FEDERAL UNIVERSITY OF MINAS GERAIS
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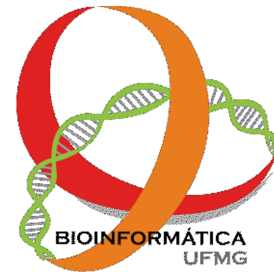

MASTER

DISSERTATION

COMPARATIVE GENOMICS AND *IN SILICO* EVALUATION OF GENES related to PROBIOTIC POTENTIAL of *Bifidobacterium breve* 110[1A]

MASTER STUDENT: **Juan Luis Valdez Baez**

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BELO HORIZONTE - MG

2021

JUAN LUIS VALDEZ BAEZ

COMPARATIVE GENOMICS AND *IN SILICO* EVALUATION OF GENES related to PROBIOTIC POTENTIAL of *Bifidobacterium breve* 110[1A]

Dissertation manuscript to the Postgraduate Interunit Program in Bioinformatics of the Federal University of Minas Gerais as a partial requirement for obtaining the title of Master in Bioinformatics.

BELO HORIZONTE - MG

2021

043 Baez, Juan Luis Valdez.
Comparative genomics and in silico evaluation of genes related to probiotic potential of Bifidobacterium breve 110[1a] [manuscrito] / Juan Luis Valdez Baez. – 2021.
126 f. : il. ; 29,5 cm.

Orientador: Dr. Vasco Ariston de Carvalho Azevedo. Coorientadora: Dra. Flavia Figueira Aburjaile.
Dissertação (mestrado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa Interunidades de Pós-Graduação em Bioinformática.

1. Biologia Computacional. 2. Genômica. 3. Probióticos. 4. Bifidobacterium. I. Azevedo, Vasco Ariston de Carvalho. II. Aburjaile, Flavia Figueira. III. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. IV. Título.

CDU: 573:004

Ficha catalográfica elaborada pela bibliotecária Fabiane C M Reis – CRB 6 - 2680

UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-graduação em Bioinformática

ATA DA DEFESA DE DISSERTAÇÃO

JUAN LUIS VALDEZ BAEZ

Às nove horas do dia **31 de maio de 2021**, reuniu-se, no aplicativo Zoom, a Comissão Examinadora de Dissertação, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho de Juan Luis Valdez Baez*,* intitulado: **"COMPARATIVE GENOMICS AND IN SILICO EVALUATION OF GENES PRESENTING PROBIOTIC POTENTIAL IN Bifidobacterium breve 1101A"**, requisito para obtenção do grau de Mestre em **Bioinformática.** Abrindo a sessão, o Presidente da Comissão, **Dr. Vasco Ariston de Carvalho Azevedo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Professor / Pesquisador	**Instituição**	**Indicação**
Dr. Vasco Ariston de Carvalho Azevedo	Universidade Federal de Minas Gerais	Aprovado
Dra. Flavia Figueira Aburjaile	Universidade Federal de Minas Gerais	Aprovado
Dr. Siomar de Castro Soares	Universidade Federal do Triângulo Mineiro	Aprovado
Dr. Rommel Thiago Juca Ramos	Universidade Federal do Pará	Aprovado
Dr. Ronnie Gustavo Gavilan Chavez	Instituto Nacional de Salud	Aprovado

Pelas indicações, o candidato foi considerado: **Aprovado**

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 31 de maio de 2021.

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Referência: Processo nº 23072.227953/2021-16 SEI nº 0757156

ACKNOWLEDGEMENTS

Firstly, I would like to express my deep and sincere gratitude to my research supervisor Dr. Vasco Azevedo for giving me the opportunity to develop this study in the LGCM laboratory, to search the possibility to get a fund for my permanence during the first months even in difficult moments and for showing us the endless desire to do research.

I appreciate and would like to thank the following people that support this research.

Co-advisor Flávia Aburjaile for her patience, respect and optimistic thinking for this work during the time of her supervision. In the same form to collaborators for their suggestions in revising the manuscript that improves for the better.

Rodrigo Profeta and Alessandra Lima for their time to explain to me basic topics in the first months, helpful comments to my questions about proceedings and adequate programs during the analysis and for their patience for guidance.

Alfonso Gala for the introduction about the region, university, the guide for paperwork, the disposition to receive me in his apartment and coexistence during the first months.

Marcus Canário for the technical assistance with some unexpected bugs with the PC and his well-thought-out comments about specific topics in Bacterial Genomics.

Vinícius Rodovalho for his calm words and interesting opinions about everything.

Wylerson Nogueira for his excellent spirit during our courses and the encouragement.

Luana Bastos for her good vibes, peaceful word and valuable support during our studies.

Glen Jasper for his direct suggestions and sincere opinion about bioinformatics questions and approaches from Metagenomics, suggestions about programming and more.

Sheila Santana and Tiago Silva of the Secretary of the Postgraduate Program of Bioinformatics (UFMG) assist me with the procedures from the beginning of the selection process until now and for their kind predisposition to solve paperwork issues.

Professor Daniella Bartholomeu who received me when I was a bunch of questions about programs of Bioinformatics in Brazil, for her time to solve my question about the Postgraduate Program of Bioinformatics (UFMG) in 2018. In the same way, to Victor

Borda, for his disposition and thoroughness to give me an overview of the program in a simple way before applying for the selection process.

My gratitude extends to the Laboratory of Cellular and Molecular Genetics (LGCM) for harbor the present study in his facilities, to the laboratory member for the reception, the comments and suggestions for the presentations to the processes of qualification and dissertation during the master.

This research study was funded with the support of the FAPEMIG (second period 2019) and CAPES (2020 - first period 2021) scholarships.

ABSTRACT

Probiotics are microorganisms with the ability to influence the composition of the intestinal microbiota and promote human health. The role of microorganisms in the human microbiota and on health has increased in importance in the last years and more approaches are used to elucidate their properties. Genomics and the bioinformatic analysis of available bacterial data have been used in the main genera of probiotics *Lactobacillus* e *Bifidobacterium. Bifidobacterium* breve is considered a safe species, dominant in newborns and it is used in probiotic products. *B. breve* to treat necrotizing enterocolitis (NEC), gastrointestinal disorders, celiac disease, pediatric obesity, and allergies in infants with positive results. In this context, the present study aims to perform the *in silico* characterization of the genome of *Bifidobacterium breve* 110^{1A} strain through a comparative genomic analysis and the identification of genes related to probiotic features. For this purpose, it was employed additional 45 available complete genomes of *Bifidobacterium breve* to (i) analyze the taxonomic and phylogenomic aspects of this genus, (ii) identify mobile elements in the 110^{1A} genome such as prophages, plasmids, IS, (iii) genomic islands (GEI), antibiotic resistance genes and (iv) analyze the pangenome. Between the results, the strain 110^{1A} was identified as *Bifidobacterium breve* and the phylogenetically closest strain was *B. breve* NRBB26. An incomplete prophage was predicted in *B. breve* 110^{1A} genome, without plasmids. Moreover, seven genomic islands (GEI) were identified: two Resistance Islands (RI) and five Genomic Islands (GI). Resistance genes present in the genome were *rpoB, iles* and *ermX*. The pangenome size was calculated in 5943 genes and the core genome in 1174 genes and it was considered an open pangenome according to previous studies. There were 63 unique genes related to the metabolism of carbohydrates, such as galactosidase and DNA binding. Also, some genes related to adherence, resistance to stress, repair and protection of DNA and proteins, production of vitamins was identified. These results reveal the probiotic potential of this bacterial strain and direct further studies *in vitro* and *in vivo* to confirm its properties.

Keywords: Pangenomic analyses, comparative genomics, probiotic, *Bifidobacterium breve*.

RESUMO

Os probióticos são microrganismos com capacidade de influenciar na composição da microbiota intestinal, devido aos seus efeitos benéficos aos hospedeiros que nos levam a explorar mecanismos de ação através de estudos *in silico* e experimentais. O papel dos microrganismos na microbiota humana e na saúde têm aumentado em importância nos últimos anos e mais abordagens são utilizadas para elucidar suas propriedades. A genômica por meio da análise bioinformática de dados bacterianos disponíveis tem sido usada nos principais gêneros de probióticos *Lactobacillus* e *Bifidobacterium. Bifidobacterium breve* é considerada uma espécie secura, dominante em recém-nascidos e é usado em produtos probióticos. *B. breve* tem sido usado no tratamento de enterocolites necrosante (NEC), desordens gastrointestinais, doenças celíacas, obesidade pediátrica e alergias em infantes com efeitos positivos. Nesse contexto, o objetivo do presente estudo é a caracterizar *in silico* do genoma de *Bifidobacterium breve* 110^{1A} por meio de análises comparativas, visando a busca de genes relacionados ao potencial probiótico. Para isso, foram empregados adicionalmente 45 genomas completos disponíveis de *B. breve* para (i) analisar aspectos taxonômicos e filogenômicos de este género, (ii) identificação de elementos móveis no genoma 110^{1A} como profagos, plasmídeos, IS, (iii) ilhas genômicas e (iv) análise de pangenoma. Os resultados da filogenômica demonstram que a nossa linhagem em estudo, *B. breve* 110^{1A}, foi clusterizada mais próxima de *B. breve* NRBB26. *B. breve* 110^{1A} apresentou um profago incompleto e nenhum plasmídeo. Além disso, sete ilhas genômicas (GEI) foram identificadas, sendo duas ilhas de resistência (RI) e cinco ilhas genômicas (GI). O pangenoma possui 5943 genes e o genoma central 1174 genes, sendo considerado como um pangenoma aberto, de acordo com estudos anteriores. Foram identificados 63 genes únicos, alguns deles relacionados ao metabolismo de carboidratos, como galactosidase, e ligação ao DNA. Também foram identificados alguns genes relacionados a aderência, resistência ao estresse, reparo e proteção de DNA e proteínas, produção de vitaminas. Estes resultados revelam o potencial probiótico desta linhagem bacteriana e direcionam á estudos *in vitro* e *in vivo* para confirmar suas propriedades.

Palavras-chave: Análises pangenômicas, genômica comparativa, probiótico, *Bifidobacterium breve*.

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PREFACE

The present dissertation was divided into six parts: (I) Introduction composed by a Book Chapter and information about *Bifidobacterium breve*, (II) Justification, (III) Objectives, (IV) Paper manuscript, (V) Conclusion and (VI) Perspectives. The first part is composed of **"Chapter 18: COMPARATIVE GENOMICS IN PROBIOTIC BACTERIA",** which is part of the **Book: "LACTIC ACID BACTERIA IN FOOD BIOTECHNOLOGY: INNOVATIONS AND FUNCTIONAL ASPECTS"**. This chapter focuses on summarizing the main concepts of probiotics, the role of Comparative Genomics and Pan-genomics for exploring new probiotic strains, developing the basic concepts about comparative genomics, and specific genes linked with probiotic features using these genomic approaches. In addition, information about the species of study and previous information about the strain *Bifidobacterium breve* 110^{1A}.

The second and the third part are referred to the development of the justification and the objectives for the present dissertation, respectively.

The fourth part develops the central study of this dissertation entitled **"COMPARATIVE GENOMICS AND *in silico* EVALUATION OF GENES related to PROBIOTIC POTENTIAL of Bifidobacterium *breve* 110^{1A}",** which present the characterization of this strain using bioinformatics to explore its general aspects at the genome level (taxonomy, phylogenomics and prediction of genomic elements), and the identification of specific genes related to its probiotic features.

Finally, the fifth and sixth part of the manuscript presents the conclusions and perspectives of this work.

1. INTRODUCTION

1.1 Chapter 18: Comparative Genomics in Probiotic Bacteria (in press)

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Abstract

With the advent of next-generation sequencing platforms, the number of bacterial genome projects has grown exponentially. In view of this significant number, it is possible to explore comparative genomics and pan-genomics of these bacteria in order to achieve a better knowledge of their probiotic features. In this scenario, probiotic bacteria are medically important organisms due to their beneficial effects in the gut and their association with better prognostic in diverse diseases. Here, we explore how comparative genomics may be used to explore the main features associated with probiosis, such as: the resistance to acidic stress and bile salts, exclusion mechanisms, and elicitation of an immune response. Also, pan-genomics analyses of probiotic bacteria compared to non-probiotic strains of the same species or related genus are discussed. Finally, we present a set of the bacterial organisms that are probiotic, such as *Bifidobacterium animalis*, *Lactobacillus acidophilus*, *Lactococcus lactis* and others, and discuss their genome analyses based on literature data.

Keywords: *Bifidobacterium*, gut microbiota, *Lactobacillus*, pan-genomics, probiotics.

1. Introduction: Probiotic Bacteria

Probiotics are known as live microorganisms, which, when administered in adequate amounts, confer a health benefit upon the host (FAO, 2001). Since 1907, Élie Metchnikoff proposed the relationship between the presence of centenarians in Bulgaria and the consumption of soured milk containing beneficial microorganisms (*Lactobacillus bulgaricus*) capable of influencing on the gut microbiota; therefore, Tissier suggested the administration of *Bifidobacterium* isolates to treat persistence diarrhea in children. Continuing with Shirota with the first probiotic product (Yakult) until the present, probiotics have been an object of study in several *in vitro* and *in vivo* evaluations that have shown varied beneficial effects on the host (human, animal, plant) and have acquired relevance as health-promoters (Siezen and Wilson, 2010).

Traditionally, probiotics have been isolated from fermented and dairy products; however, due to several of these bacteria are part of the intestinal microbiota, they were

also isolated from feces samples (Cunha et al., 2013; Karami et al., 2017; Mulaw et al., 2019). Other sources of isolation were from breast milk, human vagina, fruits, plants, environment, among other sources (Aslim and Kilic, 2006; Edalati et al., 2019; Islam et al., 2011; Jiang et al., 2016; Sornplang and Piyadeatsoontorn, 2016; Vitali et al., 2012).

The most known probiotic genera are former-*Lactobacillus* and *Bifidobacterium,* supported by a large number of studies; however, there are some strains from the genera *Lactococcus, Bacillus, Clostridium, Escherichia, Enterococcus, Propionibacterium, Pediococcus, Leuconostoc* and *Saccharomyces* that are also probiotic (Foligné et al., 2010; Holzapfel et al., 2001). Some examples of known species considered as probiotic and used in products are *Lactococcus lactis, Bifidobacterium longum, B. breve, B. animalis, B. bifidum, Lactobacillus plantarum, L. acidophilus, l. delbrueckii* and *L. casei.*

Probiotics have demonstrated positive effects in the alleviation of symptoms to lactose intolerance (Oak and Jha, 2019), anti-allergic (H. Wang et al., 2019; Yang et al., 2013) and immunoregulation (Kober and Bowe, 2015; Yan and Polk, 2011). Moreover, probiotics have exhibited other effects such as anti-obesity (Cerdó et al., 2019; Z. Wang et al., 2019), on cancer prevention (Górska et al., 2019), against inflammatory bowel disease (IBD) (Alard et al., 2018) and other disorders or conditions.

Due to the population increasing interest on healthy food and in the improvement of life quality, in the last decade, the demand of probiotic-based products has increased (Colombo et al., 2018). The world market around probiotics has predicted an increasing tendency from 3.3 to 7 US$ billion in supplements from 2015 to 2025 (Terpou et al., 2019). The food industry has developed a wide variety of products with probiotics, such as dairy-based foods (drinkable fresh milk, fermented milk, yogurt, cheese, ice cream) and non-diary-based foods (fermented products of vegetable, fruit, cereal, meat), among others (Song et al., 2017). The increasing demand leads to innovative products based on new probiotic bacteria that could present other beneficial properties for the consumers or for a new consumer group (Betz et al., 2015; Kolady et al., 2019).

2. Mechanisms of Probiotic Action

Some known mechanisms attributed to probiotic bacteria play a vital role in the survival of probiotic bacteria during the journey through the intestinal tract and the exertion of their effects as beneficial bacteria. The main mechanisms are related to acid

and bile salts resistance (Andriantsoanirina et al., 2013; Ruiz et al., 2013), adhesion to the host intestinal cells (Monteagudo-Mera et al., 2019), improvement of the intestinal epithelial barrier (Ohland and MacNaughton, 2010) and competition with pathogens (Chenoll et al., 2011; Muñoz et al., 2011). Moreover, there are other mechanisms involved in producing antimicrobial compounds (Alakomi et al., 2000; Makras et al., 2006) and immunomodulation of inflammatory processes (Cerdó et al., 2019; Plaza-Diaz et al., 2019; Sarkar and Mandal, 2016).

2.1. Acid and bile salts resistance

The bacterial route through the digestive tract implies exposure to stress conditions (Ruiz et al., 2013), where other bacteria would have been affected and with a low survival. Acidity and bile salts are two of the most harmful conditions in that environment, due to the fact that acidity could reduce the viability of bacteria affecting mainly the functionality of DNA, proteins and other cellular components that would reduce the desirable effects on the host when bacteria reach the intestinal area (Sahadeva et al., 2011). As a strategy to tolerate this stress condition, probiotic bacteria have a system of extrusion of protons that uses F0F1-ATPase to carry ions from the internal to the external environment, increasing the intracellular pH (Sánchez et al., 2007). In the same form, bile salts that participate in the solubilization and absorption of fatty food of the host are also responsible for the perturbations in the cellular membrane and in the proton motive force of bacteria (Kurdi et al., 2006). In this case, there are some strategies to tolerate these conditions, such as detoxification by bile efflux systems (Pfeiler and Klaenhammer, 2009), hydrolysis using bile-salt hydrolases (BSHs) (Kumar et al., 2006) and by the production of exopolysaccharides (EPS) (Alp and Aslim, 2010). The degree of resistance to these conditions differs depending on the strain (Champagne et al., 2005) and the evaluation of these aspects is considered necessary in selecting probiotic candidates (Andriantsoanirina et al., 2013).

2.2. Adhesion to the host epithelium cells

The adherence of bacteria represent a relevant criterion in the selection of probiotics that is key for the colonization process in the intestinal environment (Monteagudo-Mera et al., 2019) and the permanence could allow bacteria to exert their effects on the host for an extended time during their transit through that environment (Sarkar and Mandal, 2016). There are evidences that bacteria have some strategies such as specialized structures (pili, fimbria), presence of lipoproteins and production of EPS

(Gleinser et al., 2012; Guglielmetti et al., 2008; Kavanaugh et al., 2013) that mediate the bacterial adherence. Moreover, with the adherence to intestinal cells, probiotic bacteria can occupy a space, in other words, cellular binding sites, compete against pathogenic bacteria, and with this action, inhibit the colonization of these harmful bacteria in the host intestine (Gueimonde et al., 2007).

2.3. Improvement of the intestinal epithelial barrier

Probiotic bacteria, when colonizing the intestinal niche, can act as a protective barrier against pathogens avoiding their access and the delivery of their metabolites to the bloodstream. One of the protection mechanisms of some probiotics bacteria is the modulating or regulation of the proteins involved on the tight junction complex that was shown in some studies with *Lactiplantibacillus plantarum* MB452 (Anderson et al., 2010) and *Bifidobacterium infantis* (S. Guo et al., 2017). In addition to this, probiotics can stimulate the secretion of mucus that reinforces the barrier and maintain the separation between the intestinal lumen and epithelial cells, as evidenced in former- *Lactobacillus* species that promote the expression of mucin by intestinal cells (Bermudez-Brito et al., 2012).

2.4. Competition with pathogens and antimicrobial compounds

Probiotics have exhibited some strategies for competition with bacterial pathogens, for instance: inhibition of adhesion of other bacteria such as *B. breve* CNCM I-4035 against enteropathogenic bacteria (Gueimonde et al., 2007) and other pathogens (Besser et al., 2019; Collado et al., 2006) known as competitive exclusion, when bacteria compete for nutrients or mucosal adhesion sites.preventing the posterior colonization of other bacteria (Bermudez-Brito et al., 2012). Another form of inhibition is by the alteration of the pH in the environment to acid conditions, which was shown in lactobacilli, where the production of lactic acid reduces the presence of other bacteria (Yang et al., 2018). Therefore, some compounds produced by some strains, such as bacteriocins, can exert antimicrobial effects on a wide spectrum or on specific target bacteria. . There are three classes of bacteriocins in Gram-positive bacteria: Class I are heat-stable peptides (<5 kDa) with post-translational modifications, for instance, lantibiotics; Class II are peptides (<10kDa) without modifications; Class III are proteins (>30 kDa) with heat-labile conditions (Cheikhyoussef et al., 2010; Cotter et al., 2005; Martinez et al., et al., 2013a; Negash and Tsehai, 2020). Bacteriocin production in

lactobacilli and bifidobacteria was evident against pathogens such as *Bacillus, Salmonella* and *Clostridium* (Bermudez-Brito et al., 2012; Martinez et al., 2013).

2.5. Immunomodulation

Another desirable property attributed to some probiotics is the capacity to modulate immune responses related to the release of cytokines, among others, like interleukins, tumour necrosis factors and interferons (Foligné et al., 2010; Savan and Sakai, 2006). There were reported interactions between probiotics with enterocytes and dendritic cells in the intestine with posterior anti-inflammatory effects and probiotics promoting B cells' stimulation to produce IgA (Azad et al., 2018). Some examples of this modulation were an improvement in the immunity after the supply of *B. bifidum* R0071, *B. infantis* R0033, *Lactobacillus helveticus* R0052 in infants that caused changes in salivary immunoglobulin A (SIgA) levels (Xiao et al., 2017). Moreover, the alleviation of allergy symptoms to asthma has been evidenced when *B. breve* BR03 and *Ligilactobacillus salivarius* LS01 were administered due to the release of IL-10, an inhibitor of pro-inflammatory cytokine, and a balance of the levels of T-helper cells, Th1/Th2 (Drago et al., 2015).

3. Genomics in Probiosis

With advancements in DNA sequencing that have led to significant accessibility to next-generation sequencing (NGS) technologies and the growing amount of genetic information in open access databases, it is currently possible to use complete genomes of a wide variety of organisms, being microbiology one of the more benefited areas (Forde and O'Toole, 2013; Fraser et al., 2000). For instance, the Genome Online Database (GOLD) information, where bacteria represents the majority of the available genomes, 88% (Mukherjee et al., 2021), a fact that has allowed the genomic exploration of microorganisms in the last years (Gupta, 2016).

Initially, a major part of the genomic research was focused on bacteria with pathogenic implications and their relation with virulence features. However, there is a growing tendency for the use of genomics in beneficial bacteria such as probiotics (Guinane et al., 2016). This approach received the name of 'Probiogenomics' and is focused on elucidating the molecular mechanisms involved in their health-promoting properties (Ventura et al., 2012). The exploration of the genomes and the combination with *in vitro* and *in vivo* evaluations allow the unravel of functionality of these microbial

genes and could suggest possible applications for the food industry or with a biotechnological potential (Felis et al., 2017). There is a function identified for several genes and the process of characterization is facilitated by *in silico* evaluations that allow the searching for new candidates for probiotics. For instance, the exploration of the probiotic potential of *Bacillus coagulans* HS243 based on genome mining, allowed the identification of genes codifying for proteins involved in adhesion, colonization, production of bacteriocins and vitamins (Kapse et al., 2019). Besides, an *in silico* genome analysis of *Ba. velezensis* FTCo1, showed genes related to acid tolerance and lipoproteins involved in host colonization (Pereira et al., 2019). Finally, a genome-based evaluation of *Limosilactobacillus reuteri* PNW1 showed the identification of relevant elements of the assessment of safety genes (antibiotic resistance and virulence) and genes of interest for the production of lactic acid, D-lactate dehydrogenase and L-lactate dehydrogenase (Alayande et al., 2020).

Thus, genomic characterization and functional analyses are approaches that can facilitate the study and the understanding of the probiotic mechanisms of bacteria (Salminen et al., 2005; Turroni et al., 2011), such as acid tolerance, adhesion, improvement of the intestinal epithelial barrier, competition with pathogens, among others mentioned above (Chen et al., 2019). This process also permits the prospection of safety aspects, crucial criteria, antibiotic resistance genes, virulence factors, and genome stability (Guinane et al., 2016). In this context, the characterization of some mobile genetic elements such as phages and plasmids that harbour genes that could confer additional abilities (Abriouel et al., 2019) as well as undesirable features such as resistance to antibiotics and virulence factors (Bennedsen et al., 2011) are part of the process of exploration and should be investigated. It is noteworthy; the safety assessment is a relevant aspect when a candidate is proposed to be a probiotic for commercial use; some experimental tests have been done to ensure safety (Guinane et al., 2016; Sanders et al., 2010). In this respect, some restrictions are referred to probiotics strains when there is a possible transference of antibiotic resistance and virulence factors (EFSA, 2008). About this issue, the *in silico* evaluation using whole-genome information could be the standard for the assessment process of potential probiotics at this criterion (Salvetti et al., 2016).

Genomic data is also used for analysis that is considered the first step in candidates' characterization process for probiotics. The taxonomic identification and

phylogenetic analysis provide a better resolution and represent a valuable support for species identification, especially in cases of taxonomic conflicts obtained using classical molecular markers such as 16S rRNA (Suresh et al., 2019) that only permit a taxonomic application (Cao et al., 2017). Furthermore, genomic analysis enables the identification of more elements, for instance: genes responsible for the production of bacteriocins important in the bacterial competence (Goel et al., 2020), vitamins (Li et al., 2016), metabolites of interest and the searching of CRISPR-Cas systems that act as protection systems against phages and other DNA invaders such as plasmids (Barrangou et al., 2007). The latter, an important issue in starter cultures at a large scale that could be affected by viral contamination with the possibility to reduce its bacterial population (Hidalgo-Cantabrana et al., 2017). Furthermore, genomic islands influence on the diversification and adaptation of microorganisms. Also, its identification is important to infer the potential genomic plasticity of strains because they could acquire features related to antibiotic resistance, pathogenicity, or adaptive advantages to other environments (Juhas et al., 2009). Until the present, many probiotic properties were evidenced on several bacteria; however, it was not possible to identify all of these features in a single strain. Thus, some probiotic strains exhibit positive effects in the alleviation of allergy, other strains exert inhibition against pathogens and other different strains showed an immunomodulatory role (Champagne et al., 2005).

4. Pangenomics in Probiosis

Comparative genomics has advantages compared with the analysis of only a single genome, such as insights about evolutionary history and physiology. It also permits a better understanding of the possible functions of predicted genes (Fraser et al., 2000), and explores bacteria properties with different lifestyles: probiotics, symbiotic and pathogenic (Salminen et al., 2005).

Pangenome is a concept that was born with the analysis of multiple genomes of a determined pathogenic species, *Streptococcus agalactiae* (Tettelin et al., 2005) to refer to the totality of gene information of this species. Following the pangenomics approaches, it allows the comparison of the genetic repertoire of several genomes of the same species for the determination of the section composed by commonly shared genes for all analysed genomes, which confers vital properties to bacteria (core genome). Moreover, the analysis

also permits the section determination composed of genes present in some bacteria but not in all strains (dispensable genome). In this last section, identification of exclusive genes, present only in a specific strain, may help discover unknown properties for it, representing a point of interest for its potential use in the food industry (Garrigues et al., 2013; Speranza et al., 2017). While some genes involved in fundamental cellular processes usually form the core genome, some other genes with non-essential functions for the bacteria compose the dispensable genome. The dispensable genome is often related to the ability to adapt to new niches, colonization of new hosts, biochemical pathways, and metabolism of other substrates, which is considered an advantage (Tettelin et al., 2008; Vernikos, 2020). For this reason, core and dispensable genomes are known as the essence and diversity of the species, respectively (Medini et al., 2005).

The determination of the openness of a species' pangenome could represent that all genes are known when it is closed or that these species possess an incredible repertoire of genes even for discovery when it is opened. The pangenome openness is related to the species ability to acquire foreign DNA (Medini et al., 2005) and to its lifestyle (Georgiades and Raoult, 2011). The status of opened pangenome might be of special interest in species related to pathogenicity or biotechnological potential in the industry.

Although the pangenome has been applied for analyses of pathogenic bacteria (Rouli et al., 2015), in the last years, an approach named *Pan-probiosis* has been mentioned; it refers to the comparative genomic perspective to analyse bacteria and some genes related with the probiotic nature, such as adhesion, colonization, competence against pathogenic bacteria (Barh et al., 2020). According to some authors, comparing genomes and pangenomes could be considered as an opportunity for connecting genotypes and phenotypes, especially for complex or non-understood phenotypes; furthermore, we could have a large chance to select strains for starter cultures with desired characteristics (Garrigues et al., 2013).

Several studies have applied comparative genomics with bacteria to improve the understanding of the intra-specific diversity of the group of interest and its characterization to define its nature and potential. For instance, a study of comparative genome analyses of *Enterococcus faecium* was performed to determine differences between three categories: (1) pathogenic, (2) probiotic and (3) non-pathogenic non-probiotic strains, where the pangenome and its genome subsections were determined and helped to differentiate between these strains with distinct nature (Ghattargi et al., 2018).

Another pangenomics study with *Lm. reuteri* strains in porcine identified differences in the presence of gene clusters between strains with higher and lower anti-pathogenic effect (J. Y. Lee et al., 2017); another study focused on strains of *Bifidobacterium* and found differences in the presence of genes isolated from monkeys when compared with human origin (Brown et al., 2019). Comparative genomics has been applied in a wide variety of bacterial species. Between them, *Lb. johnsonii* ZLJ010 was evaluated to explore its potential probiotic ability (Zhang et al., 2019) and, in the same way, it was performed with *Lactococcus lactis* subsp. *lactis* NCDO 2118 to identify stress resistance genes accompanied by experimental trials to determine the probiotic potential (Oliveira et al., 2017). Moreover, the pangenome evaluation in *Lb. helveticus* strains combined with experimental assays about the tolerance to bile salts, low pH, and antibiotic resistance was performed (Fontana et al., 2019), and also a determination of the anti-pathogenic effect and host specificity in *Lm. reuteri* strains as a candidate to porcine probiotic (J. Y. Lee et al., 2017). In most of these studies, pangenomics analysis forms part of the characterization process of these bacteria along with identification of specific genes with function of interest.

5. Some Studies in Probiotic Bacteria

Bifidobacterium is a genus composed of Gram-positive species, non-spore-forming and that are commonly found in the GITs of animals or human (Leahy et al., 2005). Firstly, Tísser called the first strain found in feces of a breast-fed baby as *Bacillus bifidus* in 1900 (Rašić and Kurmann, 1983), and after, about 1924, a microbiologist called Orla-Jensen, classified it inside *Bifidobacterium* genus (Prasanna et al., 2014). In the online LPSN database (https://www.bacterio.net/genus/bifidobacterium), six phylogenetic groups are described, as *B. adolescentis, B. longum. B. pullorum, B. asteroides, B. pseudolongum e B. boum* (Felis and Dellaglio, 2007). Some studies reveal that these bifidobacteria are often associated with health-promoting effects as protection and immunomodulation of the intestinal microbiota, which makes them considered as probiotic bacteria (Jiang et al., 1996; Kim et al., 2007; McCarthy et al., 2003; Qiao et al., 2002).

In this section, we describe the use of genomics and pan-genomics studies of some probiotic bacteria of interest, such as: *B. animalis, B. bifidum, B. longum, Lb. acidophilus, Lc. lactis* and *Lp. plantarum.* Finally, we focus on the study of *Lb. delbrueckii*, with

information regarding the bile and acidic stresses, adhesion properties, immune response elicitation, presence of antibiotic and virulence genes, as well as virulence factors.

5.1. Bifidobacterium animalis

The *B. animalis* is a Gram-positive anaerobic bacterium, lactic acid-producing, found in the gastrointestinal tract (GIT) of various animals, including humans (Jungersen et al., 2014). This species is divided into two subspecies, *B. animalis* subsp. *animalis* (*B. animalis*) and *B. animalis* subsp. *lactis* (*B. lactis*), being only the last used to health-promoting purposes (Masco et al., 2004; Meile et al., 1997). Because it is considered a probiotic strain, *B. lactis* is commercially exploited as an active ingredient in a variety of functional foods (Jungersen et al., 2014; Duranti et al., 2020). There are several strains of *B. lactis* that are used in dairy products and have been studied for their probiotic role. Among the *B. lactis* probiotic benefits appointed by many studies are: the ability of inhibiting important gastrointestinal pathogens through production of antimicrobial substances and through competition for mucosal adhesion (Collado et al., 2007; Jungersen et al., 2014). Furthermore, the suppress body weight gain in biochemical and morphometric parameters when associated with other probiotic bacteria (Bouaziz et al., 2021; da Silva et al, 2020; Michael et al., 2021) and an antagonistic effect toward periodontopathogens, being useful as coadjutants in periodontal therapy (Valdez et al., 2021). One strain well studied is *B. lactis* Bb-12, which in clinical studies has demonstrated beneficial effects in the case of infectious diarrhea caused by viruses or bacteria and could decrease the frequency or shorten the duration of the infection (Duranti et al., 2020). Moreover, *B. lactis* Bb-12 and *B. lactis* CNCM I-2494 strains show high acid tolerance (pH 2), surviving under stomach conditions and, are able to grow in the bile-containing medium (Vernazza et al.,2006; Adouard et al., 2019). The presence of another strain *B. lactis* 420 (B420) can help to control body fat mass gain in a human intervention trial as well as glycemic control and involvement in enhancement of mucosal integrity, contributing to host resistance to pathogens (Amar et al., 2011; Putaala et al., 2008; Stenman et al., 2014; Stenman et al., 2016). The *B. lactis* CNCM I-2494 strain appears to efficiently prevent GIT disorders by restoring intestinal permeability, colonic goblet cell populations, and cytokine levels (Martín et al., 2016). The *B. lactis* V9 possesses favorable probiotic properties (Sun et al., 2010) and alleviates liver damage mediated by a reduction in hepatic fat accumulation and anti-inflammatory activity (Yan

et al., 2020). In addition, this strain modulates sex hormone levels in individuals with polycystic ovary syndrome through the gut-brain axis (J. Zhang et al., 2019).

Many *B. lactis* strains genomes were submitted to pangenome and comparative genomics analyses. An extensive comparative analysis was made by Milani et al. (2013) and found a high similarity among genome sequences of *B. lactis* commercial strains and human fecal isolates. This conservation degree is also repeated when the size and organization of *B. lactis* genomes were compared. The polymorphisms lack can appoint that these strains have an isogenic nature, presenting a closed pan-genome structure. This structure could be a consequence of the worldwide distribution of this taxon as a health-promoting bacterium and to its limited ability to colonize and persist within the human host. This might reduce the possibility of horizontal gene transfer (HGT) acquired by members of the *B. lactis* taxon (Milani et al., 2013). Despite that, there are phenotypic and genotypic differences among strains according to their origins. Some isolates from wild animals, for example, showed fingerprinting profiles identical to the commercial isolates, but, on the other side, some strains isolates from feces of a German Shepherd dog, mouflon and ovine cheese differed in their sequences of housekeeping genes, probably due to the presence of chromosomal regions required in the original ecological niche of the specific *B. lactis* strains (Bunesova et al., 2017).

In a more comprehensive study with many *B. animalis* subspecies (*B. animalis* and *B. lactis)* from the GITs of different animals, the phylogenetic analysis based on core genome sequences showed a clear differentiation between these two subspecies branch subject to genetic adaptations to environments that had a distinct glycan content (Lugli et al., 2019). More specifically, *B. lactis* taxon only hydrolyzes and metabolize a limited number of carbohydrates, either due to a high level of genetic adaptation to an ecological niche or to massive genome decay as a result of its industrial exploitation by long-term cultivation on synthetic media (Milani et al., 2016). Moreover, a gene acquisition/loss analysis based on glycosyl hydrolase (GH) genes revealed that *B. animalis* species seems to have acquired several GH enzymes through HGT, whereas *B. lactis* species appears to have suffered a loss of GH-encoding genes, confirming its less carbohydrate-metabolic ability and the evolutionary differentiation between these two subspecies in phylogenetic and genomic analyses (Lugli et al., 2019).

The phylogenomic analysis inferred a reliable evolutionary positioning of *B. lactis* within the genus *Bifidobacterium*, by placing all strains of *B. lactis* on the same cluster of *B. animalis* and all investigated *B. lactis* strains were placed on the same branch of the tree, indicating the absence of substantial amino acid sequence differences between the individual core proteins of these strains (Milani et al., 2013). Subspecies *B. lactis* have a highly synthetic genomic structure (Milani et al., 2013) that has been validated by absence of truly unique genes (TUGs) in most *B. lactis* strains, supporting the idea that they are highly isogenic in nature (Lugli et al., 2019). The genomes of the strains ATCC 27674 and CNCM I-2494 exhibited an extremely high genetic identity (99.9%) compared to that of the BB-12 strain, probably exhibiting similar probiotic characteristics (Lugli et al., 2019).

Since *B. lactis* is widely used in food and dietary supplements and colonizes well in GITs, the presence of antibiotic resistance genes in this bacterium is especially important, because they could, in hypothesis, be resistance reservoirs for intestinal pathogens *via* HGTs (Gueimonde et al., 2010; Salyers et al., 2004). *B. lactis* has the *tet*(W) gene, which confers a tetracycline resistance among its strains (Gueimonde et al., 2010) and other *Bifidobacterium* and could represent a relatively broad species-specific genetic signature in its pan-genome (Duranti et al., 2017). There is no evidence that *tet*(W) gene co-transcribed with the transposase and it transfers tetracycline resistance at date. This gene itself does not confer all resistance in *B. lactis* Bl-04 and other mutations in the genome likely contribute to the exceeding of the breakpoint, making the added level of resistance not at risk of transfer (Morovic et al., 2017). Other putative mobile antibiotic resistant gene that encodes a predicted aminoglycoside protein with an APH domain for kanamycin resistance is present in genomes of *B. lactis* (Duranti et al., 2017).

5.2. Bifidobacterium bifidum

The *B. bifidum* is a very common probiotic bacterium present in infants and adults human gut (Duranti et al., 2019). It is one of the most frequently shared bifidobacterial species between mother and infant *via* a vertical transmission route, which was displayed to persist in the human gut for up to one year of life (Milani et al., 2015). The factors for its importance include immune system modulation activity and protection against novel pathogens, including the influenza virus (Alessandri et al., 2019; Mahooti et al., 2019; Serafini et al., 2013; Turroni et al., 2013) as well as its adhesive ability (i. e. in human

epithelial intestinal cell lines, such as Caco-2 and HT29) through host-derived glycans, in particular human milk oligosaccharides (HMOs) (Serafini et al., 2013; Turroni et al., 2010, 2015) and by extracellular sialidase production, which enhances its adhesion to the mucosal surface and supports carbohydrate-assimilation (Nishiyama et al., 2017). Many extracellular proteins produced by members of the *B. bifidum* strains, such as sortase-dependent pili and the murein lytic enzyme TgaA are involved in host–microbe interactions (Guglielmetti et al., 2014). The bifidobacterial sortase-dependent pili, more than mediating adhesion/interaction with the host, also have a role on its immune system modulation (Turroni et al., 2013). The sortase-dependent pili produced by *B. bifidum* PRL2010 strain can activate macrophage signaling without triggering a detrimental inflammatory cascade in host immune cells (Alessandri et al., 2019). This is befitting with the fact that this *B. bifidum* is a neonatal and infant colonizer of the human gut and could be an essential precursor of the immune system, alerting of the immune system and enhancing the host reaction to inflammation, for a typical condition of colitis (Alessandri et al., 2019; Serafini et al., 2013; Turroni et al., 2013).

Currently, thirteen *B. bifidum* strains have whole genome available in the National Center for Biotechnology Information (NCBI) database. Some of these strains have already been used to infer phylogenomic comparisons and in species-specific pangenome. The phylogenomic evolutionary position of *B. bifidum* strains within the genus *Bifidobacterium* appears on a single branch of the same cluster, with four main sub-branches or clades A, B, C and D within the *B. bifidum* phylogenetic group (Duranti et al., 2015). The comparative genomic analyses done using only *B. bifidum* strains appointed a genomic structure highly syntenic and a closed pan-genome among some strains, showing a rather limited genetic variability within the genomes analyzed (Duranti et al., 2015). The *B. bifidum* particularities like the synthesis of different types of pili and metabolic abilities pertaining to host-derived glycans (Guglielmetti et al., 2014; Milani et al., 2017; Turroni et al., 2019) appear to be a commensal genetic strategy to maintain and stay in the human gut. When the genetic backgrounds for carbohydrate uptake in *B. bifidum* were analyzed, the taxon showed a relatively small number of genes coding for carbohydrate carriers compared with other bifidobacterial taxa that are present in the infant's intestine too (Turroni et al., 2019). However, *B. bifidum* pan-genome contrasts with other *Bifidobacterium* species (Duranti et al., 2016; Vernazza et al., 2006) because enrichment of these genes are metabolism-related, as mucin and HMOs, and support the

hypothesis of nutrient-acquisition strategy because both sugars are found in the gut in high amounts during different stages of human life-infant period (HMOs) and the adult life (mucin) (Turroni et al., 2019). Looking more widely to *Bifidobacteriaceae* glycobiome (Lugli et al., 2017), the genes encoding glycosyl hydrolases (GHs) appear to have been acquired early in the evolution of bifidobacteria, followed by a simplification that has resulted in specialization toward ecological niches in which current bifidobacterial species belongs. Members of the *B. bifidum* group, interestingly, acquired the highest number of GH-encoding genes when compared to other groups, probably in order to expand their metabolic ability towards different carbon sources present in the host (Lugli et al., 2017).

In the context of antibiotic resistance, a variable susceptibility to streptomycin among the *B. bifidum* strains was observed. They also exhibited β-lactamase encoding genes near truncated transposases. But none of these transposase-encoding genes were classified as a conjugal transposon, which could decrease the possibility of this gene mobilization by HGT (Duranti et al., 2017). However, some chromosomal regions are presumed to have been acquired by HGTs and a considerable presence of mobile elements is found in the genomes of *B. bifidum*, mainly in the LMG13195 strain genome. Another mobile element typically detected was prophage sequences and integrated plasmids. The genome of *B. bifidum* 85B contains a predicted episome, which encompasses genetic elements that are typically identified in (conjugative) plasmids and some strains genomes (S17, LMG13200 IPLA20015 and IPLA20017) are predicted to possess one clustered regularly interspaced short palindromic repeats, CRISPR (Duranti et al., 2015).

5.3. Bifidobacterium longum

Among the groups described, *B. longum* is the main bifidobacteria found. This species was found in children as young as 4- months old, which was called as *B. longum* subsp. *infantis*, and it also was found in adults, being called as *B. longum* subsp. *longum* (Avershina et al., 2013; Sun, Zhang, et al., 2015). Through genomic studies, a more gene-specific comparative methodology, the Multi-Locus Sequence Typing (MLST), was used to type and pre-screen strains of this species (LoCascio et al., 2010). This study revealed that species of *B. longum* subsp. *infantis*, with the main representative strain ATCC 15697, were associated with using human milk oligosaccharides (HMOs), and the species of *B. longum* subsp. *longum*, with the main representative strains (DJO10A and

NCC2705) were associated with the use of plant-based carbon sources. The metabolic energy of subsp. *infantis* suggests that it adapted to milk components to overcome competition in colonizing microbiota and that subsp. *longum* maintained its metabolic structure in metabolizing these carbons (LoCascio et al., 2010; Sela et al., 2008). Still about the representative ATCC 15697, some more recent studies identified through comparative and phylogenomic analysis, genes, and consequently proteins, that had probiotic relationships in the complete riboflavin biosynthesis pathway, i.e., vitamin B2, an important cofactor in redox reactions of cellular metabolism and in cell signalling processes in the bacterial quorum, between host and microbe, and between plants and bacteria (Dakora et al., 2015; LeBlanc et al., 2011; Rajamani et al., 2008; Solopova et al., 2020).

A study published in 2018 by Freitas AC and Hill JE (Freitas and Hill, 2018), with 17 species of *B. breve* and 26 species of *B. longum* (strains JCM 1217, JDM301, BBMN68, KACC 91563, DJO10A, NCC2705, 157F) used species isolated from both the vagina and the intestine. This study, talking specifically about *B. longum* from now on, confirmed what was indicated in the literature that the species of this genus had high GC content, which in this case showed about 60%. They also had a size ranging between 2.37 and 2.41 mb, and about 2137 genes on average. These species underwent average nucleotide identity (ANI) analysis, which all showed above 95%, confirming their identification as the same species (Richter and Rosselló-Móra, 2009). Through SNP alignment, a phylogeny analysis was performed, and a maximum likelihood tree was created in which the species presented an average of 9050 SNPs. The pangenome analysis of this species showed 5609 genes, with a core genome of 835 genes. The pangenome was considered open, that is, with each new sequencing, more genes will be added. According to this study, in all analyses, no clusters were formed that were related to strains isolated from the vagina or intestine, confirming the hypothesis of the work that despite some differences, they represented the same bacterial population with similar gene repertoire, and therefore were not possible to be distinguished by comparative genomics.

5.4. Lactobacillus acidophilus

Lactobacilli are bacteria that take the form of Gram-positive, microaerophilic, non-spore-forming bacilli and usually have a G+C content below 50% (Gangiredla et al.,

2018). These bacteria are normally found in the gastrointestinal microbiota of humans, and can sometimes also be found in other mammals (Canchaya et al., 2006; Vaughan et al., 2005). One of the most important species among the more than 50 belonging to the major group of lactic acid bacteria (LAB) is *Lb. acidophilus*. In the database from NCBI (National Center for Biotechnology Information), 46 genomes of this species are annotated, with an average length of 1.97 mb, about 1786 proteins and an average G+C content of 34.6%. This species is part of the human oral, gastrointestinal, and vaginal microbial flora, and is considered a very important species in maintaining the normal microbial community of the human gut, and which can also be used in the production of yogurt, cheese, and other fermented foods (Stahl and Barrangou, 2013; Sun et al., 2015).

Lb. acidophilus NCFM isolated in infant feces in 1900 showed some probiotic features that help its increased survival in the gastrointestinal tract of humans. Some of its genes encode lactacin B, an important bacteriocin, and others encode biosynthetic features with potentials to synthesize three amino acids *de novo*, such as cysteine, serine, and aspartate (Altermann et al., 2005).

Lb. acidophilus 30SC is a swine intestinal isolate with a high probiotic capacity due to a good ability to assimilate cholesterol and produce thermostable bacteriocins (Oh et al., 2011). It also showed higher acid resistance and higher bile tolerance when compared to other species used in dairy products, which gives it a higher survivability during passage through the stomach and intestine (Oh et al., 2000). When compared to other strains, as in the alignment of LA-14 and NCFM, strain 30SC proved to be different. While the other strains had about 34.7% G+C content, this one has 38.1%, which is suggested by some studies to be the presence of 1 or 2 plasmids (Stahl and Barrangou, 2013). After some phylogenetic studies with other genomes, this strain was reclassified as belonging to the *Lb. amylovorus* group (Bull et al., 2014).

There are many biochemical and phenotypic characterizations for this species that show a certain level of heterogeneity within it, however some genotypic analyses indicate that it is the smallest group with variations (Ramachandran et al., 2013; Stahl and Barrangou, 2013). Comparative and Multilocus Sequence Typing (MLST) analyses, with species such as FSI4, NCFM, La-14, demonstrated a high genomic similarity at the DNA level (Iartchouk et al., 2015), and when compared to the isolated NCFM strain, the authors realized that the difference that occurred was predominantly the result of single nucleotide

polymorphism (SNP) effects (Stefanovic et al., 2017). A pan-genome analysis shows a core genome with 1815 genes, which represents about 97.4% of *Lb. acidophilus* NCFM (Stefanovic et al., 2017). This study also identified that the strains studied had no plasmid DNA, and no presence of active phages, which may confirm the stability of *Lb. acidophilus*. However, pro-phage remnants called Potentially Autonomic Units (PAU), were identified in the NCFM strain (Altermann et al., 2005), and a new potential phage-related region was present in some strains and absent in others. PAU1 was present in all strains analysed while PAU2 and 3 were in commercial isolates with variable presence in the culture strains (Bull et al., 2014; Stefanovic et al., 2017).

5.5. Limosilactobacillus fermentum

Belonging to the group of LAB, this Gram-positive bacterium is currently the target of many studies as a tool in biotechnology and a potential probiotic. *Limosilactobacillus fermentum* is a “nomad” lifestyle bacterium and is not found regularly in the vertebrate intestine, but in different places. Because they develop in different environments, they create specificity in each strain, which, from the biotechnology point of view, leads to different possible uses, as a tool. There are already studies indicating its use as a biosurfactant for sterilizing surgical material, for example, which proves its many uses (Bing et al., 2002).

Within the species there are also some strains that are able to inhibit the growth of specific pathogens, producing bacteriocins and antifungal metabolites (Ghazvini et al., 2016; Varma et al., 2010). And, in addition, are able to survive bile salts and reduce cholesterol levels (Pereira et al., 2003; Pereira and Gibson, 2002) which is beneficial and compatible with human health. Among these strains that have probiotic potential, CECT5716 and IFO3956 isolated from human milk and fermented plant material, respectively, stand out, making them relevant as tools within biotechnology.

A study on the genetic diversity of the species (Brandt et al., 2020), revealed 9 phylogenetic clusters, using the RAxML tool to generate a tree from 38 strains of *Lm. fermentum*, based on the nucleotide alignment of phosphoglucomutase (CECT5716 and IFO3956 were in distinct but close clusters). In the same study, comparative genomics analyses were performed in an attempt to explore intraspecies differences, using BRIG

and Mauve software with only closed genomes and as reference the DSM 20052 strain, a fermented beet isolate.

In BRIG, only representative genomes from each phylogenetic clade were chosen for analysis, and three genomic islands (of approximately 180 kbp, 760 kbp and 1550 kbp in size) were identified that correlate with %GC declines and did not appear in any other genomes. Mauve was used to determine the synteny of genes between the genomes, which revealed several small syntenic blocks and many rearrangements, especially for the strains isolated from human vagina and oral cavity. The strain CECT5716, which has antimicrobial activity against Gram-positives such as staphylococci and *Listeria* and is also related to the production of B vitamins (Cárdenas et al., 2015) showed deletion regions in BRIG and inversions in Mauve (similar result occurred in the IFO3956 genome).

The pan-genome, resulting from an analysis of 11 (Including CECT5716 and IFO3956) complete genomes revealed a core genome of 400 genes, and appeared to be open, in other words, the addition of more strains, would result in a larger pan-genome (Illeghems et al., 2015), which correlates with the fact that the bacterium lives in several distinct environments, including locations in the human body. With the open pan-genome, and the ability to withstand various environments, it is clear that many new discoveries about *Lm. fermentum* and its strains may emerge.

5.6. Lactococcus lactis

Lactococcus lactis belongs to the group of bacteria capable to metabolize carbohydrates in lactic acid. Initially, the species was associated to plants, however it multiplies and shift to an active form as became supply for ruminants (Bolotin et al., 2001; Song et al., 2017). The species is non motile, spherical, Gram-positive, facultative anaerobic, homolactate, generally regarded as safe (GRAS status) and non-sporulating that can colonize the gut. The species can be isolated from dairy or non-dairy sources. The most studied strains are dairy related, although several strains have been isolated from different environments. There are six identified subspecies of *Lc. lactis*: *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* (the main subspecies), *Lc. lactis* subsp. *hordniae, Lc. lactis* subsp. *tructae* and the most recently discovered*, Lc. lactis* subsp. *garvinae* and *bovis* (McAuliffe, 2018). The differentiation among the subspecies were made according

to growth temperature, salt tolerance and utilization of arginine (McAuliffe, 2018). Also, there is the *lactis* biovar *diacetylactis* which can differ by the citrate metabolism in milk cultures and has the capacity to produce acetoin and butanediol. Further, between those subspecies there is a large number of strains and variants considering that the species is very diverse (Parapouli et al., 2013).

LAB as *Lc. lactis* are widely associated to dairy food industry. It is estimated that 2 x 10^7 tons of cheese was produced with the use of *Lc. lactis* in 2015 (Bulletin of the International Dairy Federation, 2016). The fermentation processes that these bacteria participate are essential for preventing the deterioration of the products, further, it provides a particular aspect and flavor. *Lc. lactis* has a metabolic system, which, through its complex pathways, can generate distinct products that have potential to be used as factory products and in the biotechnology industry, meantime, the challenge is to adapt the necessary engineering so that the cell can develop the compound of interest, for example, immunomodulates and vitamins (Kleerebezemab et al., 2000).

With the sequencing technology, *Lc. lactis* strains from dairy industry and plant isolated were sequenced and helped to provide a better view of the genomic diversity of this species (Bolotin et al., 2001). With the comparative genomics analyses made, it was possible to better understand the hypothesis that *Lc. lactis* dairy strains have evolutionary relationships with plant isolates (Siezen et al., 2010; Wels et al., 2019). These evolutionary relationships were better understood with the definition of genes which encodes enzymes associated to plant metabolism of sugar and polysaccharides (Wegmann et al., 2007). Those studies were performed also with the plasmids showing their strong importance for evolutionary adaptation for the species as they found markers that support this knowledge (Fallico et al., 2011).

The genome of the *Lc. lactis* subsp. *lactis* strain Il1403 has around 2Mbp with GC content of 35.4% which encodes 2310 proteins (Bolotin et al., 2001). Studies evaluating de 16S rRNA genes of *Lc. lactis* reveal that, the variability of those genes were about 0.07% among the subspecies (Pu et al., 2002). *Lc. lactis* subsp. *lactis* demonstrated higher genetic variability and larger genome compared to *Lc. lactis* subsp *cremoris* phenotype (Passerini et al., 2010; Rademaker et al., 2007).

Mobile genetic elements compose about 6% of the entire *Lc. lactis* chromosome, without including prophages, and is extremely important to the genomic variability and metabolic diversity of the species (Andersen et al., 2019). Different evolution processes are under study and an important part of them occur due to plasmids that can reach 200 kb of some strains genome (Kelleher et al., 2017; Kelly et al., 2010). Both *Lc. lactis* subspecies have diverse plasmid types which are related to adaptations about lactose and casein metabolism, however, the dairy isolated, *Lc. lactis* subspecies *cremoris*, carries higher proportion of those plasmids (Kelleher et al., 2017).

Also, due to the presence of prophages and many plasmids, this genomic flexibility makes possible the existence of different phenotypes among the species (Chopin et al., 2001; Siezen et al., 2011). This flexibility was ensured with the presence of almost 5% of transposons in *Lc. lactis* spp. *cremoris* and pseudogenes (Makarova et al., 2006), indicating an important role in evolution and adaptative capacity, which is another signal of the species diversity. Those evidences also support that the genomic size of *Lc. lactis* strains is in constriction over the time, mainly the subsp. *cremoris*, revealing the adjustment to milk factory conditions. In this process, unnecessary genes were lost while genes related to host defense system, response to stress conditions and improvement in milk growth were acquired (Ainsworth et al., 2013).

Another important feature about *Lc. lactis* is the presence of accessory genes which produce bacteriocins. Nisin is a bacteriocin produced by *Lc. lactis* subsp. *lactis* that were approved by the FDA (Food and Drug Administration) to use in dairy factory due to its high capacity in spoilage control and constrain *Clostridium botulinum* growing (Juturu and Wu, 2018). Moreover, the ingestion of nisin-producing bacteria seems to induce some beneficial effects such as protecting the intestinal flora but that still needs more studies (Beasley and Saris, 2004).

Non-dairy *Lc. lactis* strains have metabolic differences regarding to its environment and adaptative needs. *Lc. lactis* KF147 is a strain that is isolated from mung bean sprouts and has targeted genes to metabolize EPS and use carbohydrates to grown. This capability to survive from plants derivates is due to the presence of gene sets associated to plant polymer degradation (Siezen et al., 2008). Another example is the *Lc.*

lactis A12, this nondairy strain developed adaptive skills in sourdough environment to ferment carbohydrates as arabinose and α-galactosides (Passerini et al., 2013).

The diversity of *Lc. lactis* subspecies are not only related to dairy and nondairy strains. Other important division was found through multilocus sequence typing and pulsed field gel electrophoresis, and through this analysis, the evaluated variability is regarding to domesticated and environmental strains. Briefly describing, the hypothesis is that environmental strains were the first to appear and, through genetic drift, domesticated strains appeared after a single founder event where some genes are excluded so that a group is distinguished from the population. However, in case of *Lc. lactis*, it occurred with the acquisition of new genes, such as lactose metabolism, arising from plasmids. Other possible explanation of the domestication is the bottleneck event, with the large scale of cheese industries, mixed strains with important characteristics for milk production such as faster fermentation, resulted in a natural increase in strains with greater technology and a decrease in other strains (McAuliffe, 2018).

The probiotic potential of *Lc. lactis* is expressed in several ways and can vary according to the subspecies and isolates, such as the ability to grow in different concentrations of bile salts (Yerlikaya, 2019) or as the subsp. *cremoris* that has anti-inflammatory activity with high capacity for fixation and permanence in the intestinal mucosa (Oliveira et al., 2017). Other strains such as *Lc. lactis* NCDO2118 demonstrated immunomodulatory capacity, reducing the damaging effects of colitis, production of gamma-aminobutyric acid (GABA) and the strain is also being applied in studies for expression of heterologous proteins (Mazzoli et al., 2010; Miyoshi et al., 2004; Nishitani et al., 2009). *Lc. lactis* strains, have been standing out as an expression vector for protein antigens and even in DNA vaccines, since they have a safe character and also act as adjuvants, proving to be a promising strategy in the area (Azizpour et al., 2017). The antimicrobial activity is made possible by the presence of organic acids that prevent the proliferation of pathogenic and competing organisms (Yerlikaya, 2019). Studies with antioxidant, lipolytic and decarboxylation capacity, are other factors associated with the metabolism of *Lc. lactis* that are being studied and can be used as new technological resources made possible by the species (Yerlikaya, 2019).

The findings related to *Lc. lactis* through comparative genomics, reveal chromosomal and extra-chromosomal important information about the potential of the species in the dairy industry. Biotechnological approaches can be applied in many ways in order to utilize the genetic diversity and metabolic pathways of the species in the food environment.

5.7. Lactiplantibacillus plantarum

Lp. plantarum is present in the GITs of humans and animals and is also considered a safe microorganism (GRAS) and is used as a probiotic. This Gram-positive, belonging to the group of LAB can also, be found in the GITs of insects, in foods (such as cabbage and cheese) and fermented beverages and has an excellent long-term attachment capacity (Siezen and van Hylckama, 2011). Within the species, some strains have shown many probiotic properties, such as significantly improving pig growth and quality, and antimicrobial activity against various pathogens *in vitro*, including *Staphylococcus aureus, Escherichia coli, Salmonella enterica* and *Listeria monocytogenes* (Suo et al., 2012). Among the strains with probiotic potential, WCFS1, JDM1 and ST-III stand out, which have genomic regions that represent possible adaptations to lifestyle, encoding proteins for sugar transport, metabolism, and regulation. Also in this region, there is a drop in GC content, suggesting that some genes may have been acquired by HGT (Li et al., 2016).

A pan-genome study of 108 complete and incomplete genomes of different isolates, (Choi et al., 2018) using SNP's (single nucleotide polymorphism) data found in the core genes of the genomes, resulted in 1709 core genes. In the literature, there was a difficulty in inferring phylogenetic trees relating them to isolate location, because in-depth analyses were not done on the genomes. But, when a SNP analysis was done, the authors found direct correlation between isolation source and clade. In total, they formed five groups (G1-G5) and one sub-group within G1 (A and B). Thus, meat and plant strains for example were in G2 and G3, respectively.

Punctuating specific and important details from the pan-genome of the probiotic strains, it was noted an efficient system for arsenic detoxification restricted to WCFS1 regulated by the *arsR* gene and dependent on the ArsD, ArsA and ArsB proteins, which therefore defines those with only the *arsR* gene with a partial detoxification of arsenic.

Another important point to be emphasized is the presence of the nisin gene (*nsr*) in the three probiotic strains that encodes a truncated protein, which is expected, a reduced nisin activity, unlike the other strains (Evanovich et al., 2019).

A comparative genomics analysis was also performed in the study (focusing on position genes *lp_3131* to *lp_3661*, using *Lp. plantarum* WCFS1 as reference). No patterns of association between gene position and strain habitat were revealed. It was also evident that the arrangements agreed with the scores made from the pan-genome, while other arrangements were possibly from HGT. Which concludes that HGT plays a particularly important role in the development of probiotic strains of the species.

5.8. Genome-scale analysis of health-promoting Lactobacillus delbrueckii strains

The former-*Lactobacillus* is one of the most important genus of LAB group. This genus contains species of Gram-positive and rod-shaped bacteria, resistant to low pH, anaerobic or aerotolerant, and with an optimal growth temperature (30-40 ºC). Their natural habitats are high carbohydrate-containing substrates such as plants, soil, food, and the oral, genital and GI tracts of animals and humans. Besides, they are characterized by a fermentative metabolism, with lactic acid being the main metabolic product (Duar et al., 2017; Salvetti et al., 2012).

Within the genus, which recently had a new reclassification, *Lb. delbrueckii* is the type species (Zheng et al., 2020). This species includes mainly two sub-species: *bulgaricus* and *lactis*. The main interest regarding these LAB comes from their importance in the dairy industry (Rizzello and De Angelis, 2011).

Some *Lb. delbrueckii* strains present probiotic properties, conferring health benefits to the host. The beneficial effects attributed to administration of these bacteria mainly focused for GIT diseases (De Jesus et al., 2019; Rocha et al., 2014), but also for other diseases including colorectal cancer (Wan et al., 2014), diabetes (Hallajzadeh et al., 2021), arthritis (Kano et al., 2002) and depression (Qiu et al., 2021). On the other hand, these microorganisms are essential in the food industry, mainly in the production and preservation of fermented products, such as yogurt and some types of cheese and wines. They have the ability to acidify the medium, thus improving the organoleptic characteristics (texture, flavor and aroma) and nutritional value of these products (Dan et al., 2019; Liu et al., 2016).

Due to its commercial/biotechnological potential and benefits attributed to the host health, the number of sequenced and deposited genomes of former-*Lactobacillus* strains with probiotic properties has greatly accelerated. Consequently, studies have been performed to characterize the gene products functions and molecular mechanisms related to the probiotic effects attributed to these bacteria, as well as safety aspects related to animal and human consumption (Pariza et al., 2015; Ventura et al., 2012; Zhang et al., 2012).

It should be highlighted that there are few studies focusing on *Lb. delbrueckii* probiogenomics, unlike other LAB species. The genomic data related to *Lb. delbrueckii* are mainly obtained from *bulgaricus* subspecies, and has been focused on (i) molecular identification and bacteria typing; (ii) safety aspects for human and veterinary consumption, and (iii) molecular mechanism and genes products functions (Castro-López et al., 2021; Papadimitriou et al., 2015).

5.8.1. Molecular strain identification

The primary distinction between *Lb. delbrueckii* species was made only through phenotypic and biochemical characteristics (carbohydrate fermentation pattern) (Weiss et al., 1983), until the introduction of Next Generation Sequencing (NGS) technologies (Alexandraki et al., 2019).

At the molecular level, the *Lb. delbrueckii* strains have been classified to subspecies *bulgaricus* or *lactis* either based on Multi Locus Sequence Typing (MLST) or using comparative genomic analysis via 16S rRNA gene sequence (Giraffa et al., 1998; Tanigawa and Watanabe, 2011). The genome size of these species presents a variable genetic profile, being that the complete genomes of the subspecies *bulgaricus* (~ 1.8 Mb) are smaller than those of the subspecies *lactis* (~ 2.1Mb) (El Kafsi et al., 2014).

Additionally, the genomic and MLST approaches were also able to show that ND02 strain was misclassified and belongs to the subspecies *lactis*, and not to the subspecies *bulgaricus* (El Kafsi et al., 2014). In this context, unequivocal identification of bacteria (probiotic or not) can affect different areas as medical diagnosis, food industry or other important process in which these microorganisms are used or have been isolated. Thus, the correct typing of probiotic microorganisms is necessary since host health-promoting effects are strain-dependent (Hill et al., 2014).

5.8.2. Safety aspects of Lb. delbrueckii

Although many commercial probiotic strains are generally recognized as safe (GRAS), new probiotic candidates should be evaluated to characteristics related to their safety for human and veterinary consumption (Pariza et al., 2015; Z. Y. Zhang et al., 2012). Many of these characteristics have been identified through genomic analysis associated with *in vitro* and *in vivo* assays. In this context, among the main analyses carried out to assess the safety profile of new probiotics, the following stand out: (i) identification of virulence factors (Casarotti et al., 2017); (ii) genes related to antibiotic resistance of clinical relevance and production of biogenic amines (Alayande et al., 2020); and (iii) hemolytic activity (Chokesajjawatee et al., 2020).

5.8.3. Antibiotic resistance

The main concern regarding the safety of probiotics is antibiotic resistance, once these strains can transfer antibiotic resistance genes to pathogenic bacteria in the intestinal microbiota, which can represent a serious risk for the treatment of patients with bacterial infections (Ventola, 2015).

Genetic factors associated with antibiotic resistance phenotypes were already identified in genomic DNA of *Lb. delbrueckii* strains, such as genes encoding penicillin-binding proteins (PBPs), VanZ and d-alanine d-alanine ligase (Ddl) involved in ampicillin and vancomycin resistance, respectively. Additionally, aminoglycoside-modifying enzymes (streptomycin, gentamicin and kanamycin resistance)*, tet(M)* (tetracycline resistance) and *erm*(B) and macrolide efflux pumps [(*mef*(E)and *mef*(B) variants] involved with erythromycin resistance were found (Campedelli et al., 2019; Casarotti et al., 2017; H. Guo et al., 2017).

Most of these genes are associated to the intrinsic resistance mechanism of these microorganisms and, therefore, do not represent a direct clinical risk to the organisms (Gueimonde et al., 2013). However, it is important to highlight the need to identify the presence of genetic elements responsible for promoting the dissemination of these antibiotic resistance-associated genes, such as plasmids and insertion elements (transposons) (Partridge et al., 2018).

Insertion elements (IS) as ISL6 and ISL4/ISL5 were previously identified in *Lb. delbrueckii* strains genome. These IS were located on both sides of the *lac* operon (Germond et al., 2003), possibly being involved in the establishment of the operon in the genome of this strains, and allowing an advantage for their growth in an environment containing a high concentration of lactose as the only carbohydrate source. Regarding the plasmids, their presence in *Lb. delbrueckii* strains is rare (Lee et al., 2007). Thus, this property reduces the potential of these strains in the dissemination of antibiotic resistance genes and ratify the use of these microorganisms in probiotic applications.

5.8.4. Toxic metabolic products and toxins-related genes

Many proteins that have already been characterized as associated with virulence in pathogenic microorganisms have also been described in probiotic bacteria. When probiotic bacteria are consumed or inhabit the same ecological niche, they suffer the same types of stresses as pathogenic bacteria and, thus, they often use the same strategies to colonize and survive (Hill et al., 2014; Wassenaar et al., 2015). The difference between these two groups of microorganisms is that probiotic bacteria do not directly cause disease to their hosts when they colonize them, only being able to act as opportunists when the host has immunosuppression (Cohen, 2019). Therefore, these proteins in probiotic bacteria are only niche/colonization factors used for their adaptation.

In this context, additionally to the presence of genes associated to antibiotic resistance, genes related to the production of toxins (as hemolysins) and toxic metabolic products (as biogenic amines) have been also studied through genomic approach in LAB (Chokesajjawatee et al., 2020; Surachat et al., 2017).

Hemolysis is a process in which lysis of red blood cells (erythrocytes) occurs in the blood, being a common virulence factor used by many pathogens, causing both anemia and edema to the host (Vesterlund et al., 2007). The biogenic amines (putrescine, tyramine, cadaverine, histamine, among others) are organic nitrogen compounds generated by microorganisms through enzymatic activity (Doeun et al., 2017). Regarding these metabolic products, it has been reported that they cause loss of organoleptic characteristics of fermented products and high concentrations ingestion can promote intoxication manifested like diarrhea, nausea and vomiting (Alvarez and Moreno-Arribas, 2014).

Regarding *Lb. delbrueckii* strains, the hemolysin or amine biogenic genes has been reported only by PCR method and/or by *in vitro* phenotype. Using these approaches, Casarotti et al. (2017) showed that *Lb. delbrueckii* subsp. *bulgaricus* (SJRP50, SJRP76, and SJRP149 strains), isolated from water buffalo mozzarella cheese, have in their genome virulence-related factors, such as gene coding for gelatinase (*gelE, fsrC*) and hyaluronidase (*hyl*), previously identified as virulence agents in pathogenic bacteria (Lopes et al., 2006; Vankerckhoven et al., 2004). Additionally, the authors detected histidine decarboxylase (*hdc1*) and tyrosine decarboxylase (*tdc*) genes, both involved with histamine and tyramine biogenic amine production, respectively (Casarotti et al., 2017).

It was also reported that *Lb. delbrueckii* subsp. *bulgaricus* (TISTR 895) produces the histamine amine biogenic (459.1 ± 0.63 mg/L) detected by HPCL analysis (Priyadarshani and Rakshit, 2011). In the other hand, Buňková et al. (2009) showed that *Lb. delbrueckii* subsp. *bulgaricus* CCDM 364 was able to produce tyramine (1.31 ± 0.09 g/ L). Additionally, it was demonstrated α-haemolysis (partial hemolysis) to *Lb. delbrueckii* ZN7a-9 (Adimpong et al., 2012).

Unlike other LAB within *Lb. delbrueckii* species the genomic approach for genes detection involved with hemolysis and biogenic amine production has been performed to *Lb. delbrueckii* subsp. *lactis* CIDCA 133 strain, whose genes coding for hemolysin A and ornithine decarboxylase (*odc*) (involved in putrescine production) were identified (Unpublished data).

Based on the above studies, the identification of genes involved with safety process in *Lb. delbrueckii* strains with a probiotic profile and their phenotypic characteristics evaluation becomes extremely important for their biological functions and consumption safety.

5.8.5. Genetic factors involved with probiotic properties of Lb. delbrueckii

To produce many of its beneficial effects on the host health, two main properties for probiotic microorganisms must be highlighted: their ability to resist and survive to GIT stress (acid, bile salts and pancreatic secretions), and their capacity to adhere to the intestinal mucosa, promoting interactions in the host's immune response modulation (Papadimitriou et al., 2016; Vélez et al., 2007).

GIT Stress Responses. The ability of *Lb. delbrueckii* strains to resist GIT stressors allows this strain to perform better at its health-promoting site of action. Genes involved in the acid and bile stress response have been identified in *Lb. delbrueckii* strains, including *Lb. delbrueckii* DSM 20074 (Sun, Harris, et al., 2015), *Lb. delbrueckii* UFV H2b20 (Ferreira et al., 2013), *Lb. delbrueckii* 2038 (Hao et al., 2011), *Lb. delbrueckii* CAUH1 (Zhai et al., 2014), *Lb. delbrueckii* LJJ (W. Li et al., 2020), *Lb. delbrueckii* ATCC 11842 and *Lb. delbrueckii* CNRZ327 (El Kafsi et al., 2014).

These genes code for bile salt hydrolases (*bsh*) and LuxS (Sun et al., 2015) proteins; Clps proteases (*clpP*, *clpE, clpL* and *clpX*) (Ferreira et al., 2013); molecular chaperones (DnaJ, DnaK, GroeS and GroeL), HtrA (Hao et al., 2011); lysine synthesis-related genes (*dapA, dapH, lysC*) (W. Li et al., 2020); pyruvate oxidase and phosphate acetyltransferase (Zhai et al., 2014); ornithine decarboxylase and arginine deaminase (*arcA, arcB* and *arcC*) (El Kafsi et al., 2014) and F0F1-ATPase system (El Kafsi et al., 2014; Hao et al., 2011).

Mucosa Adhesion and Imunorregulation. The interaction between probiotics and the host mucosa occurs *via* different adhesion-related proteins (adhesins) that recognize and bind specific receptor regions of the host cell. The proteins with adhesion properties identified *in silico* in different probiotic bacteria include mucus-binding protein (Mubs), mannose-specific adhesin (Msa), mucus-binding factor (MBF) and sortase A-LPXTG (srtA). Additionally, S-layer protein (Slps), internalin (InlA), fibronectin-binding (FbpA), collagen-binding (CnPB), Tu elongation factor (EF-Tu) and heat shock GroEL protein were already described (Deutsch et al., 2017; Le Maréchal et al., 2015; Sengupta et al., 2013).

In addition to acting as adhesins, another function attributed to these proteins is their ability to activate the immune system (Lebeer et al., 2008), and thus positively modulate inflammatory responses, through NF-κB inhibition, IL-10 production and downregulation of pro-inflammatory cytokines. These molecular processes have already been reported for probiotic bacteria, such as *Lb. acidophilus* (Ashida et al., 2011), *Lb. helveticus* (Taverniti et al., 2013) and *Propionibacterium freudenreichii* (Le Maréchal et al., 2015).

For *Lb. delbrueckii* strains, the genes with adherence capacity and possibly immunomodulatory effects include the ones coding for aggregation-promoting factor (Apf) (Casarotti et al., 2017; Yungareva and Urshev, 2018), EPS (*epsA, epsB*, *epsC, epsD* and *epsE*) (Kanmani et al., 2018), sortase-LPXTG proteins and S-layer-associated proteins (SLAPs) (Sun et al., 2015), cell-bound proteases (PrtB) (Gilbert et al., 1996) and mucus-binding protein (Mub) (Casarotti et al., 2017).

Antibacterial Activity: Bacteriocins And Organic Acids Products. Another important characteristic attributed to probiotic bacteria is their ability to inhibit the growth of other bacteria that occupy their ecological niche, using different processes, as the production of organic compounds (lactate, hydrogen peroxide, among others), which increase the pH of the intestinal lumen, creating a hostile microenvironment for the growth of pathogenic bacteria (Bermudez-Brito et al., 2012; Plaza-Diaz et al., 2019). These compounds are produced during the carbohydrate degradation used for the growth of these bacteria (Kandler, 1983). In addition, these microorganisms are also capable of synthesize antimicrobial molecules, such as bacteriocins (Mokoena, 2017) that act generally by inducing membrane permeabilization, increasing the flow of transmembrane ions and the subsequent bacteria death.

Studies associated with the identification of genes related to bacteriocins in *Lb. delbrueckii* strains genomes is limited, but the identification of Enterolysin_A bacteriocin in this species has been reported (Sun et al., 2015). Regarding lactate biosynthesis genes involved in the conversion of pyruvate to lactate, such as L-lactate dehydrogenase and D-lactate dehydrogenase have been reported (Domann et al., 2016; Hao et al., 2011). Finally, the genetic basis for hydrogen peroxide production in *Lb. delbrueckii* is not clear, but it is believed that the production of this organic acid involves enzyme-coding genes, such as NADH oxidase and pyruvate oxidase (Marty-Teysset et al., 2000)

Altogether, these studies show the main genes involved with *Lb. delbrueckii* strains stress survival, probiosis and safety, supporting their phenotypic aspects. In addition, there is a need for further studies to be carried out for the genomic characterization of new strains of *Lb. delbrueckii*, since there is little information found in the literature for these species.

6. Conclusions

Probiotic bacteria are organisms with a promising potential in food industry and biotechnology as was mentioned above; however, traditionally, the evaluation of its properties was based in *in vitro* and *in vivo* studies that allow identifying beneficial effects. The availability of NGS technologies opened the possibility of the genome exploration of these organisms and with the development of new perspectives such as comparative genomics, it has been identified new features and differentiation between strains. A comparative genome approach may lead to a better comprehension of the potential of these microorganisms, in the context of the group of interest that could be useful for the selection of new probiotic strains. Within this perspective, the pangenome analysis of probiotic bacteria in combination with a subtractive genomic approach, to exclude genes related to pathogenic bacteria from the intestinal microbiota, could give an approximation to the identification of genes involved in beneficial bacterial processes.

This perspective represents a strategy of *in silico* exploration that should be accompanied with more studies of other type such as genome wide association studies (GWAS) or gene-trait matching (GTM) that help to show genotype-phenotype associations and the discovery of genes related with probiosis in these organisms. Furthermore, the searching for probiotic strains based on genomic studies should be complemented with studies of other areas such as metagenomics, the finding of the evidence of the influence of the community changes associated to specific probiotic strains. As well as transcriptomic, and proteomic approaches that allow the elucidation of the expression of determined proteins for specific conditions and the exploration of the interactions of crucial proteins in the relation among host-probiotic or pathogen-probiotic.

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1.2 Genus *Bifidobacterium*

The genus *Bifidobacterium* are Gram-positive bacteria, non-spore-forming, non-motile, with irregular rod-shaped cells but sometimes with Y or V shape. Bifidobacteria have been isolated from the intestinal tract of various organisms (Ventura et al., 2012). This genus is known for being the first bacterial colonizer of the gut in infants with the capacity to shape and influence the gastrointestinal tract at this early age (O'Callaghan & van Sinderen, 2016; Turroni et al., 2012). Some *Bifidobacterium* strains have positive health effects over symptoms of inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), diarrhea, and allergy (R. Tojo et al., 2014). There are some studies about *Bifidobacterium* genus and its characteristics for probiotic prospection. Although the acid tolerance in the genus *Bifidobacterium* is reduced except for *B. animalis* (Masco et al., 2004), a process of adaptation known in bacteria as acid tolerance response (ATR) improves the survival of bifidobacteria, after exposure to sub-lethal conditions of acidity (Maus & Ingham, 2003). Therefore, a mechanism of extrusion of the proton to the exterior of the bacterial cell, as a strategy to tolerate the acid conditions is performed by the F0F1-ATPase and it has been shown its relationship with acid tolerance response (ATR) and exposure to these conditions (Sánchez et al., 2007). For *Bifidobacterium,* it is known that tolerance to bile depends on the species (Sanchez et al., 2008). In this respect, some multidrug transporters (MDR) involved in bile tolerance were identified in *Bifidobacterium,* with two genes in *B. longum* (Gueimonde et al., 2009; Price et al., 2006), and one in *B. breve*, in strains UCC2003 and Bbr0838. Moreover, there is evidence in lactobacilli about the relation between hydrolases and bile tolerance. Although, there are not studies related to bifidobacterial strains, was evidenced that bile-salt hydrolases were overrepresented in *B. animalis, an* adapted strain to bile(Sánchez et al., 2007).

Another essential feature is the adherence capability, which is considered necessary in selecting probiotic bacteria (Juntunen et al., 2001). There is evidence in bifidobacteria about their capacity to adhere to mucus (Izquierdo et al., 2008). Some bifidobacteria species have been related to the ability to prevent the colonization of pathogen bacteria (Gueimonde et al., 2007), and it may be attributed to the competition for nutrients, for colonization space, or production of antimicrobial compounds (Buffie and Pamer, 2013), as well as, the colonization of a niche allows the competition for space that avoids other bacteria could occupy. The role of the exopolysaccharides was evidenced when they participate in the adhesion of bacteria with an intestinal surface

(López et al., 2012) that is confirmed with another study where an improved adherence was showed in bifidobacteria with a higher production of exopolysaccharides (Alp et al., 2010). Respect inhibitory capability, *B. breve* CNCM I-4035 showed inhibitory effects on enterotoxigenic and enteropathogenic bacteria (Gueimonde et al., 2007) and inhibition of the adhesion of some pathogens (Collado et al., 2006).

1.3 Bifidobacterium breve

Bifidobacterium breve is considered the dominant species in the gut of breastfed newborns (Turroni et al., 2012), and it is often used as a probiotic in children. This species was first isolated from infant feces, although it was found in the vagina of healthy women and human milk (Fernández et al., 2013). Some strains of *B. breve*, such as B632 and BR03, have shown antimicrobial activity against coliforms, effects on the activation of macrophages, and without cytotoxic effects on the intestinal epithelium (Aloisio et al., 2012) in *in vitro* and *in vivo* studies (Mogna et al., 2014; Simone et al., 2014). Some bifidobacterial strains such as *B. breve* UCC2003 has shown a great capacity to colonize and reduce the population of *Listeria* in inoculated mice (Sheehan et al., 2007), and BBG-01 strain has demonstrated the ability to modulate the gut microbiota and reduce the presence of pathogenic bacteria like *Campylobacter, Candida* and *Enterococcus* (Hotta et al., 1987; Tojo et al., 1987).

Studies concerning other strains of *B. breve* have demonstrated protective action against asthma, allergy, obesity, stress-related disorders, and prevention of the cognitive decline in Alzheimer disease in mice (Dinan et al., 2013; Kobayashi et al., 2017; Kondo et al., 2010; Raftis et al., 2018; Schouten et al., 2009).

1.4 A previous study with *Bifidobacterium breve* 110^{1A}

In a previous *in vitro* study, the potential probiotic of four *Bifidobacterium* strains isolated from feces from healthy children in Brazil was evaluated through some aspects, such as growth rate, oxygen tolerance, antagonism, cell wall hydrophobicity, and antimicrobial susceptibility (Souza et al., 2013). *Bifidobacterium breve* 110^{1A}, one of these strains, showed high aerotolerance, a property considered as positive for the industrial process of probiotics. Therefore, this strain showed 66.7% inhibition against tested pathogens, a required feature for a probiotic bacterium.

However, this strain showed resistance to cefoxitin, erythromycin, and metronidazole aspect that is yet a controversial topic referent to antibiotic resistance and probiotics (Souza et al., 2013). With these experimental results, exploring the genomic features of this strain may help to know about the potential capabilities and possibly support it as a future bacteria strain of interest from an *in silico* approach.

In this context, the aim of the present study is the *in silico* characterization of the *Bifidobacterium breve* 110^{1A} strain genome through a comparative genomic perspective along with the search of genes related to probiotic features to know about the potential properties of this strain.

1.5 Genes related to probiotic features

Regarding to probiotic evaluation, some characteristics could help describe a probiotic bacterium, among them survival and adhesion to GIT, competitive exclusion against pathogens and antimicrobial activity, human origin, modulation of the mucosal immune system,and production of vitamins, safe nature and good technological properties.

The relevant feature is the capacity to survive in different environments with grades of stress as in the stomach (pH: 2) in the gastrointestinal tract (GIT). Therefore, there is evidence of transcriptional responses to environmental changes through two-component regulatory systems (2CRS), surface proteins and proton efflux systems as adaptation mechanisms (Pfeiler et al., 2007). Furthermore, MDR transporter gene is another gene that plays an essential role in the probiotic bile tolerance in *Bifidobacterium longum* as *betA,* bile efflux transporter (Gueimonde et al., 2009).

The capacity to adhere to intestinal epithelial cells is crucial for these bacteria; some genes present in probiotics are essential such as *LuxS* homolog of the autoinducer-2 quorum sensing compound (Buck et al., 2009). In addition, the adhesiveness of *Bifidobacterium* to the human intestinal mucus has been evidenced (He et al., 2001) and some factors are recognized to be responsible for the adhesion in probiotics. These factors are secreted and attached to the cell wall to interface with the intestinal epithelia (Vélez et al., 2007).

Another property of probiotics is the capacity of competitiveness against pathogens. The probiotics have two mechanisms of antagonism: using chemical

compounds and as a physical barrier. On the other side, there are several antimicrobial compounds produced by probiotics for competing with pathogens as hydrogen peroxide, lactic acid, biosurfactants, immunomodulatory products and bacteriocins. These last compounds, bacteriocins, are made to inhibit the growth of similar species or closely related bacteria (Servin, 2004). Some bacteriocins were reported for *Bifidobacterium* species, such as Bifidin I in *B. infantis* BCRC146, showing inhibitory effects against strains, *Staphylococcus, Bacillus, Streptococcus, Salmonella, Shigella, E. coli* (Cheikhyoussef et al., 2010). Therefore, bifidocin B found in *B. bifidum* NCFB1454 (Yildirim et al., 1999). Another bacteriocin is lantibiotic (bisin), identified in *B. longum* DJO10A, showing inhibitory effects against *Streptococcus thermophilus ST403, Clostridium perfringens, Staphylococcus epidermidis, Bacillus subtilis, Serratia marcescens, E. coli* DH5a (Lee et al., 2011).

The complex process of interaction between the microbiota, intestinal cells and immune cells to exert a modulation of an immunomodulatory response is mediated with microorganism-associated molecular patterns (MAMPs) and patterns recognition receptors (PRRs) on the antigens-presenting cells (APCs). Studies with MAMPs in *L. casei* and *L. reuteri* demonstrated the induction of the production of IL-10 and an anti-inflammatory process (Smits et al., 2005). It was evidenced in several studies, positive effects on allergic disease, lactose intolerance, gastroenteritis, inflammatory bowel disease (IBD), and colorectal cancer (Azad et al., 2018).

2. JUSTIFICATION

Probiotics have been used to treat several diseases, including obesity, type 2 diabetes, gastrointestinal disorders and other conditions due to their capacity to influence the microbiota intestinal and exert some benefits such as anti-inflammatory, anticancer, immunomodulatory effects on the host (Markowiak & Ślizewska, 2017). The necessity of new candidates for probiotic bacteria and the search for possible new probiotic features lead to other exploratory approaches such as genomics through bioinformatics analysis, considering the current availability of NGS technologies (Lagesen et al., 2010; Land et al., 2015). The whole-genome information could give insights into the potential of the bacterial candidates regarding their gene content and potential functions.

Bifidobacterium is one of the main genera of probiotics with known species. From them, *Bifidobacterium breve* is one of the first colonizers, predominant species in the bacterial intestinal community of infants and newborns, and is considered a safe species. In a previous study, bifidobacterial strains isolated from children in Minas Gerais state, Brazil, were used for *in vitro* evaluations where respect to *Bifidobacterium breve* 110^{1A} some characteristics such as aerotolerance, inhibition of pathogens and resistance to antibiotics showing some general insights about this strain (Souza et al., 2013).

The present study developed the *in silico* comparative analysis and characterization of the *B. breve* 110^{1A} genome, based on the exploration and identification of specific genomic elements and genes related to the probiotic properties. Thus, this study allows us to find more clues from a genomic perspective and to recognize the exclusive features of this strain through a comparative approach that could support an evaluation of this strain as a potential probiotic bacterium. Furthermore, this study provides the *B. breve* 110^{1A} genome, which represents the first complete genome of *Bifidobacterium breve* from Brazil, to be available on the GenBank database of NCBI.

3. OBJECTIVES

3.1 General objective

- To characterize *in silico* the genome of *Bifidobacterium breve* 110^{1A} and evaluate genes related to the potential probiotic properties.

3.2 Specific objectives

- Perform the taxonomic identification (ANI), phylogenomic and gene synteny analysis of *B. breve* 110^{1A};

- Predict mobile elements (plasmids, prophages, insertion sequences) and genes codifying for bacteriocins and CRISPR-Cas systems in *B. breve* 110^{1A};

- Identify the antibiotic resistance genes, virulence factors and genomic plasticity in *B. breve* 110^{1A} genome;

- Characterize the pangenome for the species: core genome, accessory genome and unique genes for the *B. breve* 110^{1A};

- Identify genes involved in potential probiotic functions (stress resistance, adhesion, metabolism of carbohydrates, production of vitamins) in *B. breve* 110^{1A}.

4. RESULTS: PAPER MANUSCRIPT (in press)

Comparative genomics and *in silico* evaluation of genes related to probiotic potential of *Bifidobacterium breve* 110^{1A}

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Abstract

Bifidobacterium breve is known as the first colonizer of the intestinal tract in humans, a predominant species in the intestinal microbiota in newborns, children and a widely used species in the probiotic industry due to its beneficial properties host. Therefore, the necessity of searching for new candidates for probiotics and new properties has a trend towards increasing and NGS technologies facilitate the characterization and the potential of the bacteria using a genomic approach. The strain *B. breve* 110^{1A} was isolated from healthy children from Brazil. The present study was focused on the characterization of the genome features (phylogenomics, synteny analysis), prediction of genomic elements (plasmids, prophages, CRISPR-Cas systems, insertion sequences, genomic islands, antibiotic resistance genes and factors associated with virulence) for this strain. Furthermore, the evaluation applied a comparative genomic approach using 45 *B. breve* genomes (pangenomic analysis) and identifying specific genes related to potential probiotics properties. The 110^{1A} genome were composed of 2,371,121 bp, 1,986 CDS, 58.8% GC content and with high synteny with other genomes of this species. The closest relationship with our strain was with *B. breve* DRBB26 using ANI and phylogenomic analysis. There were no plasmids, an incomplete prophage, two non-functional CRISPR

systems and seven genomic islands (GEI). Additionally, three genes of antibiotic resistance were identified: *IleS, rpoB* and *erm(X).* In genome comparative analysis, the pan-genome was sized in 5943 genes, core genome of 1174 genes for the species and 63 unique genes, for the 110^{1A} strain, associated with processes, such as, transmembrane transport, membrane components, DNA processes and carbohydrate metabolism. Therefore, with additional analysis were identified genes related to adhesion, resistance to stress (general and acid stress) and production of vitamins. The present study pretend to use these approaches (genome characterization, comparative genomics and searching of specific genes) to explore the probiotic potential of this strain.

Keywords: Probiotic bacteria, genome sequencing, comparative genomics, pangenomic analyses, beneficial bacteria.

Introduction

Bifidobacterium species are Gram-positive bacteria, non-spore forming, non-motile, with irregular rod-shape cells but sometimes with Y or V shape (Klijn et al., 2005; Lee and O'Sullivan, 2010). Species from this genus are among the first colonizers of the intestinal tract in newborns (O'Callaghan & van Sinderen, 2016; Turroni et al., 2012) and specifically *B. breve* is the dominant species in gut of breast-fed babies (Cionci et al., 2018).

Probiotics are defined as microorganism which when administered in adequate amounts, confers a health benefit upon the host (FAO, 2001) and a considerable part of probiotics are from *Lactobacillus* and *Bifidobacterium* (Foligné et al., 2013). The principal mechanisms of action of probiotics are resistance to acid and bile salts (Ruiz et al., 2013), capacity of adhesion to the host epithelium cells (Sarkar & Mandal, 2016), improvement of the intestinal epithelial barrier (Ohland & MacNaughton, 2010), competition with pathogens and production of antimicrobial compounds (Bermudez-Brito et al., 2012), immunomodulatory effects (Azad et al., 2018), among others which are considered of relevance in the evaluation process of probiotics.

Several studies have explored about genes in bacterial genome, using *in silico* analysis, when looking for beneficial properties in new strains for probiotics. It is noteworthy to

mention that some genes are involved in the aspects mentioned above, however there are other ones related to the safety aspect (antibiotic resistance, virulence factors) and genome stability (phages, plasmids, insertion sequences). Therefore, genomic islands and CRISPR-Cas systems that are necessary to evaluate due to they could confer some additional features (Abriouel et al., 2019; Bennedsen et al., 2011; Hidalgo-Cantabrana et al., 2017). For instance, the genome evaluation of *Lactobacillus reuteri* PNW1 evidenced the presence of genes related to antibiotic resistance and allowed to test virulence factors and genes of interest among other elements (Alayande et al., 2020). Moreover, the functional analysis of *Bacillus velezensis* FTCo1 permitted the identification of genes related to adhesion and acid resistance (Pereira et al., 2019), additionally, the *in silico* evaluation of *B. coagulans* HS243 showed evidences of adaptation and probiosis (Kapse et al., 2019).

Comparative genomics is another approach that have been used in the evaluation of probiotic bacteria with the name of Pan-probiosis (Barh et al., 2020). This approach allows to work with several genomes of the group of analysis and permits the determination of the total genes in all genomes (pangenome), shared genes among all genomes (core genome), genes shared by some but not all genomes (dispensable genome) and in this last section genes present only in a specific strain (unique genes). Using this approach could be possible to connect genotypes and phenotypes and have the opportunity to select strains for cultures of interest (Garrigues et al., 2013). Some studies have applied this perspective such as, the comparative evaluation of *Lactobacillus johnsonii* ZLJ010 genome with others from the same group to understand the probiotic nature of this strain (Zhang et al., 2019). Addition to this, some *Lactobacillus helveticus* strains were analyzed finding some strains with survival properties to possibly be candidates to probiotics (Fontana et al., 2019).

In a previous *in vitro* study, *Bifidobacterium breve* 110^{1A} was evaluated with other strains of *Bifidobacterium* isolated of feces from healthy children in Brazil (Souza et al., 2013). Growth rate, oxygen tolerance, antagonism, cell wall hydrophobicity and antimicrobial susceptibility were some evaluated aspects. In that study, *B. breve* 110^{1A} showed high aerotolerance, a feature considered positive technological property during the industrial process, the high inhibition (66.7%) against the tested pathogens (*Clostridium difficile, Listeria monocytogenes, Shigella sonnei*, *Vibrio cholera* among others) and resistance to

cefoxitin, erythromycin and metronidazole, aspect in discussion related to probiotics and antibiotic resistance (Souza et al., 2013). At this respect, genomic analysis could help to elucidate some of these features, mechanisms and facilitate the exploration of others of interest.

The aim of the present study was the *in silico* characterization of the *Bifidobacterium breve* 110^{1A} genome using a comparative genome approach with 45 available complete genomes of the species along with the searching of genes related with probiotic features, in 110^{1A} strain, to explore the probiotic potential of this strain.

Materials and Methods

The *Bifidobacterium breve* 110^{1A} strain was isolated from children feces in Belo Horizonte, Brazil (Souza et al., 2013). The strain was maintained in De Man, Rogosa and Sharpe (MRS; Difco, Sparks, NV, USA) broth supplemented with 0.5% of L-cysteine for 48h in an anaerobic chamber to 37°C. The genomic DNA extraction was performed following an adapted protocol of De et al. (2010).

Whole-genome sequencing

The genomic DNA was sequenced using the Illumina Hiseq 2500 platform (Illumina, San Diego, CA, USA) with paired-end (2x150 bp). Read sequences were assembled using SPAdes v3.9.1 (Bankevich et al., 2012), the scaffolding was done with CONTIGuator v2 (Galardini et al., 2011) and gaps were closed using FGAP v1.7 (Piro et al., 2014) and CLC Genomics Workbench v7 (https://digitalinsights.qiagen.com) to obtain the complete genome. The assembled sequence data of *Bifidobacterium breve* 110^{1A} genome was deposited at GenBank under the accession number CP053655.

Annotation and synteny analysis

Forty-five complete genome sequences of *Bifidobacterium breve* available in NCBI GenBank database were downloaded in nucleotide FASTA format. These genomes and the assembled *B. breve* 110^{1A} genome were annotated using Prokka v1.14.5 (Seemann, 2014). Synteny was evaluated on 110^{1A} genome and the 45 genomes of *B. breve*, multiple whole-genome alignments were conducted using the implemented Progressive Mauve

method of Mauve v2.4 (Darling et al., 2010) using Genbank format files (.gbk) of the genomes.

Taxonomy, phylogenomics and evolutionary analysis

Average Nucleotide Identity (ANI) values were calculated for the 46 genomes of *Bifidobacterium breve* and the outgroup species (*Bifidobacterium longum* NCTC 11818, *B. bifidum* JCM 1255 and *B. animalis* subsp. *animalis* ATCC 25527) using Pyani v0.2.10 (Pritchard et al., 2016). The results were visualized with pheatmap R package (https://cran.r-project.org/web/packages/pheatmap/index.html). The phylogenomic tree was performed using the Codon Tree Test method of Pathosystems Resources Integration Center (PATRIC) web server. (http://www.patricbrc.org) with 376 genes of single copy. Support values were generated using 100 rounds of the "Rapid" bootstrapping option of RaxML. The species ougroup were *B. longum* ICIS-505, *B. bifidum* JCM1254 and *B. animalis* ATCC25527.

Prediction of mobile elements and genes codifying for bacteriocins and CRISPR-Cas systems in *Bifidobacterium breve* 1101A

The *in silico* identification of plasmids on the genome of *Bifidobacterium breve* 110^{1A} was done with PlasmidFinder 2-0 (Carattoli et al., 2014), the presence of phages was evaluated with PHASTER (Arndt et al., 2016) and the prediction of insertion sequences (IS) was performed with Insertion Sequence Semi-Automatic Genome Annotation (ISsaga v2.0) from ISfinder tool (Siguier et al., 2006). Therefore, the prediction of putative bacteriocins was performed with BAGEL4 (Van Heel et al., 2018) and the presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and Cas proteins was analyzed with CRISPRCasfinder web server (Couvin et al., 2018).

Prediction of antibiotic resistance genes and virulence factors

The prediction of antibiotic resistance genes were evaluated with ABRIcate v1.0.1 using NCBI-AMRFinderPlus (Feldgarden et al., 2019), CARD (Alcock et al., 2020), ARG-ANNOT (Gupta et al., 2014), Resfinder (Zankari et al., 2012), MEGARES 2.0 (Doster et al., 2020) databases for antibiotic resistance genes (last update of databases: April 2020). The virulence factors were predicted withVFDB database (Liu et al., 2019).

Genomic plasticity analysis

The prediction of putative genomic islands (metabolic islands and resistance islands) in the *B. breve* 110^{1A} genome such as product of possible horizontal gene transfer events were performed using GIPSy v1.1.2 (Soares et al., 2016) using *B. breve* Bifido_07 genome (ENA: FTRK01000000) as a reference genome, both genomes in genbank format. This last bacterium was present in a report in a bacteremia case (Esaiassen et al., 2017). The circular genomic maps were visualized with BRIG v0.95 (Alikhan et al., 2011). For the exploration of the genes in the islands, a functional annotation was perform with EggNOG 5.0 (Huerta-Cepas et al., 2019).

Pangenome analysis

The pangenome size calculation was performed with Roary v3.11.2 (Page et al., 2015), the visualization of core, soft core, shell, cloud genome and the matrix of presence and absence of genes were drawn with the script called "roary_plot.py" included in Roary package and Phandango web viewer (Hadfield et al., 2018), respectively. The estimation of the openness of the pangenome, was performed with Bacterial Pan Genome Analysis, BPGA v1.3 (Chaudhari et al., 2016) and it was considered the exponential parameter b of the empirical power law equation of the pangenome curve obtained with protein sequences. They were pre-processed, the posterior clustering step was done with USEARCH v11 with default parameters and an identity cutoff of 95%, considering atypical average G+C content (2*standard deviation). The functional analysis was done with Cluster of Orthologous Genes (COG) assignments based on representative sequences for core, accessory and unique gene families. The number of unique genes of *B. breve* strains were represented in a flower chart and the unique genes for *Bifidobacterium breve* 110^{1A} were processed with GOfeat in a functional annotation (Araujo et al., 2018).

Identification of genes related to probiotic features

Genes involved in mechanisms of adhesion; resistance to stress conditions (acid, bile salts, heat, osmotic); repair and protection of DNA and proteins and production of vitamins were retrieved from the literature around genera *Bifidobacterium* and *Lactobacillus* (Alayande et al., 2020; Lee & O'Sullivan, 2010; O'Flaherty et al., 2009; Ruiz et al., 2013). Protein sequences of these genes were aligned with our genome of

study with Basic Local Alignment Search Tool (BLAST) with a cutoff e-value = 1e-5, and minimal identity percentage of 70%.

Results

Genome features of *Bifidobacterium breve* 110^{1A}

The complete genome of *B. breve* 110^{1A} was a circular chromosome of 2,371,121 bp with guanine-cytosine (GC) content of 58.8%. Initially, the assembly of the genome reach 17 contigs with a coverage of 1,234x; N50 value of 643.298 bp and the after the scaffolding and closed gap process, was obtained a complete circular genome. After the annotation process, it was identified 1,986 CDS, of them, 907 CDS (45.67%) as hypothetical protein. Additionally, it was predicted 55 tRNA, 9 rRNA, 1 tmRNA. Comparing these results with the set of 45 genomes of the species, *B. breve,* from NCBI, the features on average were: GC content of 58.8%, genome size of 2.35 Mb, 1986 CDS, 55 tRNA, 8 rRNA, 1 tmRNA. Respect to the source information, most samples were from infant feces, while a few were from adult feces, vagina, environment and human milk (Table 1).

Table 1. A Genome features of the 46 complete genomes of *Bifidobacterium breve* used in the present study.

Linhagem	GC%	Size (Mb)	Annotation Prokka					Source	Host	Country	Accession Number
			CDS	tRNA	rRNA	tmRNA	CRISPR				
110[1A]	58.76	2.37	1986	55	9	1	-	Feces	Infant	Brazil	Present study
JCM 1192	58.9	2.27	1930	54	6	1	-	Feces	Infant	Japan	NZ_AP012324
NCTC11815	58.9	2.28	1927	54	9	1	-	Intestine	Infant	UK	NZ_LR134348
215W447a	59.3	2.59	2258	72	9	1	-	Gut*	Infant	Ireland	NZ_CP021558
NRBB57	59.4	2.51	2162	72	9	1	-	Gut*	Infant	Netherlands	NZ_CP021389
DRBB30	58.9	2.47	2139	56	9	1	-	Gut*	Infant	Netherlands	NZ_CP023199
CNCM I-4321	59	2.46	2142	56	6	1	-	Gut*	Infant	Netherlands	NZ_CP021559
DRBB28	59	2.46	2140	54	9	1	-	Gut*	Infant	Netherlands	NZ_CP021553
DRBB29	58.9	2.44	2132	56	6	1	-	Gut*	Infant	Netherlands	NZ_CP023198
DRBB27	58.9	2.44	2135	56	6	1	-	Gut*	Infant	Netherlands	NZ_CP021552
NRBB56	58.9	2.43	2030	55	6	1	2	Gut*	Infant	Netherlands	NZ_CP021394
UCC2003	58.7	2.42	2026	55	6	1	2	Feces	Infant (breast fed)**	Ireland	NC_020517
MGYG-HGUT-02469	58.7	2.42	2026	55	6	1	2	Gut	Human	-	NZ_LR699003
BR3	59.1	2.43	2098	55	9	1	1	Feces	Human**	Korea	NZ_CP010413
139W423	58.6	2.41	2056	54	9	1	1	Gut*	Infant	Ireland	NZ_CP021556
NRBB50	58.8	2.41	2057	55	9	1	1	Gut*	Infant	Netherlands	NZ_CP021391
LMC520	59	2.4	2050	56	9	1	-	Environment	-	-	NZ_CP019596
NRBB51	59	2.4	2001	54	9	1	2	Gut*	Infant	Netherlands	NZ_CP021392
DRBB26	58.5	2.4	2021	54	9	1	-	Gut*	Infant	Netherlands	NZ_CP021390
NRBB52	58.9	2.38	2012	53	9	1	2	Gut*	Infant	Netherlands	NZ_CP021393
NRBB11	58.7	2.38	1951	54	9	1	1	Gut*	Infant	Netherlands	NZ_CP021388
1_mod	58.8	2.36	1975	53	6	1	-	-	-	-	NZ_LR655209
JCM 7019	58.6	2.36	2017	57	6	1	1	Feces	Adult	Japan	NZ_CP006713

689b	58.7	2.33	1929	53	6	1	-	Feces	Infant	Italy	NZ_CP006715
ACS-071-V-Sch8b	58.7	2.33	1929	54	9	1	3	Vagina	Human	USA	NC_017218
NRBB04	58.7	2.32	1932	53	9	1	2	Gut*	Infant	Netherlands	NZ_CP021386
NCFB 2258	58.7	2.32	1920	53	6	1	2	Feces	Infant	UK	NZ_CP006714
lw01	58.8	2.31	1953	54	6	1	1	Feces	Infant**	China	NZ_CP034192
JR01	58.9	2.3	1959	54	9	1	-	Stool	Human	Sweden	NZ_CP040931
017W439	58.7	2.3	1955	54	6	1	-	Gut*	Infant	Ireland	NZ_CP021554
S27	58.7	2.29	1887	55	9	1	2	Feces	Infant (breast fed)	Germany	NZ_CP006716
NRBB20	58.6	2.29	1917	55	6	1	2	Gut*	Infant	Netherlands	NZ_CP023195
NRBB02	58.6	2.29	1914	55	6	1	2	Gut*	Infant	Netherlands	NZ_CP021385
NRBB27	58.6	2.29	1916	55	6	1	2	Gut*	Infant	Netherlands	NZ_CP023196
NRBB49	58.6	2.29	1917	55	6	1	2	Gut*	Infant	Netherlands	NZ_CP023197
NRBB08	58.6	2.29	1919	55	6	1	2	Gut*	Infant	Netherlands	NZ_CP023192
NRBB19	58.6	2.29	1921	55	6	1	2	Gut*	Infant	Netherlands	NZ_CP023194
NRBB18	58.6	2.29	1919	55	6	1	2	Gut*	Infant	Netherlands	NZ_CP023193
JCM 7017	58.7	2.29	1883	54	6	1	2	Feces	Infant	Japan	NZ_CP006712
082W48	58.8	2.29	1919	53	9	1	-	Gut*	Infant	Ireland	NZ_CP021555
FDAARGOS_561	58.9	2.28	1932	54	9	1	-	Clinical Isolate	Human	-	NZ_CP033841
JSRL01	58.6	2.27	1860	54	9	1	2	Feces	Baby	South Korea	NZ_CP045646
180W83	58.8	2.27	1922	54	9	1	1	Gut*	Infant	Ireland	NZ_CP021557
NRBB01	58.9	2.27	1937	54	6	1	-	Gut*	Infant	Netherlands	NZ_CP021384
NRBB09	58.7	2.27	1916	54	9	1	2	Gut*	Infant	Netherlands	NZ_CP021387
12L	58.9	2.24	1845	53	6	1	-	Human Milk	Human	Italy	NZ_CP006711

* Specific information related to the origin of sample was absent in GenBank description. These samples were reported under the project title of “Comparative genomics and methylome analysis of the gut commensal *Bifidobacterium breve* (Botaccini et al., 2018).
** Probiotic strains with demonstrated effects on studies *in vivo*.

Taxonomy and phylogenomics

ANI values calculation for the 46 strains of *B. breve* (Figure 1). ANI analysis had a fluctuating value range of 0.97 - 1 for *Bifidobacterium breve* strains, grouped all of them in the red region of the heatmap, while ANI value range among 0.76 – 0.85 for outgroup genomes: *B. longum* (strain NCTC11818), *B. bifidum* (strain JCM1255) and *B. animalis* (strain ATCC25527) in decreasing order, grouped in the external region (blue and light blue). All comparisons of 110^{1A} strain with other *B. breve* strains showed ANI values between 0.97 – 0.98, within the red region, grouped with these strains. The highest ANI value of 110^{1A} strain appeared when it was compared with *B. breve* DRBB26 (0.988), and was followed by strains 689b, ACS-071-V-Sch8b, S27, JSRL01, JR01, NRBB04 in decreasing order, with ANI values around 0.986.

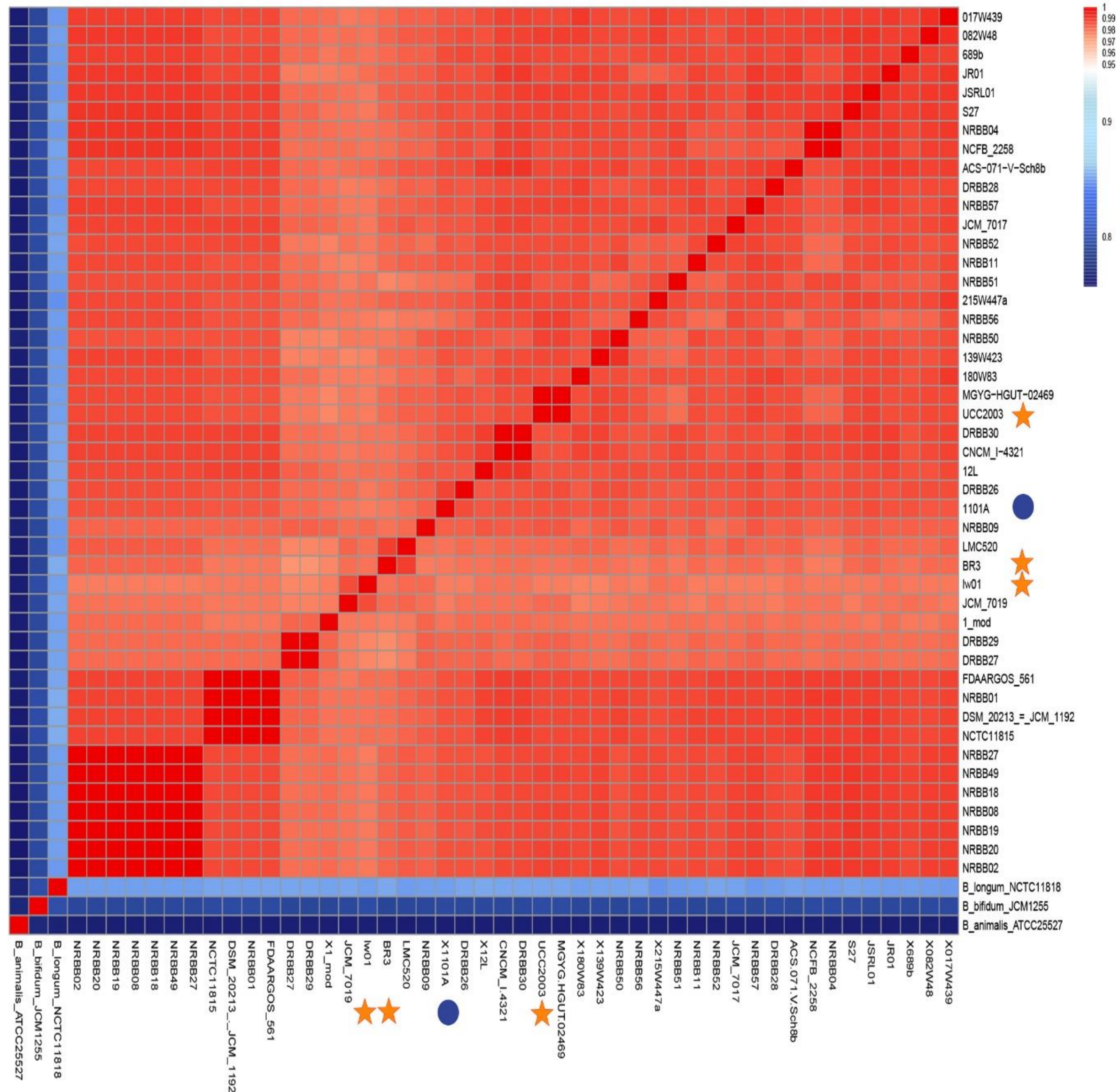

Figure 1. Heatmap of ANI values between *Bifidobacterium breve* strains with complete genome. Outgroup genomes: *B. longum* NCTC11818*, B. bifidum* JCM1255 and *B. animalis* ATCC25527. Blue circles represent *Bifidobacterium breve* 110^{1A} and stars represent demonstrated probiotic strains.

The phylogenomic tree based on 376 genes of single copy showed to *B. breve* 110^{1A} forming a strongly supported clade with *B. breve* DRBB26 (100) suggesting the closeness of this strain according to previously ANI analysis (Figure 2). Therefore, there were some strains, which formed clades with moderate to high support (74 – 100). Between them, a clade of 7 strains with strong support (100) and ANI values around 0.99: NRBB02, NRBB08, NRBB18, NRBB19, NRBB20, NRBB27, NRBB49; a clade of 4 strains with high support (100) with ANI values around 0.99: DSM20213, FDAARGOS561, NCTC11815, NRBB01 and a clade with high support of 2 strains with ANI values of 1: CNCM I-4321 with DRBB30. Strains such as UCC2003, lw01 and BR03 experimentally demonstrated probiotics, represented by stars, showed a relationship with other clades (Figure 2). The outgroup species formed a separated clade from the *Bifidobacterium breve* clade (Figure 2).

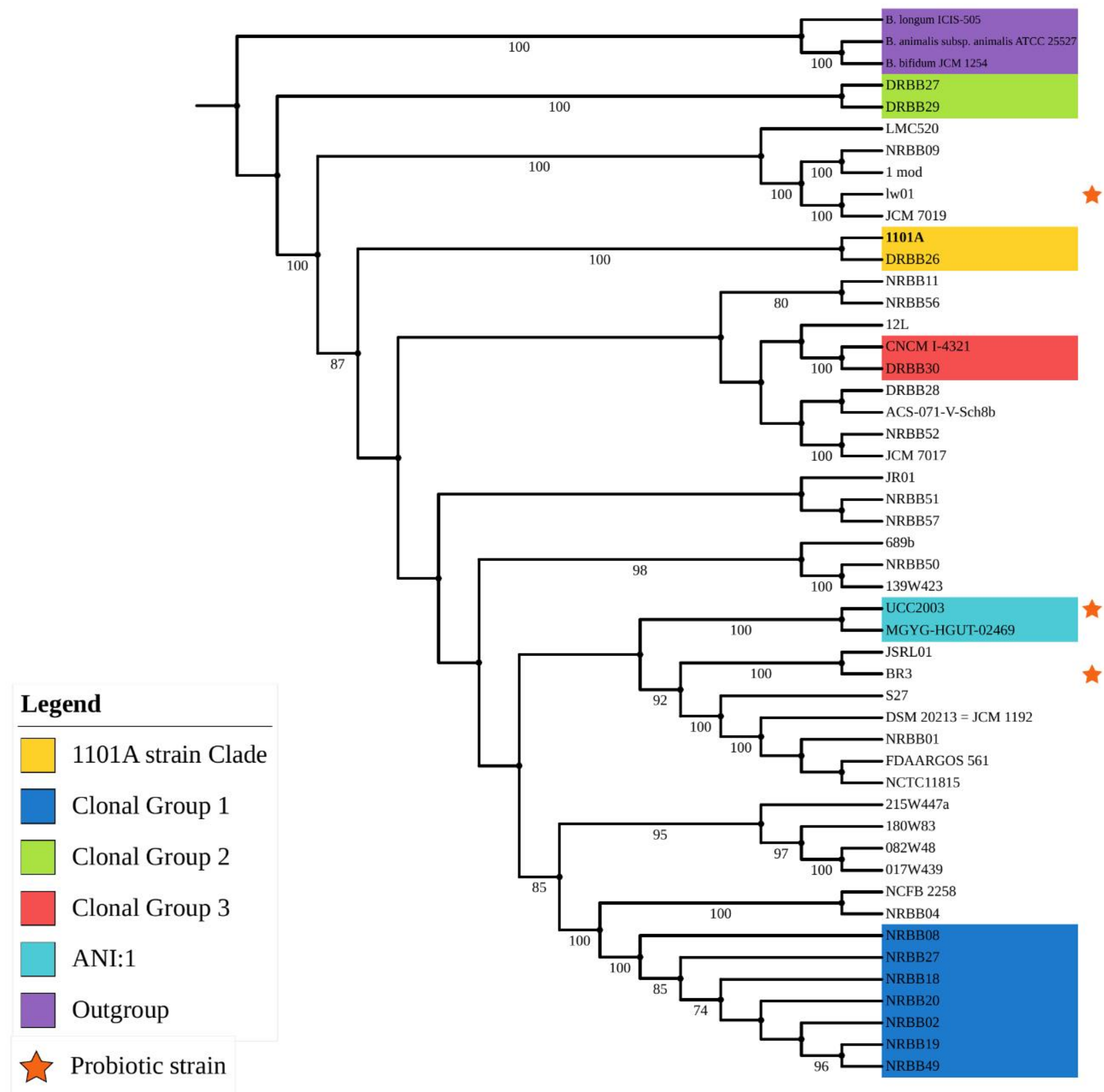

Figure 2. Phylogenomic tree of *Bifidobacterium breve* strains. Phylogenomic tree was buit based on 376 genes of single copy.Outgroup genomes: *B. longum* ICIS-505, *B. bifidum* JCM1254 and *B. animalis* ATCC25527. Stars represent demonstrated probiotic strains.

Synteny analysis

In the evaluation of conservation on the genome structure of 110^{1A} strain compared with *B. breve* strains, MAUVE showed the whole-genome alignments, considering the 110^{1A} strain as the reference (Figure 3). *Bifidobacterium breve* 110^{1A} showed a collinearity of the gene blocks with most of the evaluated genomes. (Figure 3a); in contrast, other few strains presented rearrangements over large parts of the genome (Figure 3b). At this respect, the representation of *B. breve* ACS-071-V-Sch8b showed a large inversion close to the genome center and *B. breve* JCM7017 presented a smaller inversion in the central region of its genome (around to location: 1.2Mb). In addition to this, *B. breve* BR3 seems

to have two inversion events, one over almost complete genome and the other in the terminal part of the genome (200kb of size aproximately).

Figure 3. Representation of multiple alignments of whole-genomes of 46 *Bifidobacterium breve* strains using progressiveMAUVE shows conserved synteny and collinearity among gene blocks. (a) Alignments with conserved order of gene blocks. (b) Alignments with genome rearrangements. Both graphics have as reference to *Bifidobacterium breve* 110^{1A}. Coloured boxes represent linear collinear blocks (LCB) of genes.

Prediction of mobile elements, genes codifying for bacteriocins, IS and CRISPR-Cas systems in *Bifidobacterium breve* 110^{1A}

The analysis with PlasmidFinder2.0, not identified plasmids. The PHASTER predictions identified an incomplete prophage region of 8518 bp coding region with 6 phage-like proteins and 3 hypothetical proteins (Figure 4, Table 2).

Table 2: Genes identified in the prophage region in *Bifidobacterium breve* 110[1A]. Alignments performed using Basic Local Alignment Search Tool (BLAST).

Start	End	Orientation	Description	Completeness	Percent identity
1112602	1113381	Forward	Fe-S cluster assembly ATPase SufC	100	100
1113550	1114824	Forward	Cysteine desulfurase*	100	100
1114836	1115390	Forward	SUF system NifU family Fe-S cluster assembly protein	100	100
1115398	1115982	Forward	Metal-sulfur cluster assembly factor *	100	100
1116102	1117346	Reverse	Glucose-1-phosphate adenylyltransferase	100	99.76
1117550	1118428	Reverse	RNA methyltransferase	100	100
1118671	1119468	Forward	RNA methyltransferase *	100	100
1119579	1119917	Forward	Histidine triad domain protein	100	100
1119936	1121120	Forward	PhoH family protein	100	99.75

(*)Previouly identified by PHASTER as hypothetical protein.

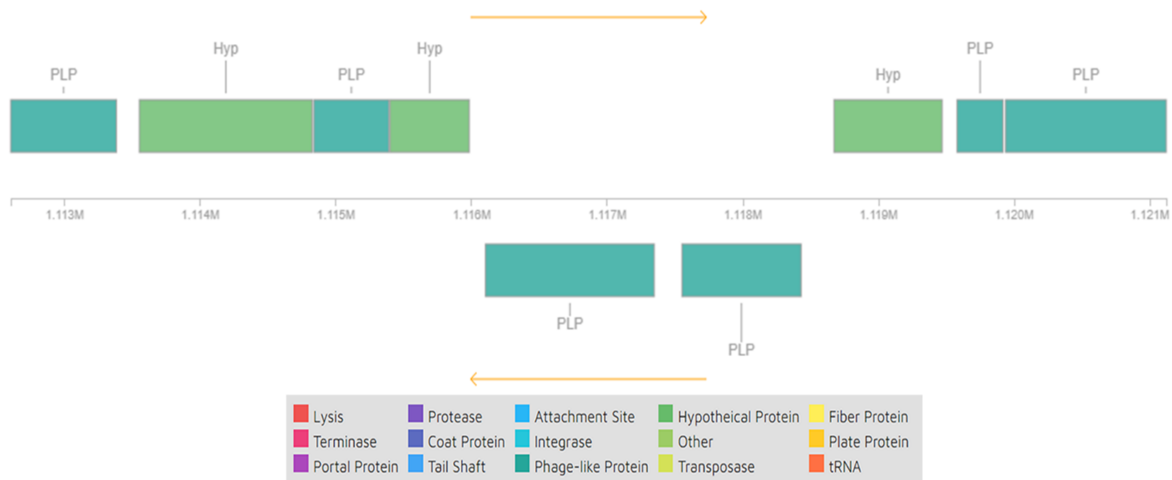

Figure 4: Representation of the incomplete prophage region in *Bifidobacterium breve* 110[1A]. The prophage showed 9 CDS: 6 CDS in dark turquoise identified as phage-like protein and 3 CDS in light green as hypothetical protein using PHASTER.

The screening of insertion sequences with ISfinder showed 29 elements, 7 of them with complete ORF. IS were classified in 6 family groups (IS3, IS256, IS21, ISL3, IS30 and IS5) being IS3 family the most represented (37.91%), followed by IS256 (20.69%) and IS21 (17.24%) (Figure 5). About bacteriocin prediction, there were not identified areas of interest (AOI) for bacteriocin-coding genes.

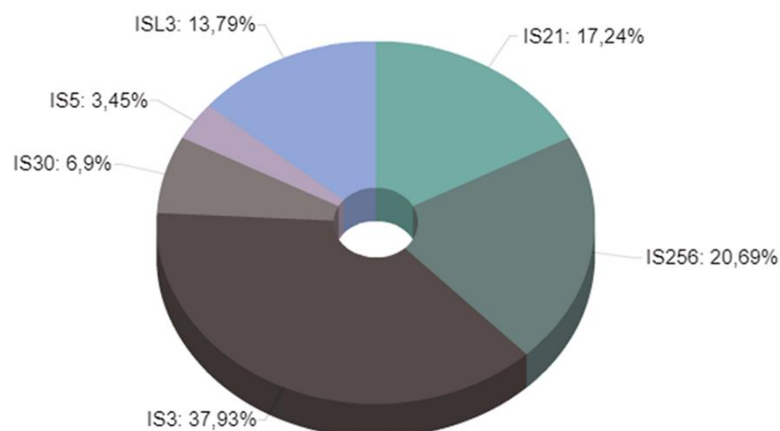

Figure 5: Percentage distribution of predicted IS family in the genome of *Bifidobacterium breve* 110^{1A}.

Considering other genomic elements, it was identified three regions related to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in the genome of *Bifidobacterium breve* 110^{1A}, with a range of 1-2 of evidence level and 1-6 spacers sequences; however, there was a lack of sequences codifying for Cas proteins (Table 3).

Table 3: Predicted CRISPR regions and related information about Repeat Sequences and number of spacers according with CRISPRCasfinder results.

CRISPRId	Start	End	Spacer	Repeat consensus	Evidence Level
110^{1A}_1	140420	140756	6	TACTGGTGGTTTTGCCCCGCTGAGG	2
110^{1A}_2	1104813	1104898	1	GCTTAGTGCAATAAATTCTCGAAAT	1
110^{1A}_3	2095179	2095325	2	AATCTCCTAAAATCCTGTCACTAAG	1

Prediction of antibiotic resistance genes

The detection of resistance genes to antibiotics revealed in total three genes: *ileS* with CARD and MEGARES 2.0 (both with 88.16 % of identity), *rpoB* only with CARD (identity: 88.56%) and *erm(X)* with ARG-ANNOT, CARD, MEGARES 2.0, NCBI-AMRFinderPlus and Resfinder with values of identity above 99.1%. The values of coverage percentage for every hit were above 99.2% (Table 4).

Table 4: Antibiotic resistance genes identified in *Bifidobacterium breve* 110^{1A} with ABRIcate using some databases.

Database	*ileS*	*rpoB*	*erm(X)*
ARG-ANNOT	-	-	99.18 (99.88)
CARD	88.16 (99.22)	88.56 (99.86)	99.18 (99.88)
MEGARES	88.16 (99.22)	-	99.88 (100)
NCBI-AMRFinderPlus	-	-	99.88 (100)
ResFinder	-	-	99.18 (99.88)

Identity values following by coverage percentage values in parentheses.

Genomic plasticity analysis

Seven genomic island (GEI) were predicted in *Bifidobacterium breve* 110^{1A}: two Resistance Islands (RI), five Genomic Islands (GI). When GIPSy software identified islands as more than one kind of island at the same time, they were considered as Genomic Islands, GI (Figure 6). Comparing with the other strain genomes, there were not identified exclusive genomic islands for our strain.

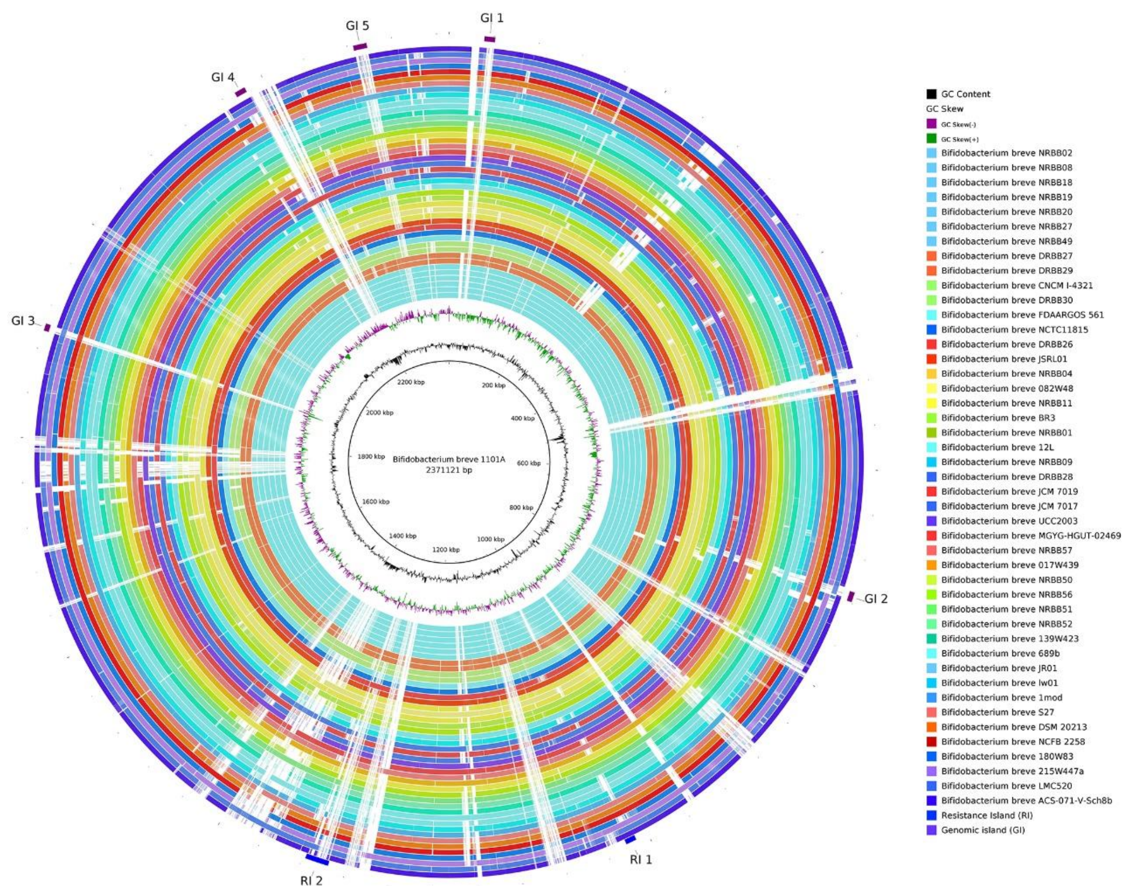

Figure 6. Circular comparative map of 46 genomes of *Bifidobacterium breve* by BRIG. The 110^{1A} strain was the reference in the central position with the first three inner rings showing its size, GC content and GC skew. The outer rings are representations of genomic islands founded with GIPSy (RI and GI)

Some genes in resistance islands (RI1, RI2) are related to antibiotic resistance (methyltransferase domain, bacterial regulatory proteins- tetR family, VanZ like family), metal ion binding as (Cupin 2 conserved barrel domain protein). Moreover, DNA binding and transcription regulator (2 genes for RelB antitoxin), DNA binding (addiction module antidote protein HigA, PemK-like, MazF-like toxin of type II toxin-antitoxin system, 2 gene helix_turn _helix domain), tranferases (HipA-like C-terminal domain, FR47-like protein), Major facilitator Superfamily (MFS). Figure S1-S2.

Gene content in Genomic Islands (GI1-GI5) were involved in processes as transport proteins (*ytfL*, transporter associated domain, ABC transporter) DNA binding (2 gene helix_turn _helix lactose operon repressor, 2 genes DNA binding to transcription regulation, transcriptional regulatory protein C terminal). Other genes involved in active transport of solutes (4 codifying for bacterial extracellular solute-binding protein, transmembrane transport), integral component of membrane (6 codifying for binding-protein-dependent transport system inner membrane component, branched-chain aminoacid transport system/permease component and branched-chain amino acid ABC transporter, permease protein). About metabolism and transport of carbohydrates (glycosyl hydrolase family 36 N-terminal domain) with α-galactosidase activity, and others (FGGY kinase family, raffinose synthase or seed imbibition protein Sip1, ABC-type sugar transport system periplasmic component), uptake and translocation of the essential macronutrient phosphorus (phosphate transporter). Among other processes (lysR substrate binding domain, DNA-binding transcription factor 2, sugar phosphate isomerase involved in capsule formation). Figure S3-S7.

Pangenome analysis

Pangenome size calculation identified 5943 genes in total, that were grouped based on their distribution in the 46 genomes and organized in 4 classes according to the number of the strains that shared them (Figure 7). Core genes were 1174 (present in 99 - 100% of the strains), soft-core genes were 163 (present in 95 - 99% of the strains), shell genes were 1197 (present in 15 - 95% of the strains) and cloud genes were 3409 (present in 0 - 15% of the strains).

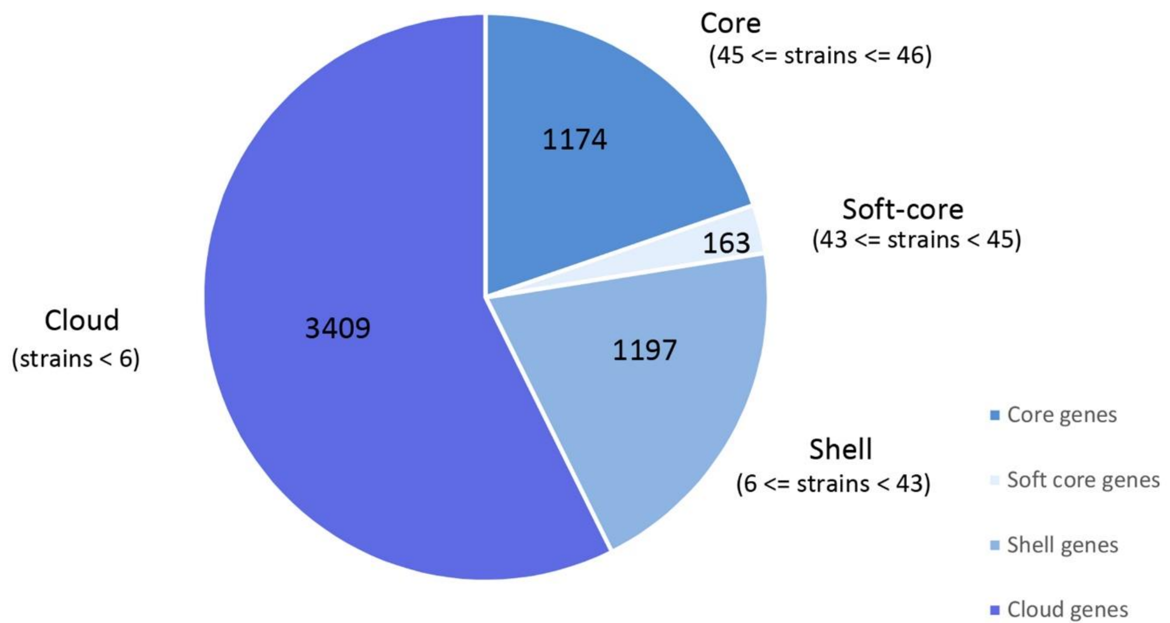

Figure 7. Pie chart representing the number of genes shared in the core, softcore, shell and cloud genome for the 46 *Bifidobacterium breve* strains.

The pangenome were visualized in a matrix that shows a block uninterrupted (core genome) and vertical bars (accessory genome) with the strains in horizontal orientation (Figure 8). There are some genomes disposed in similar form such as NRBB02, NRBB8, NRBB18, NRBB19, NRBB20, NRBB27, NRBB49. A second group with other gene disposition with few differences composed by DSM 20213, NCTC11815, NRBB01 and FDAARGOS561. Also a couple of genomes composed by CNCM I4321 and DRBB30.

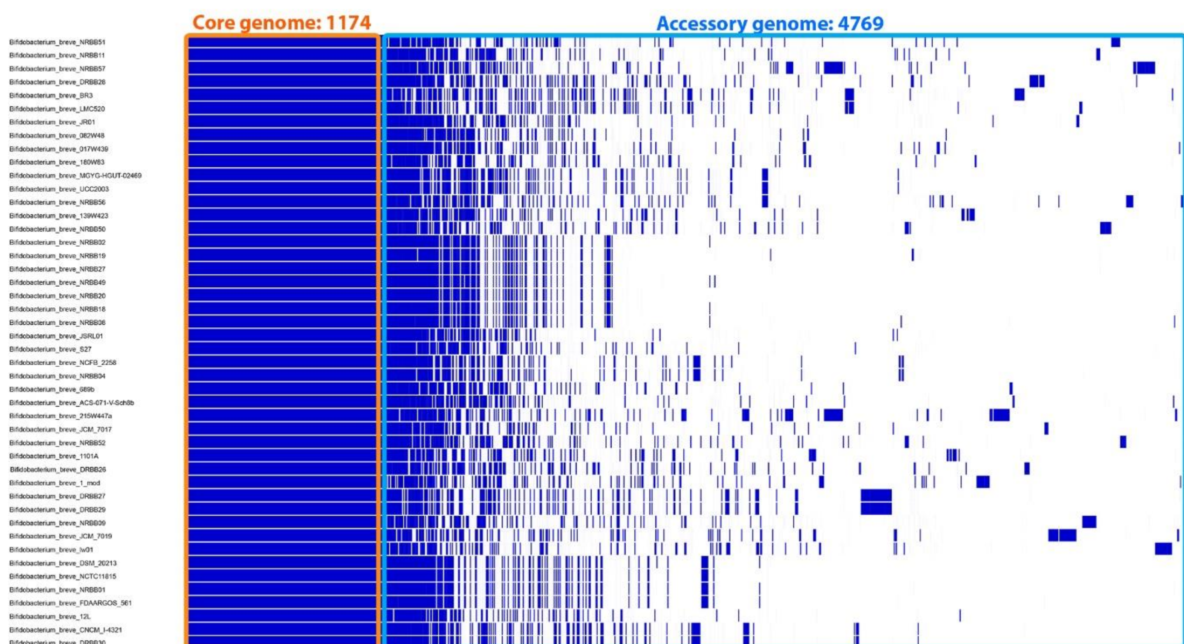

Figure 8. Matrix representation of the pangenome based on the presence-absence of genes of the pangenome using Roary.

Therefore, the estimation of the pangenome openness, with BPGA, showed the exponential parameter (*b* = 0.3047) for the empirical power law equation of the pangenome curve (orange line) that suggested to consider it as an open pangenome which have been increased in size with the addition of new genomes in the analysis (Figure 9). The core genome curve (purple line) have a curve with tendency to decrease with the addition of new genomes in the analysis. It could be due to some genes that previously were considered as core genes, were not continue when new genomes were added and they were considered part of the accessory genome.

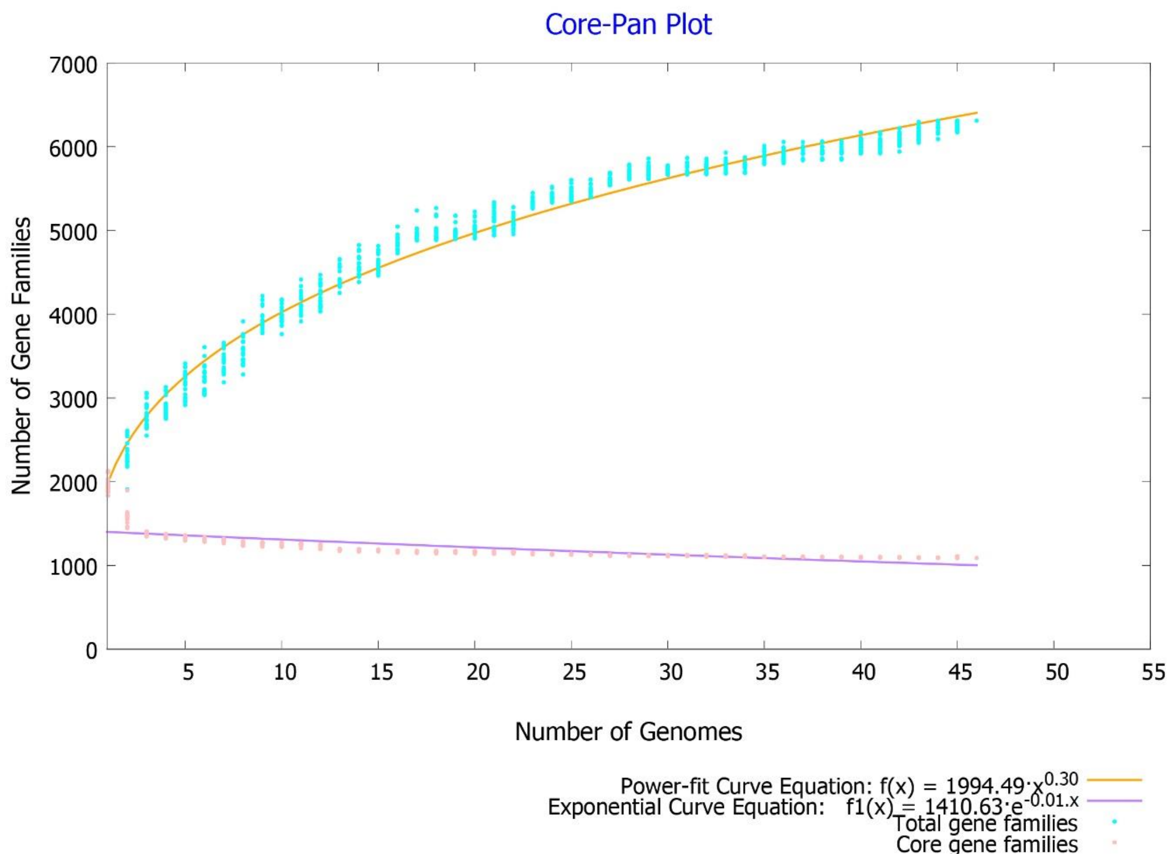

Figure 9. Pangenome-core plot with *Bifidobacterium breve* strains.
The pangenome curve (orange line) represents the new genes when new genomes are added to the analysis. The core genome curve (purple line) represents the genes in common between all the strains when new genomes are added.

The distribution of COG for the pangenome showed that the core genome present higher values were for amino acid transport and metabolism and translation, ribosomal structure and biogenesis; the accessory genome showed high values for COG terms related also with replication, recombination, repair and metabolism and transport of carbohydrates.

Furthermore, unique genes showed a concentration of genes for functions related to the process of replication, recombination and repair (Figure 10).

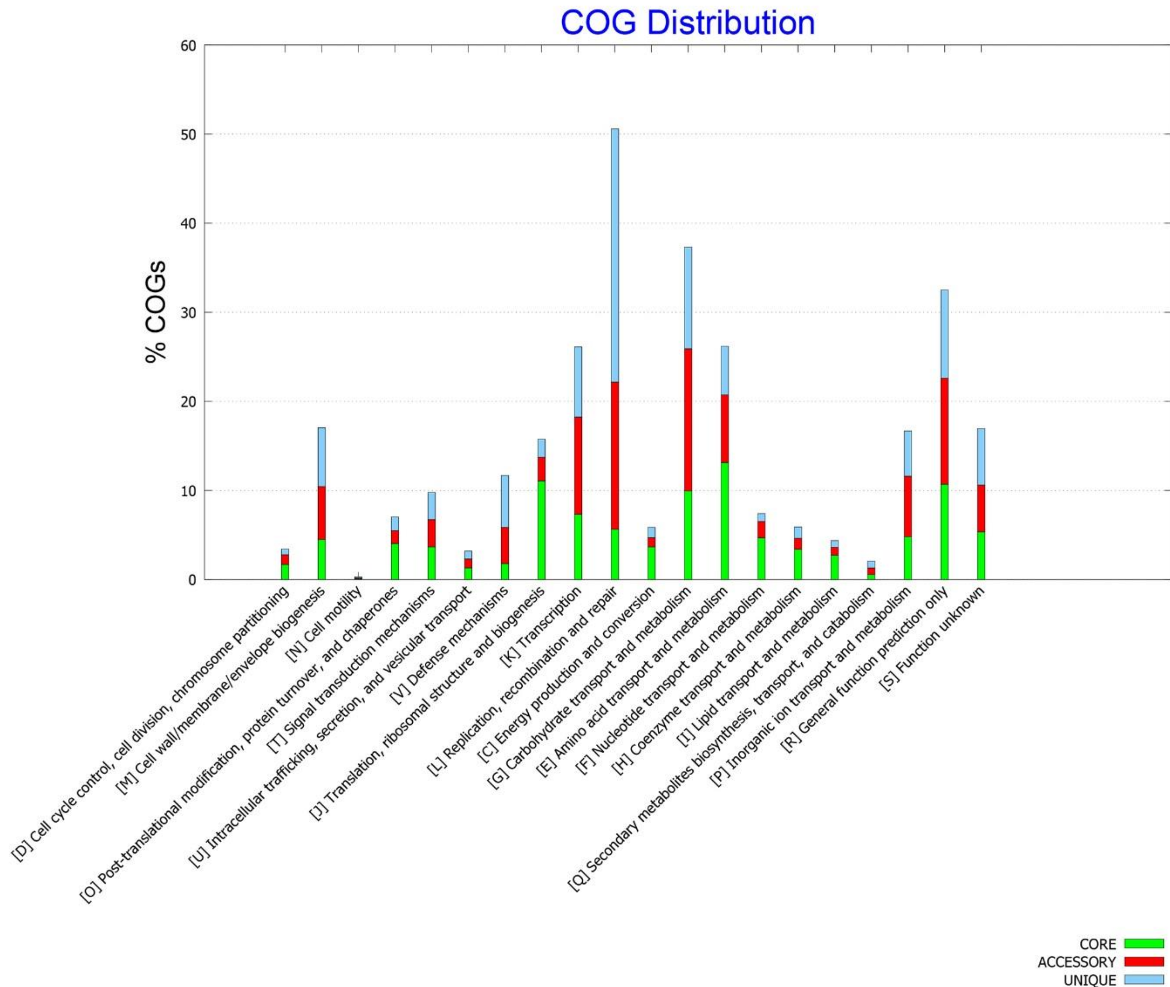

Figure 10. COG distribution of core, accessory and unique genes of *Bifidobacterium breve* strains.

With regard to the phylogenetic tree based on SNP variants from core genome, the 110^{1A} strain formed a clade with strains from other countries, such as and DRBB26, NRBB09, DRBB27, DRBB29 (Netherlands), 1 mod (non-determinated) and Asian countries, BR3 (Korea), lw01 (China), JCM7019 (Japan). Figure 11. It seems there is no a marked disposition of the leaves according to the geographical distribution, although some strains formed a clade with members from Netherlands, but are clonal strains. Only in part, Ireland strains grouped part of its members. Therefore, there were no evident differences respect to the source of isolation. A major part of samples was from feces and gut (Figure 11). However, there were few samples from other sources as 12L isolate from human milk forming a clade with gut samples (CNCMI4321 and DRBB30); ACS071VSch8b

isolate from vagina was forming a clade with a gut sample (DRBB28). The LMC520 isolate from the environment formed a clade with feces samples (BR3, lw01 and JCM 7019). In contrast, the unique sample with clinical origin was FDAARGOS561 and was rooting the tree along with samples from intestine (NCTC11815) and gut (NRBB01).

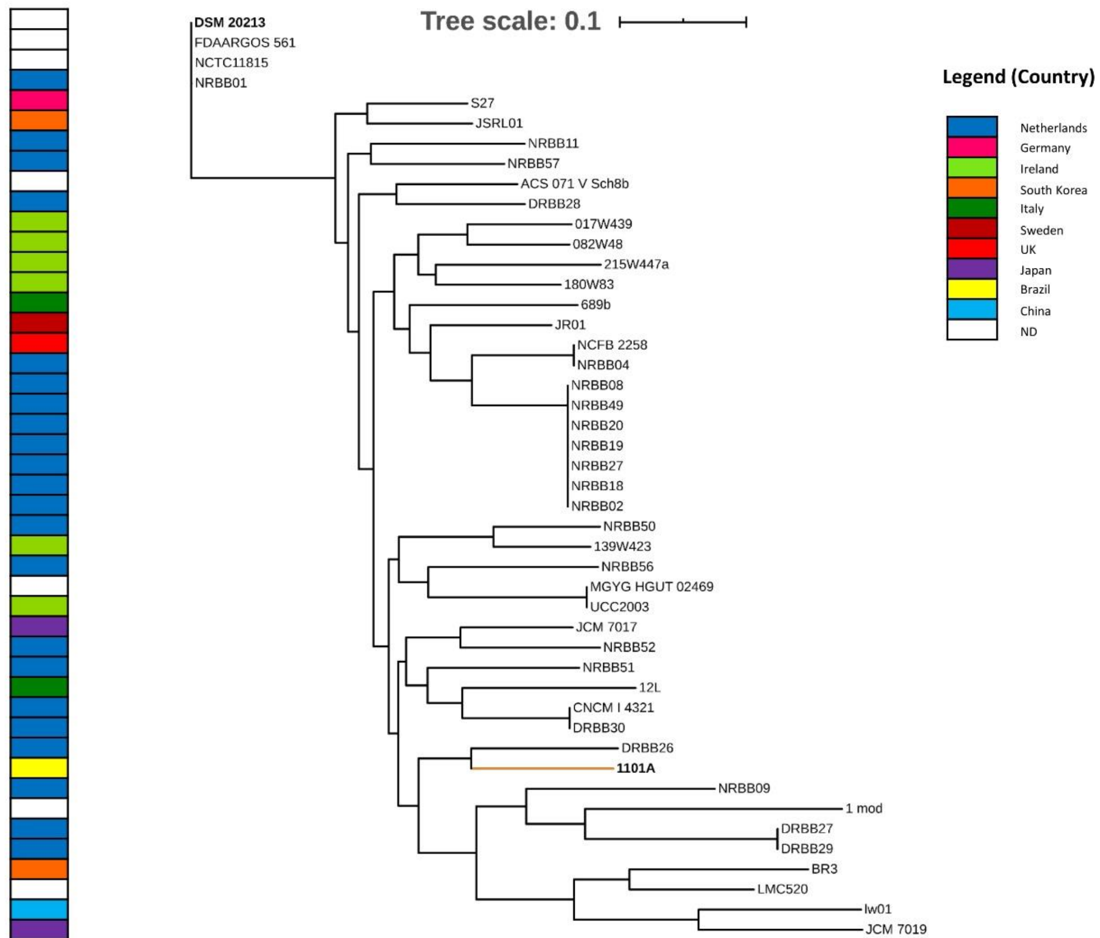

Figure 11. Phylogeny of *Bifidobacterium breve* based on variants (SNP) of core-genome using Parsnp and iTOL.

The number of unique genes was strain-specific in the pangenome analysis, they were in range of 0 – 181 (Figure 12) with a mean value of 34 genes per genome. *Bifidobacterium breve* 110^{1A} presented 63 unique genes associated with processes, such as transmembrane transport, membrane components, DNA processes, carbohydrate metabolism, among others (Table S8). Thirty genes of them were labelled as "uncharacterized protein" or without known function that represent approximately 47% of total. Of this group, 10 undetermined genes had GO terms related to integral components of membrane and 2 undetermined genes were related to DNA binding (Table S8). From unique genes with identified name, there were 5 genes involved in carbohydrate metabolism (alpha-

galactosidade, beta-galactosidase, dTDP-4-dehydrorhamnose reductase, glycosidase) and 1 gene for transport of maltose (maltose ABC transporter permease). Referring to genes related to DNA processes, there were 7 genes that received GO terms of DNA integration, transposition and DNA binding (IS3 family transposase, integrase, proteins containing domains for IS or integrase). In addition, other 2 genes related only to DNA binding and regulation of transcription were predicted (putative transcriptional regulator XRE family, transcriptional regulator *LacI* family). Moreover, 6 genes related to cellular membrane were identified (ABC transporter substrate-binding protein, ABC transporter permease protein probably xylobiose porter, MFS transporter, putative membrane protein, histidine kinase, sortase family protein, LPXTG-motif cell wall anchor domain protein) with functions, according to GO terms, such as integral component of membrane, membrane transport, plasma membrane.

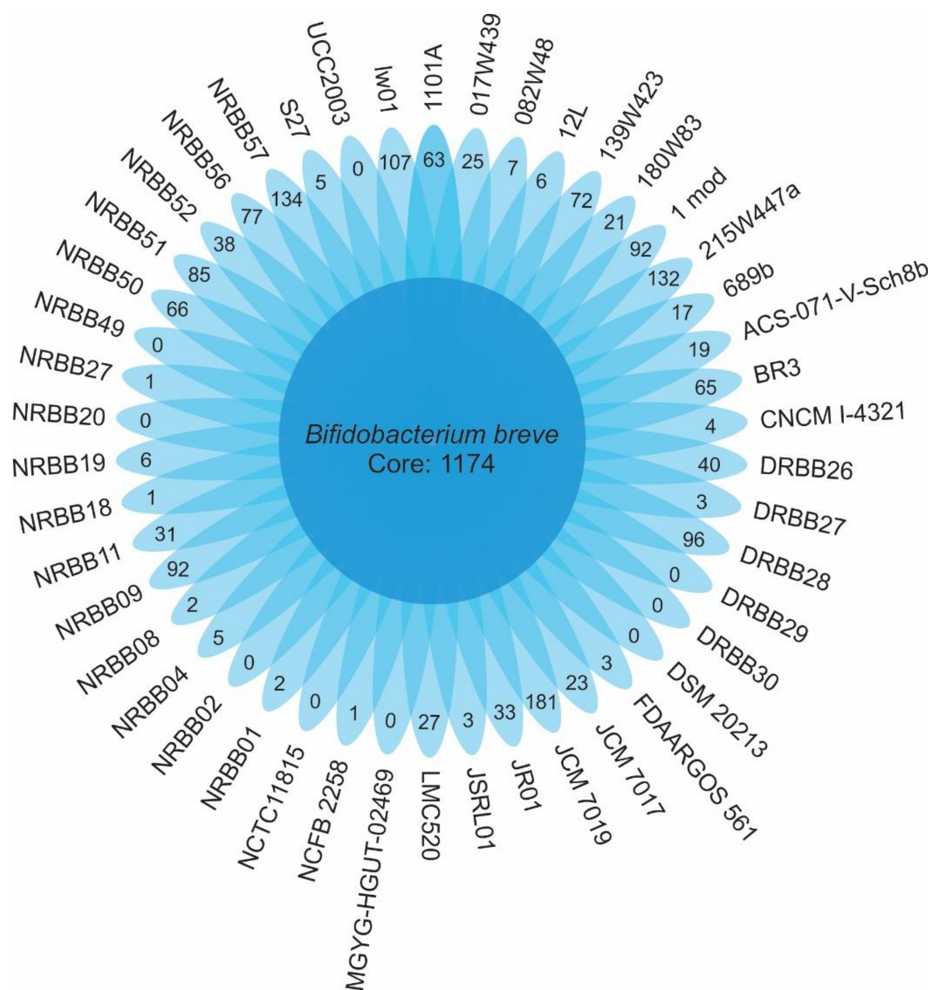

Figure 12. Flower plot diagram showing the core-genome size of all 46 *Bifidobacterium breve* strains (central area) and unique genes for each strain (flower petals)

Identification of genes related to probiotic features

The analysis found 18 genes related to adhesion, some of them were sequences codifying for sortases, related as *Tad*-like protein (A, B, C, Z), *Tad*E and *Tad*F. There were 2 sequences codifying for secretion proteins such as LPXTG-type cell surface and only 1 sequence for *lux*S or autoinducer-2 (AI-2) (Table 5). Furthermore, it was identified 39 CDS involved in resistance to general stress, among them, multiple subunits of *F0F1*-type ATPase (a, b, alpha, beta, delta, epsilon and gamma).In adition to this, There were some genes codifying for chaperons were *DnaK, GroEL* and*, GroES* as well as *DnaJ* and *GrpE*. In the same form, 2 genes for *Clp*P and *Clp*X, identified to codify for chaperons and SOS response genes such as *LexA, RuvA, RecA,* and *MutY* (Table 6). Respect to genes associated to repair and protection genes, were identified 10 sequences codifying for nucleoside triphosphate pyrophosphohydrolase (*mutT*) and DNA-binding ferritin-like protein (*dps*), methionine sulfoxide reductase (*msr*). Moreover, other sequences codifying for copper-transporting ATPase (*cop*A), subunit A of the excinuclease ATP-binding cassette ABC complex (*uvr*A) were identified in the genome (Table 7). Additionally, there were 11 genes involved in production of vitamins, such as B2, B9 and B12. Some of them, *ribF, ribU and ribF* corresponding to riboflavin (B2); *folC, folE, folK, folP* for folic acid (B9) and *cobQ* for cobalamin (B12). (Table 8).

Table 5: Identified genes potentially involved in adhesion mechanisms in *Bifidobacterium breve* 110[1A]

Sequence ID	Name	Accession number	Identity	Length	e-value
IAEJDAMG_00064	Sortase srtA2	ACD98830.1	75.714	420	0
IAEJDAMG_00138	TadZ-like protein	ABE94843.1	99.123	228	6.18E-164
IAEJDAMG_00139	TadA-like protein	ABE94844.1	98.82	339	0
IAEJDAMG_00140	TadB-like protein	ABE94845.1	98.958	192	1.42E-136
IAEJDAMG_00141	TadC-like protein	ABE94846.1	99.083	218	3.13E-157
IAEJDAMG_00142	TadE	ABE94847.1	98.947	95	2.67E-66
IAEJDAMG_00143	TadF	ABE94848.1	98.413	126	9.80E-88
IAEJDAMG_00144	Conserved hypothetical secreted protein	ABE94849.1	100	112	4.70E-80
IAEJDAMG_00149	srtA1	ACD98732.1	76.35	389	0
IAEJDAMG_00304		VUX38545.1	98.969	97	4.83E-70
IAEJDAMG_00343	LPXTG-type cell surface-anchoring secretion proteins (BLD_1637)	ACD99082.1	76.471	34	1.06E-08
IAEJDAMG_00344	LPXTG-type cell surface-anchoring secretion proteins (BLD_1638c)	ACD99083.1	100	23	6.43E-09
IAEJDAMG_00565		-	92.732	399	0

IAEJDAMG_00714	Sortase	ACD98225.1	89.091	165	4.10E-111
IAEJDAMG_00939	TadV type IV secretion peptidase	ABE95585.1	98.485	132	4.64E-88
IAEJDAMG_01041		ACS47779.1	77.528	267	1.15E-160
IAEJDAMG_02029	srtA3	ACD98903.1	91.15	226	1.94E-160
IAEJDAMG_00502	luxS	VUX37541.1	97.561	164	1.99E-121

Table 6: Identified genes potentially involved in resistance mechanisms to stress in *Bifidobacterium breve* 110^{1A}

Sequence ID	Name	Accession number	Identity	Length	e-value
IAEJDAMG_00075	hsp20	ABL75149.1	97.605	167	4.51E-127
IAEJDAMG_00129	DnaK	AAT90384.1	99.521	626	0.00E+00
IAEJDAMG_00130	DnaJ	AAT90385.1	99.559	227	5.36E-166
IAEJDAMG_00130		EEB21092.1	72.727	231	1.08E-113
IAEJDAMG_00131	GrpE	AAT90386.1	100	337	0.00E+00
IAEJDAMG_00166		WP_109057771.1	86.667	270	4.22E-175
IAEJDAMG_00297	F1F0-ATPase a	KXS24185.1	98.885	269	0
IAEJDAMG_00299	F1F0-ATPase b	WP_101673713.1	100	172	9.79E-123
IAEJDAMG_00300	F1F0-ATPase delta	KXS24188.1	99.64	278	0
IAEJDAMG_00301	F1F0-ATPase alpha	QFZ79728.1	100	543	0
IAEJDAMG_00302	F1F0-ATPase gamma	KXS24190.1	99.349	307	0
IAEJDAMG_00303	F1F0-ATPase beta	QFZ79730.1	100	490	0
IAEJDAMG_00304	F1F0-ATPase epsilon	KXS24192.1	98.969	97	4.78E-70
IAEJDAMG_00388		WP_100496451.1	96.29	566	0
IAEJDAMG_00458	MutY	VUX33106.1	98.438	320	0
IAEJDAMG_00778		WP_044088523.1	96.875	480	0
IAEJDAMG_00830	ClpP	RDX30841.1	100	227	4.30E-173
IAEJDAMG_00830		WP_026502573.1	83.26	227	4.78E-138
IAEJDAMG_00831	ClpX	VUX37037.1	99.779	453	0
IAEJDAMG_00835		AUD86824.1	99.784	462	0
IAEJDAMG_00872		WP_109057771.1	85.926	270	8.79E-171
IAEJDAMG_00944		WP_044088362.1	97.649	553	0
IAEJDAMG_01015		AAT11512.1	91.083	314	0
IAEJDAMG_01051		ABA33885.1	99.213	381	0
IAEJDAMG_01051		ADQ03125.1	96.063	381	0
IAEJDAMG_01064	RuvA	WP_052789507.1	99.038	208	2.74E-151
IAEJDAMG_01306	RecA	VEG21919.1	100	392	0
IAEJDAMG_01308	ClgR	VUX34072.1	100	172	1.82E-124
IAEJDAMG_01315		WP_015450243.1	84.211	228	7.87E-147
IAEJDAMG_01373		WP_109057771.1	89.63	270	0
IAEJDAMG_01398	LexA	WP_052789257.1	99.156	237	3.26E-178

IAEJDAMG_01485		AAW49584.1	99.885	869	0
IAEJDAMG_01486	AAW49585.1	AAW49585.1	99.688	321	0
IAEJDAMG_01493	GroEL	AAT95329.1	95.396	543	0
IAEJDAMG_01672		WP_033505167.1	85.088	342	0
IAEJDAMG_01680		KRN79975.1	48.165	218	7.70E-75
IAEJDAMG_01786	GroES	WP_106629637.1	98.969	97	5.93E-68
IAEJDAMG_01786		WP_007053011.1	95.876	97	1.65E-66
IAEJDAMG_01875	NADH oxidase	VUX32225.1	99.107	448	0
IAEJDAMG_01875	NADH peroxidase	AFJ17563.1	71.652	448	0
IAEJDAMG_01900		AAX52929.1	99.438	889	0
IAEJDAMG_02044	Thioredoxin reductase	VUX33397.1	100	339	0
IAEJDAMG_00830	Bile salt hydrolase	RDX30841.1	100	227	2.46E-173
IAEJDAMG_00041	bshA	VUW79572.1	99.153	472	0.00E+00

Table 7: Identified genes potentially involved in repair and protection of DNA and protein mechanisms in *Bifidobacterium breve* 110^{1A}

Sequence ID	Name	Accession number	Identity	Length	e-value
IAEJDAMG_00027	DNA-binding ferritin-like protein (DPS)	VEG20761.1	99.371	159	2.30E-118
IAEJDAMG_00087	mutT	AUE19745.1	100	137	4.10E-103
IAEJDAMG_00027		ADQ02354.1	98.742	159	5.91E-118
IAEJDAMG_00080	msr	BAQ99105.1	99.704	338	0
IAEJDAMG_00262	copA	VUX34953.1	99.297	853	0.00E+00
IAEJDAMG_00502		VEG23308.1	87.342	158	5.04E-108
IAEJDAMG_01035	UvrA	WP_103619798.1	99.491	982	0.00E+00
IAEJDAMG_01035		WP_044088239.1	96.036	1009	0.00E+00

Table 8: Identified genes potentially involved in biosynthesis of vitamins in *Bifidobacterium breve* 110^{1A}

Sequence ID	Name	Accession number	Identity	Length	e-value
IAEJDAMG_00138	Cobalamin, B12 (cobQ)	KXS24078.1	99.627	268	0
IAEJDAMG_00868	Riboflavin, B2 (ribZ)	VUW80559.1	99.268	683	0.00E+00
IAEJDAMG_00957		RDX31668.1	99.492	394	0
IAEJDAMG_01369	Folic Acid, B9 (folC)	VEG21982.1	99.25	533	0.00E+00
IAEJDAMG_01400		WP_003810784.1	92.5	320	0.00E+00
IAEJDAMG_01456	Riboflavin, B2 (ribU)	VUX35278.1	98.165	218	4.44E-156
IAEJDAMG_01631	Folic Acid, B9 (folK)	QFV12943.1	99.254	536	0.00E+00
IAEJDAMG_01632	Folic Acid, B9 (folP)	QHP50922.1	95.862	290	0
IAEJDAMG_01633	Folic Acid, B9 (folE)	QHP52232.1	99.515	206	4.01E-153
IAEJDAMG_01712	Riboflavin, B2 (ribF)	VUX29772.1	99.198	374	0.00E+00

Discussion

Taxonomy, phylogenomics, and synteny analysis

Our strain, 110^{1A}, is confirmed as *Bifidobacterium breve* based on the genomic analysis (ANI) and it has been closely related to DRBB26 strain that was isolated from an infant in Netherlands in 2014 (Table 1). The more proximal *Bifidobacterium breve* genomes, in decreasing order according to ANI values, when compared with *B. breve* 110^{1A} corresponded to strains, 689b from infant feces (Italy), ACS-071-V-Sch8b from human vagina (USA), S27 from infant feces (Germany), JSRL01 from feces of baby human (South Korea), JR01 from human stool (Sweden), NRBB04 from gut (Netherlands). No correlation was evident between these strains respect to the countries of origin. Noteworthy of mention, only 3 strains were involved in experimental studies to prove their probiotic condition with complete genome: UCC2003 (Fanning et al., 2012), lw01 (Li et al., 2021; Wang et al., 2019) and BR3 (Kwak et al., 2015; Lee et al., 2013). Any of them showed a close relationship with the clade of 110^{1A} strain. However, due to these results, the proximity of these probiotic strains with other ones could suggest the possible condition of probiosis of, especially, JCM 7019, MGYG-HGUT-02469 and JSRL01 strains; although, also of the members of the clades closely related, so experimental studies are necessary and should be performed.

As was shown in our phylogenetic results, the closer relationship of *B. breve* strains with the *B. longum* strain than other outgroup strains was mentioned in other studies, when *B. breve* fall into the *B. longum* clade based on orthologous genes and a multilocus approach (Bottacini et al., 2014a; Ventura et al., 2006). The strain of study, *B. breve* 110^{1A}, formed a clade with DRBB26 strain and contrasting our results with a previous phylogenetic study (Bottacini, Morrissey, Roberts, et al., 2018), this last strain is placed in the group 5 of that tree, and thus, our strain 110^{1A} could be placed in that clade. Moreover, it is important to mention that there are some clades that remain and other ones that are different when our results and the other are compared. For instance, at Botaccini study, the DRBB26 strain formed a clade with NRBB01, however, this last strain appeared in another clade in our phylogenomic analyses. Some clades that remain were the clonal strains (clonal group 1, 2 and 3) Figure 2.

The *Bifidobacterium* genus has shown a highly conservative level of synteny (Ventura et al., 2007); even so, there is a degree of diversity related to every species. The synteny of gene blocks was present in most of the evaluated genomes of *Bifidobacterium breve*, including 110^{1A} strain that was showed in the existence of locally collinear blocks (LCBs) on multiple alignment analysis. Nevertheless, three genomes presented a different genome organization such as inversions on regions of different size. Two genomes of them (ACS-071-V-Sch8b and JCM7017) were reported previously in a comparative study of this species, *Bifidobacterium breve* (Bottacini et al., 2014a), where was discarded the possibility of genome assembly errors in both cases. In addition to this, the strain BR3 presented two inversions and more evaluation should discard an error of assembly. Although, the genus *Bifidobacterium* exhibits a high degree of synteny, there are genomic regions affected by inversions and events of insertion and deletion (Ventura et al., 2007).

Prediction of mobile elements and genes codifying for bacteriocins and CRISPR-Cas systems in *Bifidobacterium breve* 110^{1A}

Regarding mobile elements, it is known that plasmids provide new characteristics to probiotic bacteria that increase the possibility to survive in other environmental conditions, acquire additional properties in bacterial metabolism, adherence, movement (Abriouel et al., 2019) and antibiotic resistance, this last one, an undesired attribute for a probiotic bacterium. Although plasmids were not detected in *B. breve* 110^{1A}, they were reported in the genus *Bifidobacterium* (Cui et al., 2015) previously. With regard to studies in *B. breve,* plasmids were detected in 40% of 42 analyzed strains (Iwata & Morishita, 1989); in contrast, they were absent in 106 evaluated isolates of this species in another study (Sgorbati et al., 1982). There is a reduced number of complete genomes of *Bifidobacterium breve* with plasmids in GenBank corresponding to only two strains, as NCFB 2258 (O'Riordan & Fitzgerald, 1999) and BR3 (Kwak et al., 2015), a fact that coincide with the absence of plasmids in our 110^{1A} strain.

With respect to the predicted prophage in 110^{1A} genome, the region was incomplete and did not contain typical genes that codify for structural proteins (such as tail, capsid), DNA regulation, integrases, lysis and others for a functional bacteriophage but some prophage-like proteins. Instead, this prophage region could be considered as a defective prophage and it presented some genes involved in the response to oxidative stress (Fe-S cluster assembly ATPase SufC, cysteine desulfurase, SUF system NifU family Fe-S cluster

assembly protein), biosynthesis of glycogen (glucose-1-phosphate adenylyltransferase), among other processes (RNA methyltransferase, histidine triad domain protein, PhoH family protein). Prophages could represent a future event of lysis for the bacteria or they could provide some additional properties, as a double-edged sword (Mahony et al., 2018). The frequency of integrated prophages is common in bacterial genomes (Casjens, 2003) and a considerable part of them is defective possibly as a product of the phage domestication event (Bobay et al., 2014). About insertion sequences (IS), previous studies have reported the presence of the mentioned IS families in the *Bifidobacterium* genus (Bottacini et al., 2014; Mancino et al., 2019). Some IS elements, such as IS3, and more specifically the frequency in our results, were reported previously and it was referred as the IS family with the most widespread and the major number of identified IS in *Bifidobacterium* (Mancino et al., 2019). Contrasting, the study of the mobilome of *Bifidobacterium breve* showed the IS30 as the most frequent IS family (Bottacini et al., 2014a).

Genes codifying for bacteriocins were not identified in the analyzed *Bifidobacterium breve* 110^{1A}. These molecules allow the competence against other bacteria, being specific for an intra-genus or less specific for inter-genus strains, in the gut environment and it is a desirable feature for a probiotic bacteria. Some studies have reported some produced bacteriocins in the genus *Bifidobacterium* (Liu et al., 2015; Sarkar & Mandal, 2016). However, in other studies, the production of these compounds in *Bifidobacterium* was relatively rare (Walsh et al., 2015) or no reported in gut samples (Zheng et al., 2015). Although, any gene for bacteriocin was not predicted, the antagonism showed in *in vitro* test (Souza et al., 2013) could be due to other mechanisms of the strain that was evident in probiotic bacteria such as adhesive capability which allows the inhibition of pathogens colonization by competitive exclusion (Plaza-Diaz, 2019) or reduction of pH in the environment with the glucose fermentation and production of short chain fat chain (SCFA) seen in bifidobacteria (Den Besten et al., 2013; Duncan et al., 2009). In reference to the identification of CRISPR regions, these components are important for the bacteria to deal with phage sequences from the environment, especially in the gut tract where there is a viral community. Phages can lyse bacteria and it can affect drastically the survival of bacterial population in the process of production of probiotics, fermentation time, taste, among other parameters (Yang et al., 2020). Considering that phages are resistant to the pasteurization process and its elimination is difficult, the search of probiotics with the

ability to be protected from phages and other DNA invaders such as plasmids (Hidalgo-Cantabrana et al., 2017) is important in the terms of production. In our results, 3 CRISPR loci were identified in 110^{1A} genome, although there were not any Cas proteins. These orphan CRISPR regions were considered as no functional and it was not possible to determine the type and subtype of the systems due to the necessary presence of Cas proteins for its classification. The occurrence of CRISPR-Cas systems in the genus *Bifidobacterium* was of 77% of the 48 analyzed species, in a previous study, representing a high presence in the genus (Briner et al., 2015).

Prediction of antibiotic resistance genes

The antibiotic resistance genes founded in 110^{1A} genome were *IleS, rpoB* and *erm(X)*. *IleS* gene confers resistance to mupirocin (Gueimonde et al., 2013) and *rpoB* gene confers resistance to rifampicin (Lokesh et al., 2018). Both genes were detected in the other 45 genomes of *Bifidobacterium breve* in an extension of this predictive analysis. These results were supported by other studies where resistance to mupirocin is considered as intrinsic resistance in bifidobacteria (Gueimonde et al., 2013; Serafini et al., 2011). The *erm(X)* gene was identified in *Bifidobacterium breve* 110^{1A} using different resistance databases. This gene has the capacity of enzymatically modify the DNA sequence of 23S rRNA gene by methylation to avoid the binding of macrolides, lincosamides, streptogramin B and conferring resistance (Chen et al., 2007).

The presence of homologous to gene *erm* was reported in *B. longum* and in some strains of *B. breve* (BR-14 and DPC6330) and it was suggested the acquirement by events of horizontal gene transfer, HGT (Martínez et al., 2018). More specifically, *erm(X)* was reported in *B. thermophilum* and *B. animalis subsp. lactis* strains from pigs, *erm(X)* was founded in the Tn5432-like transposon, similar transposon founded also in opportunistic pathogenic bacteria as corynebacteria, arcanobacteria, propionibacteria (van Hoek et al., 2008). This antibiotic resistance gene were founded in two strains of *B. breve* in another study (Bottacini et al., 2018): NRBB51 (three copies) and DRBB26 (two copies) interleaved by genes codifying transposases. In the present study, only a transposase IS1249 (IS256 family) was detected in the vicinity of *erm(X)* and both within the putative resistance island 2 (RI2), it could suggest the island was acquired by HGT. Contrasting these *in silico* results with the experimental essays of antibiotic susceptibility in *B. breve* 110^{1A} (Souza et al., 2013), this suggests the identified *erm(X),* in this study, could be the

responsible gene for the showed resistance to erythromycin. Currently, the species *Bifidobacterium breve* is considered with QPS status, Quality Presumption of Safety (Andreoletti et al., 2012) due to not be related with any infective process. Although the presence of genes of resistance is not considered a *per se* safety issue is necessary to determine the possible transference of this resistance (Lokesh et al., 2018).

Genome plasticity analysis

The analysis to predict genomics islands (GEIs) in *Bifidobacterium breve* 110^{1A} showed seven islands: Respect to genes in resistance islands (RI1 and RI2), some of them were involved in resistance to tetracycline resistance (bacterial regulatory proteins- tetR family) and vancomicin (VanZ like family). The rest of the genes had a variety of functions such as DNA binding, transferases and membrane transport. Gene content in Genomic Islands (GI1-GI5) are concerning to transport of solutes (up to 4 genes), components of membrane, DNA binding, transport and metabolism of carbohydrates such as genes related to galactose and raffinose.

Pan-genome analysis

In a previous study, an analysis with MCL to perform a pan-genome analysis with *Bifidobacterium breve*, the estimation of pangenome size was 3667 gene families and the core genome was composed by 1307 gene families using 13 genomes with eight complete and five draft (Bottacini et al., 2014). In another study, the pangenome size estimation was 6138 gene families and the core genome size was 1282 gene families using 73 genomes with 37 complete and 36 draft (Bottacini, Morrissey, Esteban-Torres, et al., 2018). A recent study, the pangenome of the species were evaluated with 55 genomes with 46 draft originally from China, 6 complete and 3 draft from GenBank, resulting in a pangenome composed by 6707 gene families and the core genome composed by 1111 gene families (Liu et al., 2020). Considering our results with 46 complete genomes of *Bifidobacterium breve,* with a pangenome size of 5943 genes and a core genome size of 1174 are closer to the second study (Bottacini, Morrissey, Esteban-Torres, et al., 2018) even with the considerable difference in number of used genomes. At this respect, the genome assembly and annotation are two main factors that determine the performance of a pan-genome analysis, as well as, an adequate number of complete genomes (Xiao et al., 2015). Furthermore, it is important to mention that draft genomes are useful on some

studies; however, due to the underrepresentation of some genes it could affect comparative studies, because of low read coverage or assembly errors (Veras et al., 2018; Klassen & Currie, 2012)

Respect to the result of the matrix representation of the pangenome, the similarity of group composed by NRBB02, NRBB8, NRBB18, NRBB19, NRBB20, NRBB27, NRBB49; the second group composed by DSM 20213, NCTC11815, NRBB01 and FDAARGOS561; and the third group composed by CNCM I4321 and DRBB30, concord with the ANI values = 0.9999 between member of each group. Moreover, the first 2 groups were reported as clonal strains (Bottacini, Morrissey, Roberts, et al., 2018). In reference to the estimation of the curve parameters of the pangenome suggested to consider it as an opened pangenome yet, because it has a tendency to continue increasing in size with the addition of new genomes in the simulations (Figure 9). Results that agree with a previous study with thirteen genomes considered the pangenome as opened (Bottacini et al., 2014) and with the seventy-three genomes as opened (Bottacini, Morrissey, Esteban-Torres, et al., 2018) of *B. breve* pangenome. However, the last reference (R. Liu et al., 2020) considered the pangenome as not fully closed but gradually saturated. The distribution of COG showed a high percentage of genes related to the process of replication, recombination and repair processes concentred in the core and accessory genome. In the second place, considerable genes involved in carbohydrates transport and metabolism in core and accessory genome. The COG items were distributed according to basic (core genome) and adaptative processes (accessory genome) respectively.

According to the phylogenetic tree results from SNPs of core genome, there was no a marked distribution of the strains according to geographical distribution and source of isolation. Referring to *B. breve* 110^{1A}, the closest strain was DRBB26 isolated from gut in Netherlands and without additional information. A comparative perspective and the pangenomic analysis allows itssegmentation and the identification of core, accessory and unique genes (Rouli et al., 2015). This last part, relevant to know the genetic repertoire strain-specific that could represents adaptive features to different niches, metabolic advantages or even genes related to pathogenicity. Comparative approach has been used in industrial starter cultures previously (Garrigues et al., 2013). The results about mean value of unique genes per genome, in this study, was of 34 genes considering 46 complete

genomes; contrasting with a previous study for the species of 53 unique genes per genome using 8 complete genomes (Bottacini et al., 2014). From the pool of unique genes, 47% were considered as uncharacterized proteins in this study that coincide with the predominant presence of genes with this label in a previous *B. breve* genomic comparison (Bottacini et al., 2014). More generally, genome projects identified hypothetical protein that range 30-40% of total identified proteins in prokaryotes (Naveed et al., 2018) that partially interfere in the characterization of the strain.Moreover, mobile elements were present in minor frequency (Bottacini et al., 2014), represented by transposases and integrases. It could be related with the unique genes and the form of acquirement by horizontal gene transfer (HGT). Therefore, unique genes involved in the metabolism and transport of carbohydrates (alpha-galactosidade, beta-galactosidase, dTDP-4-dehydrorhamnose reductase, glycosidase; and maltose ABC transporter permease) that could represent additional features that allow utilization of a wide variety of sources of energy, were found. Especially, alpha-galactosidade and beta-galactosidase are enzimes of interet due to they are used commercially to improve symptoms of lactose intolerance and the use of this carbon source (Roel et al., 2012). These enzimes were identified in bifidobacteria and the use of probiotic was considered to the alleviation of lactose intolerance (De Vrese et al., 2001; Han et al., 2014). Other genes codify for sortase family protein and LPXTG-motif cell wall anchor domain protein which were reported to be involved in cellular adhesion that represent a desirable characteristic in potential candidates for probiotic bacteria in the gut colonization stage (Alayande et al., 2020). Moreover, a MFS transporter also was predicted in the unique gene group and its overexpression was reported under acid bile exposition (Pfeiler & Klaenhammer, 2009).

Identification of genes related to probiotic features in *B. breve* 110^{1A}

Some of these genes were related to adhesion mechanisms, among them: sortases (*srtA1, srtA2* and *srtA3*), LPXTG-type proteins that probably has a role in the attachment to cells or mucus in the gut (Lee & O'Sullivan, 2010). Other identified protein was *Tad* (ABCEFZ), important for the pili assembly, structure necessary for the bacterial colonization (Motherway et al., 2011). Therefore, *luxS* has been involved with acid tolerance and adherence to intestinal epidermal cells in *Lactobacillus plantarum* (Jia et al., 2018).

About genes related to stress resistance, some were identified such as *F0F1*-type ATPase, working as proton pumps in gram-positive bacteria. The transcription and activity of this was evidenced in acid conditions in *Bifidobaterium* species (Hamon et al., 2011; Kullen & Klaenhammer, 1999; Matsumoto et al., 2004). Also identified genes (*DnaK, GroEL, GroES*) codifying for chaperones are involved in a general response to stress by protection, remotion of damage proteins among other related functions. Moreover, *DnaJ* and *GrpE*, identified in the analysis, have shown response (upregulation) to acid environments (Pfeiler et al., 2007). Some other genes (*LexA, RuvA, RecA,* and *MutY*) involved in SOS response. *LexA* have showed to be induced under temperature stress in *B. breve* and *recA* showed an upregulated response in heat stress (Zomer et al., 2009). Moreover, reduced survival in *L. reuteri* was demonstrated in mutation in *clp*, in exposition to bile (Whitehead et al., 2008) indicating its importance for the survival in conditions of acidity and general stress. In addition, it was identified 10 CDS with functions related to repair and protection of DNA or proteins such as DNA-binding ferritin-like protein (*dps*) and nucleoside triphosphate pyrophosphohydrolase (*mut*T) that act in the reparation due to the oxidative damage removing hydroxyl radicals (Lee & O'Sullivan, 2010). Other identified genes, such as mutants of methionine sulfoxide reductase (*msr*), have showed a reduced capacity in stress conditions in *L. reuteri* 100-23 (Walter et al., 2005) and mutants of copper-transporting ATPase (*cop*A) in *L. plantarum* WCFS1 have showed a reduced competitive ability in mouse (Bron et al., 2004). Therefore, *cop*A are involved in the nucleotide reparation in acid conditions in *L. helveticus* CNBL 1156 (Cappa et al., 2005). Furthermore, there were 11 genes involved in biosynthesis of vitamins. Some of them form part of operons for determinated vitamin. The integrity of the operons for vitamins are of interest for consider the functional its production by the *Bifidobacterium breve* 110^{1A}.

Conclusion

Probiotics are known for its beneficial health properties and there is an increasing tendency to look for and to select new candidates for human usage. The present study applied the genomic approach to explore features of *Bifidobacterium breve* 110^{1A} from healthy children feces. Furthermore, forty-five complete genomes and publicly available of this species were evaluated to identify exclusive genes for the strain through a

comparative analysis and a specific search for genes with relation to probiotic properties to identify the potentiality of this strain.

The analysis of this strain showed positive characteristics to be considered as a candidate based on the identified genes related to probiotic properties (adhesion, resistance to stress for acidity and heat, and production of vitamins) that suggest the survival of the strain in the gastrointestinal tract. Furthermore, some unique genes for 110^{1A} are involved in adhesion and metabolism of some carbohydrates that could represent a desired condition for bacteria to consume different sources of energy from the niche. Therefore, the reason for the antagonistic against pathogens on previous *in vitro* studies could be for other factors (i.e., competence by adherence, reduction of pH) due to the absence of bacteriocin genes. On the other hand, considering the safety criteria, a crucial point to elucidate is whether it is possible the transference of the antibiotic resistance gene *erm(x)* in experimental essays. Moreover, the evaluation of unique genes without identified function and the exploration of metabolic pathways. *In vitro* and *in vivo* studies with *Bifidobacterium breve* 110^{1A} should continue to provide supportive evidence of this strain as a probiotic bacteria considering that is the first strain with complete genome from Brazil for this species.

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5. CONCLUSIONS

- The closest relationship of *Bifidobacterium breve* 110^{1A} was with DRBB26 from the Netherlands. The evaluation of conservation of genome structure showed a high level of synteny of *Bifidobacterium breve* 110^{1A};

- The *Bifidobacterium breve* 110^{1A} genome presented an incomplete prophage and any plasmids. Antibiotic resistance genes were predicted, such as *ileS, rpoB*, corresponding to an intrinsic resistance and *erm(X)* with only a close transposase that could compromise its mobility;

- Pangenome calculation suggests considering the *Bifidobacterium breve* pangenome as opened considering the 46 complete genomes. Furthermore, the size pan-genome was estimated in 5943 genes, and the core genome was composed of 1174 genes. Therefore, the pangenome was considered opened;

- *Bifidobacterium breve* 110^{1A} genome showed 63 unique genes, a significant part of them involved in membrane processes (components and transport) and DNA integration (binding and integration), and some related to carbohydrate metabolism (alpha-galactosidase, beta-galactosidase, rhamnose, glycosidase), this last one could represent an advantage to degrade more sources of carbon;

- Genes related to adhesion, resistance to acidity conditions and general stress, reparation of DNA that could help the strain to resist and survive in the gastrointestinal tract, and genes codifying to the biosynthesis of vitamins were predicted could represent features desired for a probiotic bacterium.

6. PERSPECTIVES

- The evaluation of unique genes with uncharacterized function after the functional annotation to have the complete set of genes and exclusive properties of the 110^{1A} strain;

- The identification of operons and evaluation of its integrity to know whether some genes could be functional and confer some attribute to the strain;

- Evaluate metabolic ways of carbohydrates and vitamins in the study strain to know the spectrum of possible sources of energy that could be used and the potential production of beneficial compounds for the host, both considered positive features for a bacterial probiotic;

- Evaluation of possible mobility (inside and between genomes) of the identified genes involved in antibiotic resistance determines its potential horizontal transference (safety requirement).

7. SUPPLEMENTARY MATERIAL

Table S1: Functional annotation of genes in RI1 of *B. breve* 110[1A] using EggNOG.

Query	e-value	Preferred name	Best tax	Description
IAEJDAMG_00857	1.2e-233	XK27_00240	Bifidobacteriales	Fic/DOC family
IAEJDAMG_00858	7.2e-59	yccF	Bifidobacteriales	Inner membrane component domain
IAEJDAMG_00859	2.3e-159	ksgA	Bifidobacteriales	Methyltransferase domain
IAEJDAMG_00860	4.2e-67		Bifidobacteriales	Cupin 2, conserved barrel domain protein
IAEJDAMG_00861	2e-52		Bifidobacteriales	PemK-like, MazF-like toxin of type II toxin-antitoxin system
IAEJDAMG_00862	1.8e-37		Bifidobacteriales	RelB antitoxin
IAEJDAMG_00863	2.1e-243		Bifidobacteriales	HipA-like C-terminal domain
IAEJDAMG_00864	2.2e-15		Bacteria	addiction module antidote protein HigA
IAEJDAMG_00865	4.4e-220		Bifidobacteriales	Transmembrane secretion effector
IAEJDAMG_00866	2.7e-118		Bifidobacteriales	Bacterial regulatory proteins, tetR family
IAEJDAMG_00867	1.3e-11		Bifidobacteriales	

Table S2: Functional annotation of genes in RI2 of *B. breve* 110[1A] using EggNOG.

Query	e-value	Preferred name	Best tax	Description
IAEJDAMG_01133	1.1e-126		Bifidobacteriales	Major facilitator Superfamily
IAEJDAMG_01134	1.9e-186		Bifidobacteriales	VanZ like family
IAEJDAMG_01135	1.8e-65		Bifidobacteriales	Aminoacyl-tRNA editing domain
IAEJDAMG_01136	4.9e-42		Bifidobacteriales	Toxic component of a toxin-antitoxin (TA) module
IAEJDAMG_01137	1.9e-18	relB	Bifidobacteriales	RelB antitoxin
IAEJDAMG_01138	8.5e-87	MA20_25245	Bifidobacteriales	FR47-like protein
IAEJDAMG_01140	4e-26		Bifidobacteriales	Helix-turn-helix domain
IAEJDAMG_01141	7.8e-161	mocA	Bifidobacteriales	Aldo/keto reductase family

IAEJDAMG_01142	1.3e-13		Bifidobacteriales	Antitoxin component of a toxin-antitoxin (TA) module
IAEJDAMG_01143	1.3e-80		Bifidobacteriales	ABC transporter
IAEJDAMG_01144	6.5e-71		Bifidobacteriales	FtsX-like permease family
IAEJDAMG_01145	1.1e-53		Bifidobacteriales	Histidine kinase
IAEJDAMG_01146	3.8e-74		Bifidobacteriales	Bacterial regulatory proteins, luxR family
IAEJDAMG_01147	1.7e-19		Bifidobacteriales	
IAEJDAMG_01148	4.4e-86		Bifidobacteriales	Acetyltransferase (GNAT) domain
IAEJDAMG_01149	5.5e-50		Bifidobacteriales	
IAEJDAMG_01150	8.8e-121		Bifidobacteriales	
IAEJDAMG_01151	3.1e-161	ksgA	Corynebacteriaceae	Belongs to the class I-like SAM-binding methyltransferase superfamily. rRNA adenine N(6)-methyltransferase family
IAEJDAMG_01152	2e-111		Bacteria	
IAEJDAMG_01153	5.1e-228		Corynebacteriaceae	Transposase and inactivated derivatives
IAEJDAMG_01156	6.2e-214		Bifidobacteriales	Transposase, Mutator family
IAEJDAMG_01157	1.7e-16		Bifidobacteriales	Histidine kinase

Table S3: Functional annotation of genes in GI1 of *B. breve* 110^{1A} using EggNOG.

Query	e-value	Best tax	Preferred name	Description
IAEJDAMG_00028	4.1e-216	Bifidobacteriales	ytfL	Transporter associated domain
IAEJDAMG_00029	1.5e-195	Bifidobacteriales		AAA ATPase domain
IAEJDAMG_00030	2.2e-137	Bifidobacteriales		helix_turn _helix lactose operon repressor
IAEJDAMG_00031	5.3e-210	Bifidobacteriales	msmE	Bacterial extracellular solute-binding protein

IAEJDAMG_00032	1.2e-147	Bifidobacteriales	amyD	Binding-protein-dependent transport system inner membrane component
IAEJDAMG_00033	7.5e-131	Bifidobacteriales	amyC	Binding-protein-dependent transport system inner membrane component
IAEJDAMG_00034	5e-293	Bifidobacteriales		Glycosyl hydrolase family 36 N-terminal domain

Table S4: Functional annotation of genes in GI2 of *B. breve* 110^{1A} using EggNOG.

Query	e-value	Best tax	Preferred name	Description
IAEJDAMG_00578	3.2e-169	Actinobacteria	ugpQ	Domain of unknown function
IAEJDAMG_00579	5.9e-239	Actinobacteria	ugpB	Bacterial extracellular solute-binding protein
IAEJDAMG_00580	6.8e-142	Bifidobacteriales	ugpE	Binding-protein-dependent transport system inner membrane component
IAEJDAMG_00581	5e-163	Bifidobacteriales	ugpA	Binding-protein-dependent transport system inner membrane component
IAEJDAMG_00582	7.6e-161	Actinobacteria		Phosphate transporter family
IAEJDAMG_00583	2.2e-190	Bifidobacteriales		helix_turn _helix lactose operon repressor
IAEJDAMG_00584	2.3e-142	Bifidobacteriales		LysR substrate binding domain
IAEJDAMG_00585	9.9e-102	Bifidobacteriales		LysR substrate binding domain

Table S5: Functional annotation of genes in GI3 of *B. breve* 110^{1A} using EggNOG.

Query	e-value	Best tax	Preferred name	Description
IAEJDAMG_01653	1.9e-179	Bifidobacteriales		

IAEJDAMG_01654	5e-107	Bifidobacteriales	ytrE	ABC transporter
IAEJDAMG_01655	1.4e-179	Bifidobacteriales		Putative peptidoglycan binding domain
IAEJDAMG_01656	1.9e-105	Bifidobacteriales		
IAEJDAMG_01657	6.3e-56	Bifidobacteriales		
IAEJDAMG_01658	1.2e-117	Bifidobacteriales		Transcriptional regulatory protein, C terminal
IAEJDAMG_01659	4.2e-210	Bifidobacteriales	qseC	GHKL domain

Table S6: Functional annotation of genes in GI4 of *B. breve* 110^{1A} using EggNOG.

Query	e-value	Best tax	Preferred name	Description
IAEJDAMG_01906	9.4e-45	Ruminococcaceae		sugar phosphate isomerase involved in capsule formation
IAEJDAMG_01907	2.2e-58	Lachnoclostridium		Branched-chain amino acid transport system / permease component
IAEJDAMG_01908	1.1e-72	Clostridia		branched-chain amino acid ABC transporter, permease protein
IAEJDAMG_01909	6.2e-120	Lachnoclostridium		ATPases associated with a variety of cellular activities
IAEJDAMG_01910	3.6e-79	Alphaproteobacteria		ABC-type sugar transport system periplasmic component
IAEJDAMG_01911	3e-167	Actinobacteria	xylB	Belongs to the FGGY kinase family
IAEJDAMG_01912	1.8e-75	Actinobacteria	xylR	ROK family
IAEJDAMG_01913	1.1e-36	Bifidobacteriales		

Table S7: Functional annotation of genes in GI5 of *B. breve* 110^{1A} using EggNOG.

Query	e-value	Best tax	Preferred name	Description
IAEJDAMG_01984	1.9e-296	Bifidobacteriales	rafA	Raffinose synthase or seed

				imbibition protein Sip1
IAEJDAMG_01985	0.0	Bifidobacteriales		Alpha-amylase domain
IAEJDAMG_01986	7.1e-147	Bifidobacteriales		Binding-protein-dependent transport system inner membrane component
IAEJDAMG_01987	2.3e-190	Bifidobacteriales	malC	Binding-protein-dependent transport system inner membrane component
IAEJDAMG_01988	2.8e-249	Bifidobacteriales	msmE	Bacterial extracellular solute-binding protein
IAEJDAMG_01989	1.2e-232	Actinobacteria		Protein of unknown function (DUF2961)
IAEJDAMG_01990	8.9e-232	Bifidobacteriales	msmE	Bacterial extracellular solute-binding protein
IAEJDAMG_01991	1.5e-189	Bifidobacteriales		helix_turn _helix lactose operon repressor
IAEJDAMG_01992	2.7e-188	Bifidobacteriales		Periplasmic binding protein-like domain

Table S8: Functional annotation information about the 63 unique genes of *Bifidobacterium breve* 110^{1A} using GO FEAT.

Order	Locus tag	Length	Product	Completeness	Gene onthology
1	IAEJDAMG_00028	393	Transporter associated domain-containing protein	86.18% [393/456]	GO:0016021 - integral component of membrane| GO:0050660 - flavin adenine dinucleotide binding
2	IAEJDAMG_00029	355	AAA family ATPase	92.21% [355/385]	
3	IAEJDAMG_00030	243	Transcriptional regulator LacI family	71.47% [243/340]	GO:0003677 - DNA binding| GO:0006355 - regulation of transcription DNA-templated
4	IAEJDAMG_00031	361	ABC transporter substrate-binding protein	85.34% [361/423]	GO:0055085 - transmembrane transport
5	IAEJDAMG_00032	275	ABC transporter permease protein probably xylobiose porter	89.00% [275/309]	GO:0005886 - plasma membrane| GO:0016021 - integral component of membrane| GO:0055085 - transmembrane transport
6	IAEJDAMG_00033	248	Maltose ABC transporter permease	84.64% [248/293]	GO:0005886 - plasma membrane| GO:0016021 - integral component of membrane| GO:0055085 - transmembrane transport
7	IAEJDAMG_00034	491	Alpha-galactosidase	67.35% [491/729]	GO:0016052 - carbohydrate catabolic process| GO:0052692 - raffinose alpha-galactosidase activity
8	IAEJDAMG_00208	50	Uncharacterized protein	68.49% [50/73]	GO:0003677 - DNA binding| GO:0006310 - DNA recombination| GO:0015074 - DNA integration
9	IAEJDAMG_00403	705	Beta-galactosidase	96.71% [705/729]	GO:0004565 - beta-galactosidase activity| GO:0006012 - galactose metabolic process| GO:0009341 - beta-galactosidase complex
10	IAEJDAMG_00416	226	Acyltransferase	96.17% [226/235]	GO:0016746 - transferase activity transferring acyl groups
11	IAEJDAMG_00417	327	Glyco_trans_2-like domain-containing protein	97.03% [327/337]	
12	IAEJDAMG_00418	399	Uncharacterized protein	98.28% [399/406]	GO:0016021 - integral component of membrane
13	IAEJDAMG_00424	482	dTDP-4-dehydrorhamnose reductase	97.57% [482/494]	GO:0008830 - dTDP-4-dehydrorhamnose 35-epimerase activity| GO:0008831 - dTDP-4-dehydrorhamnose reductase activity| GO:0019305 - dTDP-rhamnose biosynthetic process
14	IAEJDAMG_00437	439	Uncharacterized protein	91.84% [439/478]	GO:0016021 - integral component of membrane
15	IAEJDAMG_00549	46	Uncharacterized protein	47.92% [46/96]	
16	IAEJDAMG_00642	177	Uncharacterized protein	98.33% [177/180]	
17	IAEJDAMG_00643	108	Uncharacterized protein	98.18% [108/110]	
18	IAEJDAMG_00644	437	Uncharacterized protein	98.20% [437/445]	
19	IAEJDAMG_00649	56	Uncharacterized protein	98.25% [56/57]	

20	IAEJDAMG_00650	206	HTH IS21-type domain-containing protein	98.10% [206/210]	
21	IAEJDAMG_00651	206	Uncharacterized protein	98.10% [206/210]	
22	IAEJDAMG_00652	569	DUF4091 domain-containing protein	98.27% [569/579]	
23	IAEJDAMG_00653	130	Uncharacterized protein	97.74% [130/133]	GO:0043565 - sequence-specific DNA binding
24	IAEJDAMG_00654	200	Integrase catalytic domain-containing protein	98.04% [200/204]	GO:0015074 - DNA integration| GO:0032196 – transposition
25	IAEJDAMG_00723	400	Site-specific recombinase phage integrase family	98.28% [400/407]	GO:0003677 - DNA binding| GO:0006310 - DNA recombination| GO:0015074 - DNA integration
26	IAEJDAMG_00849	721	Two-component system sensor histidine kinase	85.33% [721/845]	GO:0000155 - phosphorelay sensor kinase activity| GO:0016021 - integral component of membrane| GO:0046983 - protein dimerization activity
27	IAEJDAMG_00920	87	PadR family transcriptional regulator	63.97% [87/136]	
28	IAEJDAMG_00921	123	Uncharacterized protein	54.91% [123/224]	GO:0016021 - integral component of membrane
29	IAEJDAMG_00922	175	Integrase	41.87% [175/418]	GO:0003677 - DNA binding| GO:0015074 - DNA integration
30	IAEJDAMG_00986	387	MFS transporter	97.97% [387/395]	GO:0016021 - integral component of membrane| GO:0022857 - transmembrane transporter activity
31	IAEJDAMG_01087	174	Uncharacterized protein	97.75% [174/178]	
32	IAEJDAMG_01088	155	Uncharacterized protein	96.88% [155/160]	GO:0016021 - integral component of membrane
33	IAEJDAMG_01093	164	Uncharacterized protein	86.32% [164/190]	GO:0016021 - integral component of membrane
34	IAEJDAMG_01094	107	Uncharacterized protein	86.99% [107/123]	
35	IAEJDAMG_01095	162	Uncharacterized protein	87.10% [162/186]	GO:0004222 - metalloendopeptidase activity| GO:0005524 - ATP binding
36	IAEJDAMG_01096	19	Uncharacterized protein	16.96% [19/112]	GO:0016021 - integral component of membrane
37	IAEJDAMG_01098	135	Uncharacterized protein	75.84% [135/178]	
38	IAEJDAMG_01099	108	Uncharacterized protein	96.43% [108/112]	
39	IAEJDAMG_01100	96	Putative transcriptional regulator XRE family	92.31% [96/104]	GO:0003677 - DNA binding
40	IAEJDAMG_01102	129	Uncharacterized protein	81.13% [129/159]	GO:0016021 - integral component of membrane
41	IAEJDAMG_01103	42	Uncharacterized protein	47.73% [42/88]	GO:0016021 - integral component of membrane
42	IAEJDAMG_01104	391	Integrase catalytic domain-containing protein	84.45% [391/463]	GO:0003677 - DNA binding| GO:0015074 - DNA integration| GO:0032196 – transposition
43	IAEJDAMG_01105	437	Uncharacterized protein	98.20% [437/445]	

44	IAEJDAMG_01106	108	Uncharacterized protein	98.18% [108/110]	
45	IAEJDAMG_01107	18	IS3 family transposase	18.56% [18/97]	GO:0015074 - DNA integration
46	IAEJDAMG_01108	68	Uncharacterized protein	49.28% [68/138]	
47	IAEJDAMG_01109	133	Sortase family protein	40.92% [133/325]	GO:0016021 - integral component of membrane
48	IAEJDAMG_01110	165	LPXTG-motif cell wall anchor domain protein	30.22% [165/546]	GO:0016021 - integral component of membrane
49	IAEJDAMG_01111	194	Uncharacterized protein	23.57% [194/823]	GO:0016021 - integral component of membrane
50	IAEJDAMG_01112	21	Transposase	63.64% [21/33]	
51	IAEJDAMG_01113	74	Uncharacterized protein	53.24% [74/139]	
52	IAEJDAMG_01114	173	DnaJ-like protein	60.70% [173/285]	
53	IAEJDAMG_01115	54	Uncharacterized protein	72.97% [54/74]	
54	IAEJDAMG_01116	190	Hydrolase	95.48% [190/199]	GO:0006807 - nitrogen compound metabolic process| GO:0016787 - hydrolase activity
55	IAEJDAMG_01222	652	Putative membrane protein	96.45% [652/676]	GO:0016021 - integral component of membrane
56	IAEJDAMG_01227	2161	Cell surface protein	91.65% [2161/2358]	
57	IAEJDAMG_01229	315	Uncharacterized protein	92.11% [315/342]	GO:0016021 - integral component of membrane
58	IAEJDAMG_01235	108	Uncharacterized protein	98.18% [108/110]	
59	IAEJDAMG_01243	69	Helix-turn-helix domain-containing protein	97.18% [69/71]	
60	IAEJDAMG_01654	229	Lipoprotein-releasing system ATP-binding protein LolD	90.51% [229/253]	GO:0005524 - ATP binding| GO:0016887 - ATPase activity
61	IAEJDAMG_01659	432	Histidine kinase	98.18% [432/440]	GO:0000155 - phosphorelay sensor kinase activity| GO:0016021 - integral component of membrane
62	IAEJDAMG_01984	609	Alpha-galactosidase	98.23% [609/620]	GO:0003824 - catalytic activity
63	IAEJDAMG_01985	556	Glycosidase	98.23% [556/566]	GO:0005975 - carbohydrate metabolic process| GO:0008788 - alphaalpha-phosphotrehalase activity