzae	(TOHNO et al., 2013)
W. diestrammenae	(OH et al., 2013)
W. uvarum	(NISIOTOU et al., 2014)

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 according to the stress condition to which the bacterium is submitted. In relation to the organization, they can also be found in pairs, alone or in small chains (COLLINS et al., 1993; HUYS et al., 2012).

Bacteria from the genus Weissella are chemoragotrophic, facultative anaerobic, Gram-positive, non-spore forming, catalase negative (COLLINS et al., 1993) and has no motility with except to W. beninenses that has peritrichal flagella (PADONOU et al., 2010). All the microorganisms of the genus are obligatory heterofermenters producing lactic acid, carbon dioxide, ethanol and/or acetic acid from carbohydrate fermentation (GARVIE, 1986). They are characterized as lactic acid bacteria (LAB) and use the phospho-ketolase and the hexose monophosphate pathways to perform the carbohydrate fermentation (BJÖRKROTH; DICKS; ENDO, 2014; BJÖRKROTH; DICKS; HOLZAPFEL, 2009).

The bacteria of Weissella sp. have 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, panthotenic acid) for their development (COLLINS et al., 1993; FUSCO et al., 2015; GARVIE, 1967). Although, 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.

METABOLIC CHARACTERISTICS

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 and 45 °C, being their optimum temperature of multiplication between 20 and 30 °C (BJÖRKROTH; DICKS; HOLZAPFEL, 2009). These bacteria can be able to be applied in fermented dairy products like yoghurt whose temperature of incubation of the microorganisms used is 42 °C and fermented meat sausages with an incubation temperature of 25 °C.

Different species produces dextran, hydrolyzate esculin and produces ammonia from arginine. Dextran is an exopolysaccharide and these are important for the manufacture of products in which it is desired to have viscosity, such as yogurt. In addition, as these bacteria are able to 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.

Most EPS produced by strains of the genus Weissella are homopolysaccharides (HoPS) such as dextran, fructan, galactan, levan and inulin for exemplo. 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 (MOZZI; RAYA; VIGNOLO, 2016). 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 acceptor (HIJUM et al., 2006).

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, that are secreted, and cell wall proteins. It has been found that many of the secretome proteins were large containing multiple domains and greater than 1000 amino acids. Also showed that W. cibaria and W. confusa have a similar number of secretory proteins as Lactobacillus rhamnosus GG, which is a known probiotic strain.

W. cibaria MG1 possesses all the necessary genes for the use of the phosphoketolase pathway and metabolyze the galactose via the Leloir pathway. It is also capable of using maltose, fructose, ribose, xylose, sucrose and gluconate as carbon sources. In addition a β-galactosidase has been detected indicating 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 the main sugar.

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 have the ability to metabolize D-xylose, glucose, D-fructose, D-mannose, sucrose, D-maltose and cellobiose and also showed β-glucosidase and β-galactosidase activity (LÓPEZ-HERNÁNDEZ et al., 2017).

ECOLOGY

Although Weissella 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 (CHEN; YANAGIDA; SHINOHARA, 2005), sediments of swamps (ZAMUDIO-MAYA; NARVÁEZ-ZAPATA; ROJAS-HERRERA, 2008), lake water (YANAGIDA; CHEN; YASAKI, 2007), milking machines (KANDLER; SCHILLINGER; WEISS, 1983), being identified, mainly, in fermented foods as cheese made with raw milk (AYENI et al., 2011; MASOUD et al., 2012), fermented milk (JANS et al., 2012; MATHARA et al., 2004), vegetables (CHAO et al., 2008, 2009; LEE et al., 2005; WOUTERS et al., 2013) and sausages (PEREIRA et al., 2009; URSO; COMI; COCOLIN, 2006) (Table 2).

Lynch and colleagues studied the genome of the W. cibaria species and according to the study of pan-proteome and core-proteome at the species level, 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 its ability to survive in several ecological niches where they were found (LYNCH et al., 2015).

Some strains of Weissella such as W. cibaria MG1 have the ability to hydrolyze arginine, which favors its survival in environments where it is subjected to stress. As for example providing a greater amount of ATP when the carbon source is scarce or producing ammonia by protecting from acid stress (LYNCH et al., 2015).

Table 2. 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 et al., 2011)
W. confusa	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	(FHOULA et al., 2013)
	Fermented sausage	(PEREIRA et al., 2009)
W. hellenica	Vegetable forage crops	(TOHNO et al., 2012)
	Croatian cheese fermented from raw milk	(FUKA et al., 2013)
W. kandleri	Desert Plants	(HOLZAPFEL; WYK, 1982)
W. uvarum	Wine grapes	(NISIOTOU et al., 2014)
W. paramesenteroides	Raw milk cheeses	(MASOUD et al., 2012)
	Fermented sausage	(URSO; COMI; COCOLIN, 2006)

Because they are autochthonous of 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 (SRIONNUAL et al., 2007; TANASUPAWAT et al., 2000), while the strains W. cibaria, W. confusa and W. koreensis were detected in fermented foods of vegetal origin (BJÖRKROTH et al., 2002; JANG et al., 2002; LEE et al., 2002). 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 are manufactured without the presence of these strains.

LIMITING FACTORS FOR THE APPLICATION OF WEISSELLA IN FOODS

These bacteria are also found in habitats associated with the human and animal body, such as the digestive and urogenital system, human milk (FUSCO et al., 2015), human gallbladder, liver (BJÖRKROTH et al., 2002; ELAVARASI et al., 2014), brain, and spleen of primates (VELA et al., 2003). Although the bacteria W. confusa being considered a member of the normal human intestinal microbiota (STILES; HOLZAPFEL, 1997), this was found, along with W. cibaria, in clinical samples of human and animal origin associated with some diseases (BJÖRKROTH et al., 2002). The W. cibaria strain was isolated from human bile and feces, canary liver and infected dog ear material (BJÖRKROTH et al., 2002).

W. confusa bacteria were isolated from feces of children with bacteremia (GREEN; WADOWSKY; BARBADORA, 1990) undergoing liver transplantation (GREEN; BARBADORA; MICHAELS, 1991). Infections usually associated with bacteria of the genus Weissella in humans are due to the fact that these microorganisms are resistant to vancomycin and mainly affect immunosuppressed patients (HUYS; LEISNER; BJÖRKROTH, 2012).

W. ceti was identified as the causative etiological agent of the disease called "weissellosis" (WELCH; GOOD, 2013) which affects rainbow trout (Oncorhynchus mykiss) causing septicemia in animals with a high mortality rate (COSTA et al., 2014). Weissella was associated with outbreaks of rainbow trout (Oncorhynchus mykiss) grown in China, Brazil, and North Carolina in the United States, with the most severe impairment in the largest fish approaching the ideal weight to be marketed (WELCH; GOOD, 2013).

Abriouel et al. (2015) studied the pathogenic potential of Weissellas and showed the presence of several virulence determinants such as collagen adhesins, aggregation substances, hemolysin, mucus binding proteins and staphylococcal surface protein present in strains of W. ceti, W. cibaria, W. confusa, W. halotolerans, W. hellenica, W. koreensis, W. oryzae, W. paramesenteroides and W. thailandensis.

Although LAB are considered safe and beneficial to humans, their safety concerns are increasing, since some strains of different genera presented virulence factors, resistance to antibiotics or were associated with development of diseases. Thus, to ensure the safety of a strain to be applied to food, studies of source specifications, absence of harmful enzyme activity, susceptibility to antibiotics, verification of virulence factors and production of biogenic amines are required.

SOME APPLICATIONS OF BACTERIA FROM GENUS WEISSELLA IN FOODS

Due to its many characteristics, the genus Weissella has great potential in its application in food and for this reason it has been studied for this purpose.

Some strains of Weissella have already been studied due to their antagonistic activity, this activity is due to the production of several compounds like bacteriocins, organic acids, hydrogen peroxide, among others.

The strain Weissella 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 of fruits and vegetables (TRIAS et al., 2008).

Nam et al., (2002), showed that Weissella 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. Weissella 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 with the aim of combating H. pylori.

Some strains present antimicrobial activity due to the production of compounds such as bacteriocins (Table 3). The first new bacteriocin produced by Weissella strains to be discovered was Weissellicin 110 in the year 2007. This compound is produced by strain Weissella 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 lost its activity when exposed to proteinase K and trypsin (SRIONNUAL et al., 2007).

Table 3. Bacteriocins produced by Weissella strains.

Bacteriocin	Producing species	Reference
Weissellicin 110	W. cibaria 110	(SRIONNUAL et al., 2007)
Weissellin A	W. paramesenteroides DX	(PAPAGIANNI; PAPAMICHAEL, 2011)
Weissellicin L	W. hellenica 4-7	(LEONG et al., 2013)
Weissellicin D	W. hellenica D1501	(CHEN et al., 2014a)
Weissellicin M	W. hellenica QU 13	(MASUDA et al., 2012)
Weissellicin Y	W. hellenica QU 13	(MASUDA et al., 2012)

In the year 2014, Chen et al. 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, Listeria monocytogenes and Escherichia 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 presence of volatile antimicrobial compounds and bacteriocins (CHEN et al., 2014b).

Besides of Weissellicin D, others isolate of W. hellenica demonstrated the production of Weissellicins L, M and Y which showed antagonist activity against L. monocytogenes and Bacillus coagulans (LEONG et al., 2013; MASUDA et al., 2012). As bacteriocins are natural antimicrobial compounds, there is an interest in their use by the food industry for the purpose of application as bioconservants, i.e. natural preservatives and possible substitutes for chemical preservatives (CLEVELAND et al., 2001). Some characteristics that make bacteriocins produced by lactic acid bacteria safe to be used at industrial level are their non-toxicity to eukaryotic cells, inactivation by digestive proteases, little influence on the intestinal microbiota, tolerance to different temperatures and pHs, action against pathogens and microorganisms spoilage of food, and do not generate cross-resistance to antibiotics (GÁLVEZ et al., 2007).

Bacteriocins are also of great importance in the food industry, for example in the production of biodegradable food packaging with antimicrobial properties. 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).

Exopolysaccharides are structures of high molecular mass, composed of carbohydrates, which when added in the food behave mainly as thickening and emulsifying agents. The ability to produce exopolysaccharides (EPS) is a common feature of LAB. The polysaccharides that confer thickening and gelling properties are invaluable in the food industry formulation. In addition to its ability as probiotics, EPS imposes highly desirable rheological changes in the food matrix, such as viscosity increase, improved texture and reduced syneresis (BADEL; BERNARDI; MICHAUD, 2011). They can be used instead of corn starch in manufacture of puddings, for example, where these characteristics are desired. Most of the strains of Weissella have the capacity to produce the EPS dextran and can also produce other EPS as shown in Table 4.

Table 4. Exopolysaccharides produced by Weissella strains.

Species	Product	Reference
Weissella cibaria RBA12	Dextran	(BARUAH et al.,2016)
W. confusa	Dextran	(KAJALA 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 et al., 2015)

The EPS dextran has great efficacy as soluble dietary fiber since it has the capacity to be fermented by the prebiotic intestinal microbiota and also due to its low digestibility when compared to commercial prebiotic inulin. 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 (BARUAH et al.,2016; KAJALA et al., 2016).

The strains that have the capacity to produce more than one exopolysaccharide have great interest in the food industry due to their synergistic effect on texture and nutritional improvement (MALANG et al., 2015). Some EPS produced, such as galactan, has good emulsifying and stabilizing capacity which provides its future use in cosmetic and food emulsions (DEVI; KAVITAKE; SHETTY, 2016; KAVITAKE; DEVI; SHETTY, 2016).

Due to the production of exopolysaccharide, the application of W. cibaria as an adjunct 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, due to the fact that W. cibaria MG1 produces exopolysaccharides (dextran) and oligosaccharides (glucooligosaccharides), it 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 Brazil infant formulas are heated before consumption because of the dubious quality of the water. In this way, the addition of probiotics is impossible and the solution is the addition of prebiotics. The oligosaccharides produced by Weissella can be a source of prebiotics for the food industry.

Rosca et al. (2018) verified that W. confusa produces 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 also have been shown to have great potential as probiotics such as the W. cibaria strain isolated from goat's milk. This strain has good probiotic properties such as resistance to 1% bile salt and tolerance to pH 3.0 (ELAVARASI et al., 2014). In addition, W. cibaria, together with Lactobacillus plantarum, showed in vitro tests high antioxidant capacity, survived simulated gastric and intestinal transit, and tolerated bile acids and salts. Furthermore, the two strains were administered to male Wistar albino rats and showed an improvement in liver and kidney damage caused 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 in relation to 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 that there was an increase in the production of lactic acid when xylan hydrolyzed was used.

Some Weissella strains producing exopolysaccharides such as W. confusa AI10 and W. cibaria 142 showed great potential as probiotic strains due to high tolerance to bile salts and low pH, besides having antimicrobial activity against E. coli (PATEL et al., 2012).

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

On the other hand, the EPS glucansucrase produced by Weissella sp. TN610 has the ability to 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 yoghurt fermented with dextran producer W. cibaria MG1 was developed and the concentration of EPS (40 mg/L) guaranteed the high water retention capacity and viscosity (0.57 mPa s) of the final product.

The EPS dextran, levan and ropy capsular polysaccharide, produced by W. confusa were evaluated in breads and the delaying of the deterioration by fungus, as well as improving of the texture were observed (TINZL-MALANG et al., 2015).

In addition to the production of EPS, some strains of W. cibaria and W. confusa as well as producing lactic acid also have the capacity to produce folate (vitamin B9), which allows the nutritional improvement of fermented products that use these strains in the fermentation process (DEATRAKSA et al., 2018).

CONCLUSION

The microorganisms of the genus Weissella have a wide range of applications in food products, since they are capable of producing a very wide variety of compounds from the production of EPS, bacteriocins, even vitamin such as B9. However a few studies have been conducted yet. The industrial application of Weissella strains it is still not a reality. Besides that there are still some obstacles that prevent the use of Weisella as a starter culture in the food industry, 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 Weissella strains have a potential for opportunistic infection in humans, the food industry should always be vigilant for safety testing of any strain before its technological application.

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CHAPTER 2: BIODIVERSITY AND TECHNOLOGICAL POTENTIAL

Biodiversity and technological potential of the Weissella strains isolated from different regions producing artisanal cheeses in Brazil

ABSTRACT

The species belonging to the genus Weissella were isolated from several food-related environments. The present study aimed to identify the microbial biodiversity and evaluate the technological potential of strains isolated from regions producing artisanal cheeses in Brazil. Firstly, the PFGE and Rep-PCR technique ware performed to analyzed the biodiversity of the regions. It was observed a good diversity since the three strains of Weissella isoalated were present in a very wide variety of environments. It was also noted that the PFGE technique provides a better resolution of heterogeneity between strains of the same species. After selecting the representative strains of each cluster, analyzes of antagonism and bacteriocins against the pathogens Listeria monocytogenes ATCC 15313, Staphylococcus aureus subsp. aureus ATCC 6538, Salmonella enterica ATCC 14028 and Escherichia coli ATCC 11229 were performed. Most of the isolates tested showed broad inhibition against the strains of pathogens used as targets having a percentage of inhibition greater than 60% in the antagonism test with a decrease in the pH of the medium after 24 h of incubation due to acid production. However, it did not demonstrate inhibition through the production of bacteriocins. For the technological tests coagulation capacity, diacetyl production, acidification capacity, extracellular proteolytic activity and exopolysaccharide production (EPS) were all tested in 10% (w/v) reconstituted skimmed milk (RSM). Some strains were found to be positive for most tests except for exopolysaccharide production. The strain isolated from cheese of the southern of Pará was highlighted because of good acidification ability and decreased milk pH, and positive results for the production of diacetyl, proteolytic activity and formation of homogeneous clots. In conclusion, this study showed how the Weissella strains are distributed in different regions producing artisanal cheeses in Brazil and the technological characterization helped to understand the role of these strains in artisanal cheeses showed a favorable potential for future use as initial cultures and for the study of prevention of cheese defects.

1. INTRODUCTION

The genus Weissella belongs to the group of lactic acid bacteria (LAB) due to its ability to produce lactic acid from the fermentation of carbohydrates and phylogenetic position (BJÖRKROTH; DICKS; HOLZAPFEL, 2009). It is composed of Gram-positive, facultative anaerobic, catalase-negative, non-spore forming microorganisms that have cocoide or bacilli morphology (COLLINS et al., 1993).

This genus is composed by 19 validated species W. soli (MAGNUSSON et al., 2002), W. diestrammenae (OH et al., 2013), W. koreensis (LEE et al., 2002), W. kandleri (HOLZAPFEL; WYK, 1982), W. oryzae (TOHNO et al., 2013), W. cibaria (BJÖRKROTH et al., 2002), W. confusa (Holzapfel e Kandler, 1969 apud COLLINS et al., 1993), W. thailandensis (TANASUPAWAT et al., 2000), W. hellenica (COLLINS et al., 1993), W. paramesenteroides (GARVIE, 1967), W. ceti (VELA et al., 2011), W. halotolerans, W. minor (KANDLER; SCHILLINGER; WEISS, 1983), W. viridescens (NIVEN; EVANS, 1957), W. uvarum (NISIOTOU et al., 2014), W. beninenses (PADONOU et al., 2010), W. fabalis (SNAUWAERT et al., 2013), 2013), W. fabaria (DE BRUYNE et al., 2010) and W. ghanensis (DE BRUYNE et al., 2008).

The bacteria belonging to this genus have been isolated from several ecological niches, being considered autochthonous bacteria of many of them, such as soil (CHEN; YANAGIDA; SHINOHARA, 2005), sediments of marshes (ZAMUDIO-MAYA; NARVÁEZ-ZAPATA; ROJAS-HERRERA, 2008), lakes (YANAGIDA; CHEN; YASAKI, 2007), and many fermented foods such as cheeses and vegetables (AYENI et al., 2011; CHAO et al., 2008, 2009; LEE et al., 2005; MASOUD et al., 2012; WOUTERS et al., 2013).

Many positive aspects may be related to the use of some species of Weissella in the food industry. In fact, different characteristics have been attributed to the strains belonging to this genus, among them activities as starter culture (CHOI et al., 2012), antimicrobial activity (AYENI et al., 2011; SRIONNUAL et al., 2007), production of exopolysaccharides, among others (ZANNINI et al., 2013). In particular, W. paramesenteroides and W. hellenica, found in dairy products, are widely studied as to their antimicrobial potential, since the production of bacteriocins by strains of these species has already been identified (CHEN et al., 2014a; LEONG et al., 2013; MASUDA et al., 2012; PAPAGIANNI; PAPAMICHAEL, 2011). W. hellenica was found in dairy products such as croatian cheese fermented from raw milk (FUKA et al., 2013) and W. paramesenteroides was isolated from raw milk cheeses (MASUDA et al., 2012) and in others artisanal cheeses in different countries (COPPOLA et al., 2006; GERASI; LITOPOULOU-TZANETAKI; TZANETAKIS, 2003; MAS et al., 2002).

Artisanal cheeses have a complex microbiota and although there are not many studies about the genus Weissella along with this microbiota, this one can contribute to the development of the characteristics of these products since it has been found in several of them. Therefore, the present study aimed to identify the microbial biodiversity and to evaluate the technological potential of strains isolated from regions producing artisanal cheeses in Brazil.

2. MATERIAL AND METHODS

2.1. Microorganisms and culture conditions

For this study, a total of 57 strains of Weissella were used. The strains previously sequenced provided by the laboratory of milk and dairy products from INOVALEITE at Federal University of Viçosa were isolated from samples of artisanal cheeses, raw milk, manufacturing utensils, soil, silage and pasture in different regions producing artisanal cheeses in Brazil, such as Campo das Vertentes (Minas Gerais), Marajó Island (Pará), southern Pará and Guanambi (Bahia). Bacteria, which were already identified at the level of the species by sequencing the 16S rDNA, were stored at -60 °C in MRS (Man Rogosa and Sharpe) broth (Kasvi, Curitiba, Paraná, Brazil) supplementd with 30% (v/v) glycerol. Prior to analyses, the strains were transferred in MRS broth and incubated at 30 ºC for 14 h.

2.2. Biodiversity by molecular cluster analysis

2.2.1. Characterization of Weissella strains by PFGE

The analysis of Pulsed-Field Gel Electrophoresis (PFGE) were performed as described by Chuat and Dalmasso (2015), with modifications.

Weissella strains were grown to an optical density (OD650) of 0.3 in MRS broth (Kasvi). Cells were obtained by centrifugation (3500 × g, 10 min) from 10 mL of culture, the supernatant was discarded and TES buffer (10 mM Tris, 1 mM EDTA, pH 8.0, 0.5 M sucrose) was added to the pellet and homogenized. The pellet was again obtained by centrifugation of the mixture and to this pellet was added lysis solution to a final concentration of 10 g/L of lysozyme (Sigma, St. Louis, MO, USA). After incubation at 37 °C for 1 h, the suspension was heated at 55 °C for 1 min, and added 700 μL of 1% (w/v) ultrapure agarose (Gibco-BRL, paisley, UK) in 125 mM EDTA (pH 7.0) at the same temperature. After the mixture was added to the molds and solidified at 4 °C for 10 min, the block was transferred into a solution of deproteinization buffer (10 mM Tris, 100 mM EDTA, pH 8.0, 10% SDS, 20 g/L Proteinase K Qiagen) for 2 h at 55 °C.

The agarose blocks were washed 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 conserved at 4 °C in TE Buffer (pH 8.0) until digestion.

For the digestion, the DNAs in the blocks of agarose was cleaved with 10 U of restriction enzyme SmaI (Promega Corporation, Madison, WI, USA) and transferred to 1% (w/v) agarose gel. The DNA fragments were separated by PFGE in 0.5x TBE at 14 °C in the CHEF DR II apparatus (Bio-Rad, USA) with alternating pulses between 2 s and 25 s at 200 V for 18 h at 14 °C. The gel was stained with GelRed (3x in 0.1 M NaCl solution) (Biotium Inc., Miami, EUA) for 15 min and the image recorded under ultraviolet light.

Photographs of PFGE gels were scanned, and the band profiles were analyzed using the software BioNumerics, version 6.6 (Applied Maths, Kortrijk, Belgium). Comparisons between the normalized band profiles were made using the Dice similarity coefficient (1%). The compiled matrix was used for cluster analysis using the unweighted pair group method with the arithmetic average (UPGMA) clustering algorithm.

2.2.2. DNA extraction

The pellet of the activated culture of Weissella strains was obtained by centrifugation at 10.000 x g for 10 min. The DNA of the bacteria was isolated by DNA Purification Wizard® Genomic kit (Promega Corp., Madison, WI, USA). The quality and the concentration of the extracted DNA was measured by the equipment NanoDrop™ Lite Spectrophotometer (Thermo Scientific, Massachusetts, EUA) and the concentration was standardized to 100 ng/μL**.**

2.2.3. Characterization of Weissella strains by Rep-PCR

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) to a final volume of 25 μL. PCR amplification was carried out in a thermal cycler and the cycle used was 95 °C for 5 min as initial step, 95 °C for 30 s, annealing at 40 °C for 30 s and 65 °C for 8 min for the next 30 cycles, 65 °C for 16 min concluded 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, SP, Brazil). The bands profile was analyzed using BioNumerics software 6.6 (Applied Maths). The similarities between the profiles were calculated using the Pearson correlation. Dendrograms were constructed using the UPGMA method.

2.3. Technological characterization

2.3.1 Antagonism analysis

From the PFGE analysis, a representative of each cluster (80% of similarity) was selected to perform the antagonism and bacteriocin tests. And for the technological tests a representative of each cluster was also selected at a similarity level of 72%.

Weissella isolates were cultured in MRS broth (Kasvi) at 30 °C for 24 h and cell-free supernatant was obtained by centrifugation (10,000 x g for 10 min) and filtration (Ø=0.45 μm).

The sensitivity of Listeria monocytogenes ATCC 15313, Staphylococcus aureus subsp. aureus ATCC 6538, Salmonella enterica ATCC 14028 or Escherichia coli ATCC 11229 to cell-free supernatants derived from each strains of Weissella selected in the PFGE test (80% of similarity) was examined. Sterile MRS broth along with the pathogen was used as control.

For each pathogen, wells of a sterile polystyrene 96-well microplate with round bottoms (Microtiter MIC 2000, Thermo-Labsystems, Franklin, MA) were filled with 100 μL of BHI containing the specific pathogen in a concentration of 10^4 CFU/mL and 50 μL of Weissella sp. supernatant. Microplates were incubated at 30 °C for 24 h and reading the OD at 650 nm with a microplate reader with intervals of 30 min (TURCOTTE et al., 2004). The experiment was performed with two replicates.

The percentage of inhibition for each pathogen was calculated according to Equation 1.

$$\% = 100 - \frac{\text{Mean OD}}{\text{Mean OD control}} \times 100 \qquad \text{(Eq. 1)}$$

2.3.2 Bacteriocins production

The supernatant of the Weissella strains was obtained according to section 2.3. After measuring, the pH of the supernatant was adjusted to 6.0–6.5 with 1 M NaOH to eliminate the effects of acids produced, and treated for 10 min at 80 °C to eliminate the possible peroxides produced (CAVICCHIOLI et al., 2015).

The analysis of bacteriocin was performed in wells of a sterile polysestirene 96-well microplate, similarly as described to antagonism analysis (section 2.3). The presence of bacterioncin in Weissella sp surpernatant, was pereformed against the same pathogen before tested.

2.3.3 Diacetyl production

Diacetyl production was determined according to King (1948). Activated culture of Weissella strains (1% v/v) were inoculated in 10 mL of reconstituted skimmed milk (RSM, 10% v/v, Molico Nestlé, São Paulo, Brazil) and incubated at 30 °C for 24 h. In an aliquot of 1 mL of culture was added 0.5 mL of α-naphthol (1% w/v) and KOH (16% w/v) (Sigma-Aldrich) and incubated at 30 °C for 10 min. Diacetyl production is indicated by the formation of a red ring at the top of the tubes. The experiment was performed in duplicate with two replicates.

2.3.4 Coagulation test

The test was procedure according to Salama et al. (1995) with modifications. Activated culture of Weissella strains (1% v/v) were inoculated in 10 mL of RSM (10% v/v, Molico Nestlé) and incubated at 30 °C for 24 to 48 h. The evaluation of the results occurred through empirical analysis. The formation of clot was observed and classified as: uniform, uniform with presence of whey on the lower part, uniform with fragile predominance (appearance), crushed with the presence of whey in the lower part and absence of clot. The experiment was performed in duplicate with two replicates.

2.3.5 Extracellular proteolytic activity

Extracellular proteolytic activity was determined according to Franciosi et al. (2009) with modifications. Activated culture of Weissella strains (1% v/v) were inoculated in chopped form in Petri dishes containing Plate Count Agar (PCA, HiMedia, India) with addition of RSM (10% v/v, Molico Nestlé) and incubated at 30 °C for 24 to 48 h. Proteolytic activity was indicated by a clear zone around the colonies. The experiment was performed in duplicate with two replicates.

2.3.6 Acidifying activity

One hundred microliters of the strains were revitalized in 10 mL of RSM (10% w/v, Molico Nestlé) and incubated at 30 °C for 24 h. For the acidifying activity test, tubes containing 10 mL of sterile RSM (10% v/v, Molico Nestlé) were inoculated (1% v/v) with activated strains and incubated at 30 °C. The titratable acidity expressed as lactic acid and the pH was measured after 6, 12 and 24 h. The data are expressed as the mean of duplicate analysis (DAL BELLO et al., 2012).

2.3.7 Exopolysaccharide production

The production of EPS from lactose present in milk was evaluated according to Dal Bello et al. (2012). In this analysis, Weissella strains were inoculated individually into RSM (10%, Molico Nestlé) and incubated at 30 °C for 48 hours. After the incubation time the EPS production was determined by qualitatively measuring the degree of stringiness. The culture was considered to be EPS positive if the coagulated culture could be tied to a string with an inoculating loop.

3 RESULTS

3.3 Microorganisms and culture conditions

For this study, were selected microorganisms isolated in four different regions based on their importance in artisanal cheese production which were Campo das Vertentes (Minas Gerais), Marajó Island (Pará), southern Pará, Guanambi (Bahia). Among the different isolates from these environments, 57 isolates were chosen based on morphological characteristics compatible with the genus Weissella and genetic sequencing of the rDNA 16S. The strains were isolated from raw cow's milk, manufacturing utensils, Marajó cheese and fermented pasta, cheese from the south of Pará, curd cheese from the backlands, soil, silage, and pasture being them, W. cibaria (n = 38), W. paramesenteroides (n = 12), and W. confusa (n = 7) (Table 1). In milk, strains of the three species were identified, whereas in soil, silage and pasture was only W. cibaria. In manufacturing utensils, were isolated W. confusa and W. paramesenteroides, whereas in Marajó cheese and fermented pasta was only W. confusa and in cheese from the south of Pará and curd cheese from the backlands was only W. paramesenteroides.

Table 1. Strains, biotope and geographical origins and species identified for Weissella strains used in this study.

Strain Number	Origins	Species
1, 2, 3 and 4	Utensil/ Marajó Island	
5 and 6	Fermented pasta/ Marajó Island	W. confusa
7 and 8	Cow's milk/ Marajó Island	
9	Utensil/ Marajó Island	
10	Marajó cheese/ Marajó Island	
11, 12, 13, 14, 15, 16 and 17	Cheese / South of Pará	W. paramesenteroides
18	Curd cheese from the backlands/ Guanambi	
19	Cow's milk /Guanambi	
20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30	Pasture / Campo das Vertentes	
31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44 and 45	Soil / Campo das Vertentes	W. cibaria
46, 47, 48, 49 and 50	Silage / Campo das Vertentes	
51, 52, 53, 54, 55, 56 and 57	Cow's milk / Campo das Vertentes	

3.4 Biodiversity by molecular cluster analysis

3.4.1 Characterization of Weissella strains by PFGE

Restriction digestion of chromosomic DNA of 57 isolates of Weissella sp. with the enzyme SmaI yielded 51 different restriction patterns (pulsotypes); of these, 8 pulsotypes represented W. confusa, 32 W. cibaria and 11 represented W. paramesenteroides. Clonal differentiation by PFGE led to the identification of different pulsotypes among isolates from the same origin. No identical pulsotypes were detected among the different regions used in this study. The Weissella isolates were grouped in 23 clusters, 14 of them constituted by single strain, at 80% similarity level (Fig. 1) clusters 4, 5, 6, 18, 20 and 23 are W. cibaria, clusters 2, 8, 9, 11, 13, 21 and 22 are W. paramesenteroides and clusters 1 and 14 are W. confusa. There are four distinct clusters containing only W. cibaria strains, clusters 15, 16, 17 and 19 and one cluster containing only W. paramesenteroides.

Cluster 7 contained only isolates from Marajó Island, clusters 15, 16, 17 and 19 from Campo das Vertentes and cluster 10 from southern Pará.

If it is considered a coefficient of similarity equal to 56% we have the formation of two major groups, one containing practically all strains from the Marajó Island and another containing most of the strains isolated in Campo das Vertentes.

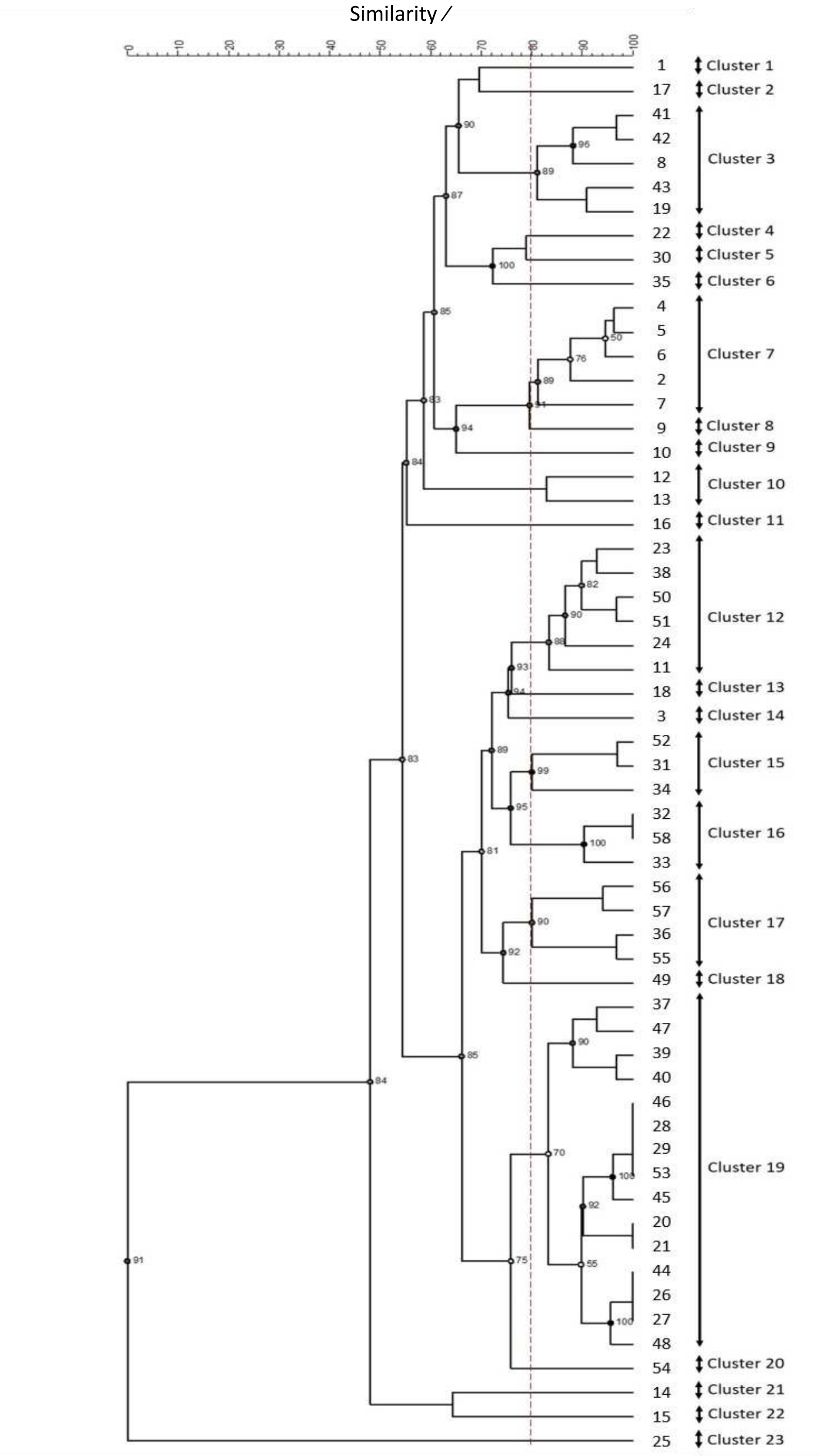


Figure 1. Dendrograms based on UPGMA clustering (Dice coefficient) of PFGE profiles of Weissella sp. strains, isolated from regions producing artisanal cheeses in Brazil.

3.4.2 Characterization of Weissella strains by Rep-PCR

The Rep-PCR amplified into bands ranging in size from 500 to 5000 bp. The dendrogram generated 29 clusters for an 80% similarity level (Fig. 2). This same similarity level enabled the observation of a large cluster, named cluster 3, comprising 13 of the 57 isolates being all of them W. cibaria and 19 clusters constituted by single strain, clusters 19, 20 and 24 are W. confusa, clusters 11, 12, 14, 17, 18, 21, 22, 23, 26 e 28 are W. paramesenteroides and clusters 2, 6, 8, 9, 10 and 16 are W. cibaria. There are eight distinct clusters containing only W. cibaria strains, clusters 1, 3, 4, 5, 7, 15, 25 and 27 and one cluster containing only W. confusa. The similarity between the most distant isolates was 27.8%. Six clusters of identical pattern (100% similarity) were observed, formed by 18 isolates five of them composed by W. cibaria and cluster one composed by W. confusa. In relation to the isolating regions of the strains, at a similarity level of 80% clusters 13 and 29 contained only isolates from Marajó Island and clusters 1, 3, 4, 5, 7, 15, 25 and 27 from Campo das Vertentes. If it is considered a coefficient of similarity equal to 55% there is the formation of one major groups containing most of the strains isolated in Campo das Vertentes.

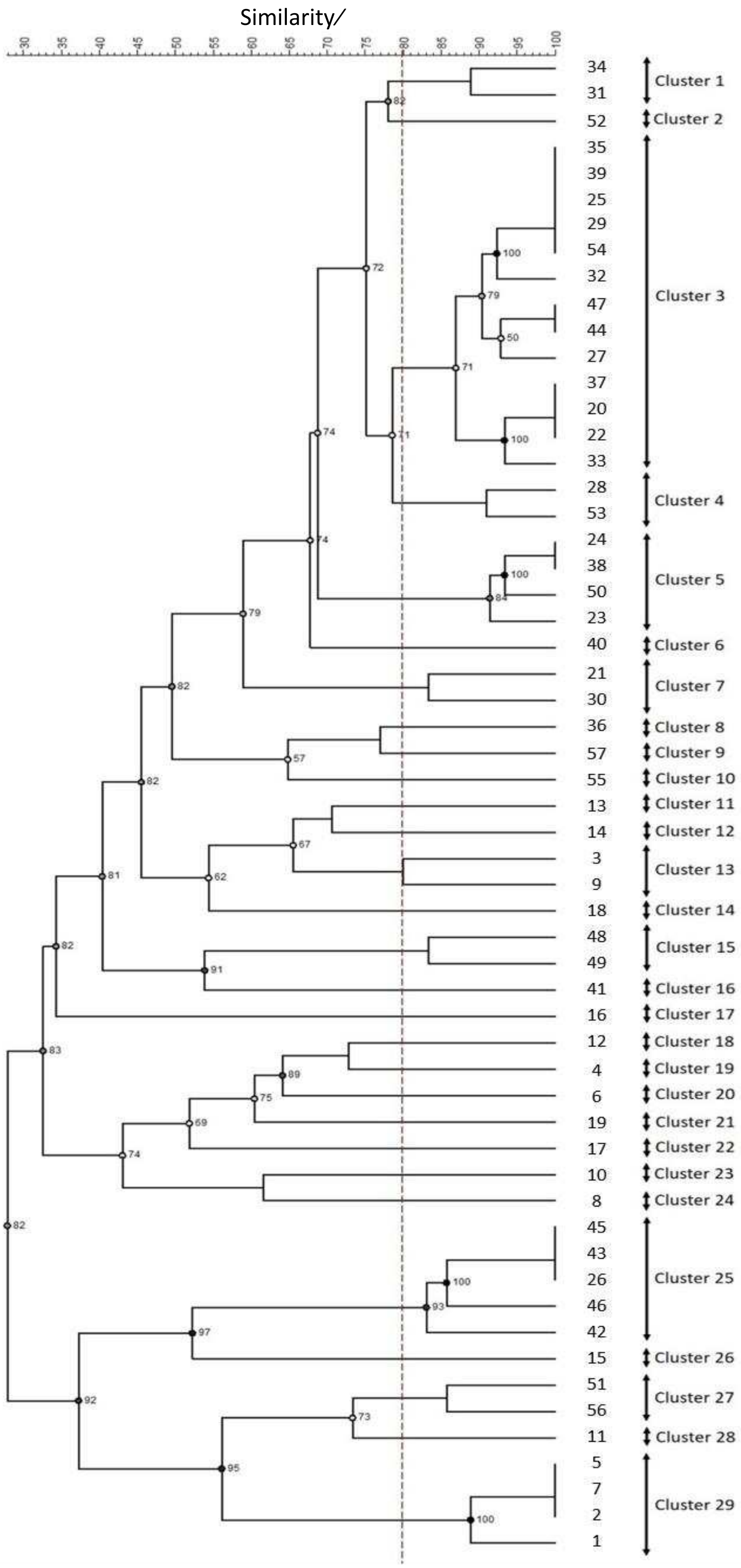


Figure 2. Dendrograms based on UPGMA clustering (Dice coefficient) of Rep-PCR profiles of Weissella sp. strains, isolated from regions producing artisanal cheeses in Brazil.

3.5 Antagonism analysis

The pH of the supernatant of the Weissella culture measured after filtration and the data obtained using the microplate test to verify the antimicrobial activities of the selected isolates are presented in Tables 2 and 3 respectively. The growth curves obtained through the microplate test are shown in Figures 3 and 4.

Table 2. pH values of Weissella sp. supernatant.

Strain Number	pH	Species
1	4.12	W. confusa
3	4.35	
5	4.03	
8	3.95	
9	4.03	W. paramesenteroides
10	4.04	
11	3.94	
12	4.04	
14	4.01	
15	4.74	
16	4.36	
17	4.00	
18	4.17	
19	3.94	
21	4.79	W. cibaria
22	4.49	
25	4.41	
30	4.67	
32	4.71	
35	4.20	
42	3.92	
49	4.72	
51	4.01	
52	4.46	
54	4.75	
56	4.39	

The pH of the MRS medium used to grow the Weissella strains was 6.5 before addition of the cultures. It is noted that after 24 h of incubation at 30 °C there was a decrease in pH due to acid production.

Table 3. Percentage inhibition of target pathogens by Weissella supernatants in the antagonism test.

Strain Number	Target pathogen				Species
	L. monocytogenes (%)	S. aureus (%)	S. entérica (%)	E. coli (%)	
1	89.2	53.0	80.6	67.5	W. confusa
3	88.1	37.0	77.3	71.1	
5	88.7	86.4	78.1	86.1	
8	85.3	85.2	76.6	87.9	
9	62.4	25.0	50.9	29.2	W. paramesenteroides
10	89.4	85.1	81.9	84.1	
11	90.3	89.2	83.0	85.6	
12	87.1	79.8	77.5	83.6	
14	88.0	86.6	79.5	87.3	
15	60.1	45.0	26.7	49.5	
16	31.8	36.8	43.4	46.3	
17	88.0	84.0	79.5	85.4	
18	88.1	68.8	78.1	65.6	
19	88.6	88.1	82.0	86.1	
21	98.5	87.1	76.8	82.8	W. cibaria
22	39.4	39.9	15.2	63.4	
25	44.0	22.2	49.9	18.6	
30	48.7	28.9	26.3	40.2	
32	26.6	37.0	4.2	21.8	
35	86.8	65.9	74.7	70.0	
42	89.1	88.5	80.3	86.2	
49	85.2	27.4	0.7	20.6	
51	86.7	63.8	78.4	74.0	
52	11.4	4.0	8.5	2.2	
54	78.5	30.5	70.7	28.0	
56	87.9	48.4	77.2	56.6	

Most of the tested isolates demonstrated broad inhibition against the pathogens strains used as targets presenting percentage of inhibition greater than 60%. L. monocytogenes strains were especially sensitive to all antagonistic strains of Marajó Island and Guanambi and only the 16 strain didn’t present antagonistic activity above 60% among all the isolates from southern Pará. S. aureus, S. enterica and E. coli were highly inhibited by almost all isolates from Marajó Island and southern Pará and by all isolates from Guanambi. However, the strain that presented the highest percentage of inhibition was 21 (W. cibaria) isolated from Campos das Vertentes, and its inhibitory activity was against L. monocytogenes. The strain 11

(W. paramesenteroides) isolated from southern Pará presented the highest percentage of inhibition against S. aureus and S. enterica.

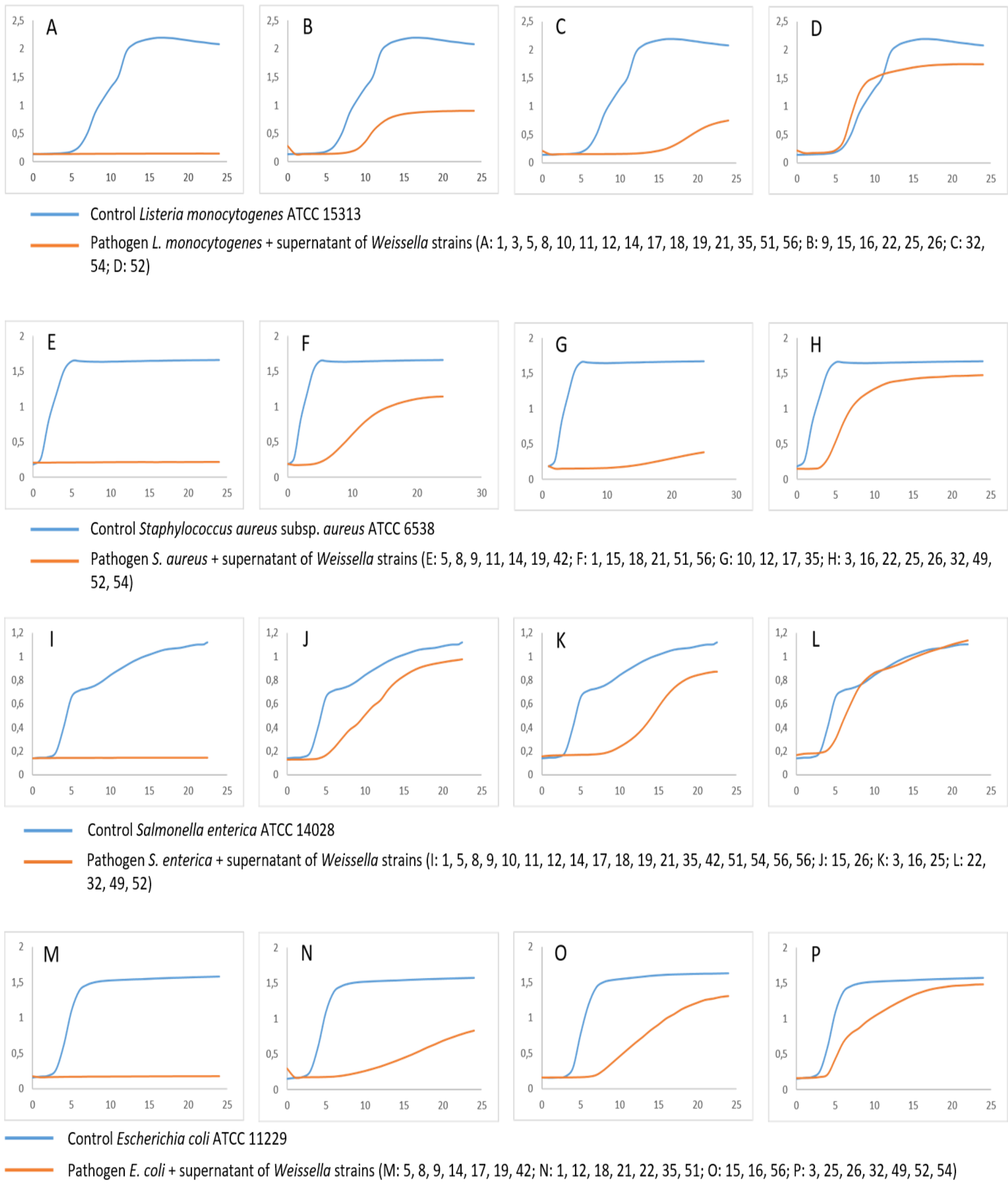
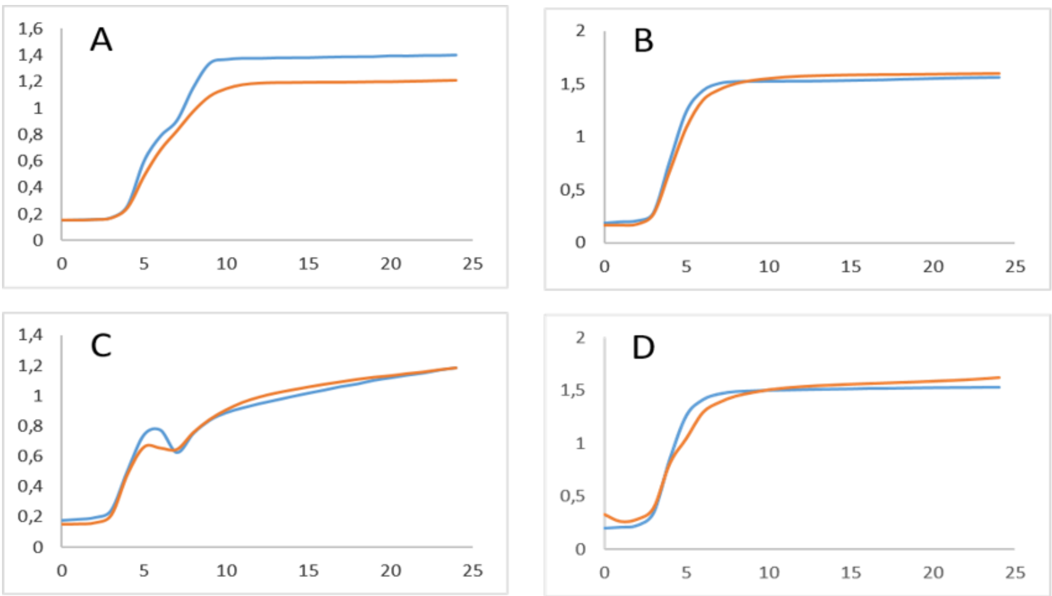


Figure 3. Typical growth curve obtained by the microplate test with the pathogens L. monocytogenes ATCC 15313 (A, B, C and D), S. aureus subsp. aureus ATCC 6538 (E, F, G and H), S. enterica ATCC 14028 (I, J, K and L) and E. coli ATCC 11229 (M, N, O and P).

According to the growth curves (Figs. 3), after the pathogens were cultured in BHI broth for 24 hours at 37 °C together with the supernatant of the isolates, it is noted that the supernatant of the isolates from graphic A, E, I and M inhibited the multiplication of all pathogens, maintaining its growth curve constant and with approximate DO of 0.15. However, for the supernatants from graphics B, C, F, G, K, N and O it is noted that there was an increase in the lag phase for all pathogens being less pronounced for the supernatants of the graphs B, F and O and little increase in OD compared to the growth curves of the pathogens without the Weissella supernatants.

3.6 Bacteriocins production

After the supernatants of Weissella isolates with antimicrobial activity of more than 60% were treated for peroxide elimination and acidity correction, new growth curves were obtained to verify the bacteriocinogenic activities of the selected isolates (Fig. 4).



Control *Listeria monocytogenes* ATCC 15313 (A), *Staphylococcus aureus* subsp. *aureus* ATCC 6538 (B), *Salmonella enterica* ATCC 14028 (C) and *Escherichia coli* ATCC 11229 (D).

Characteristic curve of each supernatant with its respective pathogen. A: *Listeria monocytogenes* ATCC 15313, B: *Staphylococcus aureus* subsp. *aureus* ATCC 6538, C: *Salmonella enterica* ATCC 14028 D: *Escherichia coli* ATCC 11229.

Figure 4. Typical growth curves obtained by the bacteriocin test with the supernatants obtained by antagonistic test.

The inhibition percentages obtained in this test are shown in Table 4. All supernatants tested did not show good inhibition against the target pathogens in the test conditions.

Table 4. Percentage inhibition of target pathogens by Weissella supernatants in the bacteriocin test.

Strain Number	Target pathogen				Species
	L. monocytogenes (%)	**S. aureus (%)**	**S. entérica (%)**	**E. coli (%)**	
1	12.8	--	21.9	0.0	W. confusa
3	9.4	2.7	9.3	3.8	
5	12.1	0.0	18.0	0.0	
8	13.9	0.0	0.0	0.0	
9	1.4	--	0.0	0.0	W. paramesenteroides
10	4.2	2.8	9.7	4.2	
11	0.0	--	--	--	
12	--	--	--	--	
14	--	--	--	--	
15	19.5	--	--	--	
16	13.0	--	0.0	--	
17	11.1	0.9	0.0	0.0	
18	--	--	--	0.0	
19	0.0	--	0.0	--	
21	11.5	1.4	0.0	0.6	W. cibaria
22	--	--	--	--	
25	--	--	--	--	
30	--	--	--	--	

32	13.0	0.0	0.0	0.0
35	10.8	0.6	0.0	0.0
42	9.7	0.0	0.0	0.0
49	5.6	2.4	9.8	3.4
51	0.0	--	--	--
52	14.0	0.0	0.0	0.0
54	9.8	5.5	11.1	5.9
56	11.5	0.7	0.0	0.0

3.7 Technological characterization

3.7.1 Diacetyl production and coagulation test

Among the thirteen Weissella strains tested, six presented diacetyl production (12, 14, 15, 16, 22, and 25) (Table 5), the strain 25 being the one with the highest intensity of rosacea color. The remaining seven strains tested negative. It was also observed that four (12, 14, 15 and 16) of the six strains that tested positive for diacetyl were isolated from cheese of the southern Pará and only two from pasture of Campo das Vertentes.

In this study, four of the Weissella strains tested showed good milk coagulation (16, 17, 22 and 25). Two of them presented homogeneous coagulation after 24 h of incubation at 30 °C (16 and 25) and the other two strains showed a homogeneous clot, but after 48 h of incubation at 30 °C. The other strains did not show coagulation even after 48 h of incubation.

Table 5. Results of the technological tests carried out with Weissella sp.

Strain Number	Acidifying activity								Diacetyl production	EPS production	Extracellular proteolytic activity	Clot type
	pH drop in skim milk				Percentage of acidity							
	0	6h	12h	24h	0	6h	12h	24h				
1	7.05	6.50	6.44	6.15	0.14	0.15	0.13	0.19	-	-	+	-
5	7.05	6.59	6.26	6.15	0.14	0.11	0.16	0.19	-	-	+	-
10	7.05	6.62	6.40	6.33	0.14	0.13	0.11	0.20	-	-	+	-
12	7.05	6.60	6.42	5.46	0.14	0.13	0.13	0.23	+	-	+	-
14	7.05	6.63	6.45	6.27	0.14	0.12	0.14	0.18	+	-	+	-
15	7.05	6.62	6.43	6.36	0.14	0.13	0.17	0.16	+	-	+	-
16	7.05	5.41	4.65	4.60	0.14	0.37	0.57	0.77	+	-	+	uniform
17	7.05	6.53	6.14	5.84	0.14	0.12	0.20	0.27	-	-	+	uniform
18	7.05	6.69	6.53	6.37	0.14	0.12	0.13	0.17	-	-	+	-
19	7.05	6.57	6.40	6.24	0.14	0.13	0.15	0.18	-	-	+	-
22	7.05	6.30	5.78	5.35	0.14	0.17	0.29	0.38	+	-	+	uniform
25	7.05	6.47	5.58	5.00	0.14	0.15	0.35	0.60	+	-	-	uniform
54	7.05	6.77	6.73	6.66	0.14	0.12	0.13	0.12	-	-	-	-

3.7.2 Acidification, extracellular proteolytic activity and exopolysaccharide (EPS) production

A test of the ability of each Weissella strain to acidify skim milk showed that all weakly reduced the pH over a 24 h period of incubation at 30 °C except the strain 16 (Table 5) isolated from cheese of southern Pará, according to Cogan (1997). The strain 12, 22 and 25 were found to be more efficient acidifiers than the others. The results of acidification percentage confirm the results obtained with the pH measurement (Table 5).

In this study, proteolytic activity was observed in ten Weissella strains (1, 5, 10, 12, 14, 15, 16, 17, 18, 19 and 22). Two Weissella strains appeared to lack proteolytic activity (Table 5). Of the eleven strains that presented proteolytic activity, five were from southern Pará cheese (12, 14, 15, 16 and 17), and the others were isolated from Marajó cheese (10), utensils used to make Marajó cheese (1), fermented pasta from Marajó Island (5), curd cheese from the backlands of Guanambi (18), cow's milk from the Marajó Island (19) and pasture from Campo das Vertentes (22).

Exopolysaccharide (EPS) production from lactose was determined qualitatively and all strains proved to be EPS negative (Table 5).

4 DISCUSSION

According to the results observed by the molecular characterization exemplified by the generated dendograms (Figs 1 and 2), it can be observed that the PFGE and Rep-PCR techniques are able to differentiate the studied strains. Nisiotou et al. (2014) also achieved differentiation between the strains studied using the same techniques in the identification and characterization of strains of W. uvarum isolated from grapes from the Nemea region of Greece.

Comparing the molecular techniques used in this study, it was noticed that the Rep-PCR presented both a greater number of profiles and clusters in comparison to the PFGE. This result was also observed by other authors who used these same two techniques with other lactic bacteria such as Perin and Nero (2014) and Cavichiolli et al. (2015). These differences can be explained due to the sensitivity of the methods used. PFGE has the highest sensitivity in detecting deletions, insertions and genetic modifications in chromosomal DNA due to the cleavage of specific sites in it by the enzyme used (CHUAT; DALMASSO, 2015), whereas the Rep-PCR technique analyzed the sequence and extent of polymorphism of regions amplified by primers (MALATHUM et al., 1998). Freitas et al. (2015) also compared the use of PFGE with another molecular technique called random amplified polymorphic DNA (RAPD), confirming that the PFGE method provides better resolution of heterogeneity between strains of the same species. Thus, in this study the PFGE technique was chosen to be used as screening to select representatives from each cluster to perform antimicrobial activity tests and technological tests.

Considering the studied regions, a good diversity was observed since the three strains of Weissella isolated were present in a very wide variety of environments, being milk, cheese, pastage, utensils and others. Other studies have also identified strains of this genus in dairy environments such as Fuka et al. (2013) that isolated strains of W. paramesenteroides and W. hellenica in Croatian cheese derived from raw ewe's milk cheeses. Masoud et al. (2012) also isolated the same species in raw milk and cheeses.

Each region represents a specific ecological niche, since isolated strains in different regions did not present similar PFGE restriction profiles (100% similarity), in the same way that Freitas et al. (2015) considered each farm studied in Campo das Vertentes a different ecological niche from the lactic acid bacteria.

Within the same region are strains that have the same PFGE profile and clones can be considered. These strains, besides belonging to the same region (Campo das Vertentes) were isolated from very close and/or similar environments, such as 20 and 21 strains (100% similarity to each other) that were isolated from pasture on the same farm in the region and strains 44, 26 and 27 (100% similarity between each other) that were isolated from soil and pasture on the same farm. Vela et al. (2003) also obtained the same PFGE restriction pattern by isolating W. confusa from primates infected by this strain and as Costa et al. (2014) isolated W. ceti from Brazilian rainbow trout with weissellosis.

The highest susceptibility of L. monocytogenes to Weissella supernatants is justified since antimicrobial activity among related microorganisms is common in Gram-positive bacteria. E. coli was used as Gram-negative target microorganism and was also highly inhibited by supernatants. Ortolani et al. (2010) also obtained LAB antagonist activity isolated from raw milk and soft cheese against the foodborne pathogens L. monocytogenes and S. aureus. Espeche et al. (2009), isolated 6 strains of W. paramesenteroides from bovine milk samples and one of them also showed antimicrobial activity against the pathogens Streptococcus dysgalactiae ATCC 27957 and E. coli.

It is noteworthy that in this test only cells were removed from Weissella sp. supernatants, and these were obtained under optimum multiplication conditions, which does not prevent the production of more than one antimicrobial compound that will be present in the supernatant. LAB can produce a variety of antimicrobial compounds such as hydrogen peroxide, organic acids (lactic acid, acetic acid, formic acid, for example), carbon dioxide, bacteriocins, ethanol, diacetyl, among others (LEROY; DE VUYST, 2004).

According to the bacteriocin production test, these isolated strains did not demonstrate the production capacity of this compound under the multiplication conditions (MRS broth at 30 °C for 24 h). On the other hand, according to Papagianni and Papamichael (2011), W. paramesenteroides DX is able to produce the bacteriocin weissellin A when grown in M17 + 2% glucose broth at 30 °C for 60 h. Srionnual et al. (2007) incubated W. cibaria 110 also in MRS broth at 30 °C and obtained production of the bacteriocin weissellicin 110. However, during the incubation time of 16 h it was noted that the highest bacteriocin titers were obtained after 10 h of incubation and after this time there was a decrease of the same.

The decrease in pH indicates that there was acid production during the incubation time in MRS. Since this medium has in its formulation dextrose and ammonium citrate, it can be assumed that the acids produced by the strains are lactic acid and acetic acid, through the glycolytic and phosphoketolase pathway, and acetic acid through the diacetyl/acetoin pathway (LYNCH et al., 2015). These organic acids have antimicrobial capacity due to alteration of the pH of the cytoplasmic medium of the pathogenic bacteria. According to Russell (1992) the non-dissociated form of organic acids is liposoluble, being able to passively pass through the cytoplasmic membrane of the cells. Inside the cell, it changes the pH by modifying the proton gradient and the electric charge by interfering with the amino acid transport system and inactivating enzymes.

Silva (1998) states that in order for the amount of organic acids produced to be sufficient to reduce microbial growth, the pH of the medium should be less than 5.5, a result obtained in this work whose highest pH value was 4.79.

Weissella sp. are autochthonous of some artisanal cheeses in Brazil made from raw milk and of producing environments. The fact that they present antimicrobial activity against the main foodborne pathogens indicates a possible interference of this microbiota with the pathogens present in the raw milk leading to their inhibition. According to Jay (1996) the effect of autochthonous microorganisms against pathogens is well established and this effect is greater in products of animal origin containing a greater amount of autochthonous microbiota.

The six strains producing diacetyl, two are strains of W. cibaria and the other four are W. paramesenteroides. Study have revealed that strain W. cibaria MG1 isolated from pozol has the ability to produce diacetyl via the diacetyl/acetoin pathway (LÓPEZ-HERNÁNDEZ et al., 2017) and Pakdeeto, Naranong and Tanasupawat (2003) observed the production of diacetyl by W. confusa strains under incubation conditions using modified MRS broth. In the metabolism of heterofermentative LAB, pyruvate, in addition to becoming lactic acid can also lead to the generation of other metabolites such as diacetyl. This is a volatile compound that contributes to the typical taste of certain products like butter and fermented creams, for example (LEROY; DE VUYST, 2004).

Of the fourth strains capable of coagulating milk, two are W. cibaria. Some studies also obtained positive results for the fermentation of lactose and of galactose by some strains of this species (BOUNAIX et al., 2010; LYNCH et al., 2014). According to Lynch et al. (2015), W. cibaria MG1 possesses all the necessary genes for the use of the phosphoketolase pathway and metabolyze the galactose via the

Leloir pathway. Milk coagulation is important for the manufacture of various fermented products such as yoghurt and cheeses. Coagulation occurs due to the lowering of the pH of the milk due to the production of acids by the LAB. Upon reaching the isoelectric point of casein, this is added expelling the serum, forming the gel in yoghurts and curd in cheese (ROBINSON, 2002; TAMIME; ROBENSON, 2007).

The ability to degrade milk proteins was observed by almost all strains selected for the test. Lynch et al. (2014) also obtained positive results for the proteolysis test performed with the strains of W. cibaria MG1, stating that the enzymatic activities are strain specific. The proteolytic and lipolytic activity of LAB in cheese lead to generation of aroma and flavor, besides contributing 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), a strain with good acidification capacity is one that can reduce the pH of the milk in 1.3 after 6 h of incubation. In this study, strains can be classified as poor in producing acid except for the 16 strain, which was the only one that managed to reduce the pH to 5.41 after 6 h of incubation at 30 °C, producing a large amount of acid at the end of 24 h of incubation. The 25 strain also produced a large amount of acid at the end of 24 h, but was able to reach pH below 5.75 only after 12 h.

The strain 16 was isolated from samples of cheese from the southern region of Pará, justifying its good acidification capacity because it was more adapted. In addition, we can infer that this strain may play an important role in the production of these cheeses since it presented positive results not only for acidification and decrease of the pH of the milk, but also positive results for the production of diacetyl (generating aroma), activity proteolytic (texture) and homogeneous clot formation.

5 CONCLUSION

This research shows the genetic diversity of Weissella isolated in different sources of different producing regions of artisanal cheeses in Brazil, showing the importance of the molecular technique to be used to characterize Weissellas diversity. The technological characterization helped to understand the function of these strains in the artisanal cheeses and demonstrated a favorable potential for future use as initial cultures and for the study of the prevention of cheese defects.

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GENERAL CONCLUSION

It was concluded that the 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.