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MICROBIOLOGY

In silico evaluation of genomic characteristics of *Streptococcus infantarius subsp. infantarius* for application in fermentations

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Abstract: This study aims to evaluate the *in silico* genomic characteristics of *Streptococcus infantarius subsp. infantarius*, isolated from Coalho cheese from Paraíba, Brazil, with a view to application in lactic fermentations. rRNA sequences from the 16S ribosomal region were used as input to GenBank, in the search for patterns that could reveal a non-pathogenic behavior of *S. infantarius subsp. infantarius*, comparing mobile genetic elements, antibiotic resistance genes, pan-genome analysis and multi-genome alignment among related species. *S. infantarius subsp. infantarius* CJ18 was the only complete genome reported by BLAST/NCBI with high similarity and after comparative genetics with complete genomes of *Streptococcus agalactiae* (SAG153, NJ1606) and *Streptococcus thermophilus* (ST106, CS18, IDCC2201, APC151) revealed that CJ18 showed a low number of transposases and integrases, infection by phage bacteria of the *Streptococcus* genus, absence of antibiotic resistance genes and presence of bacteriocin, folate and riboflavin producing genes. The genome alignment revealed that the collinear blocks of *S. thermophilus* ST106 and *S. agalactiae* SAG153 have inverted blocks when compared to the CJ18 genome due to gene positioning, insertions and deletions. Therefore, the strains of *S. infantarius subsp. infantarius* isolated from Coalho cheese from Paraíba showed genomic similarity with CJ18 and the mobility of genes analyzed *in silico* showed absence of pathogenicity throughout the genome of CJ18, indicating the potential of these strains for the dairy industry.

Key words: Comparative Genomics, dairy industry, mobile genetic elements, *Streptococcus infantarius subs. infantarius.*

INTRODUCTION

Species of *Streptococcus* spp. are well known for their contribution to food fermentation, biopreservation using antimicrobial metabolites and the development of sensory characteristics by synthesizing aromatic compounds. They are characterized as Gram-positive, immobile cocci, occurring in pairs or in short chains, non-sporulated and catalase negative (Du-Toit et al. 2014, Santos et al. 2020a) and differ according to their physiology, biochemistry, molecular characteristics, applications and origins. *Streptococcus thermophilus*, *Streptococcus gallolyticus subsp. macedonicus* and *Streptococcus infantarius subsp. infantarius* are present in traditional fermented foods (Domínguez-Ramírez et al. 2020).

Jans et al. (2013) and Wullschleger et al. (2013) isolated predominant *S. infantarius subsp. infantarius* in African fermented dairy products such as suusac, gariss and fènè. Since then, knowledge about the potential of *S.*

infantarius subsp. infantarius in fermentations has grown and new insights into its evolution and adaptation to the dairy environment have been reported.

According to Jans et al. (2013) the growth ability of *S. infantarius subsp. infantarius* in fermented dairy products was identified in studies of phenotypic and genotypic adaptations in lactose metabolism. Lactose uptake is mediated by the galactose-lactose system encoded in the operon gal-lac by the LacS/Z genes, a resource that, according to the authors, is present in *S. thermophilus*.

On the other hand, *S. infantarius subsp. infantarius* is a member of the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC), a heterogeneous group of bacteria that can be part of the gastrointestinal microbiota of animals and humans, but can also grow as opportunistic pathogens, some strains of *S. infantarius subsp. infantarius* being associated to different diseases in animals and humans (Jans et al. 2016). Santos et al. (2020a) showed that *S. infantarius subsp. infantarius* K1–4 and K5–1 may be considered safe for technological applications as they have virulence factors.

Genetic mobility has been used as a good way to differentiate pathogenic and non-pathogenic strains. Santos et al. (2020b) used mobile genetic elements to differentiate strains of *Enterococus faecium* 141V and 137V from pathogenic, non-pathogenic and non-probiotic bacteria.

Mobile genetic elements (MGEs) such as transposases, integrases, conjugative transposons, phages and antibiotic resistance genes (ARGs) are types of genetic material that can move within a genome. They can also rearrange genes, cause duplication in the host genome, and lead to mutations that underlie the evolution of species (Singh et al. 2014).

In the study carried out by Brito et al. (2020) it was determined that strains of *Streptococcus infantarius subsp. infantarius* isolated from Coalho cheeses from northeastern Brazil have *in vitro* biotechnological potential for lactic acid fermentations. However, their use in foods is still not regarded as safe due to their relationships with pathogenic members of the SBSEC complex. Therefore, the aim of this study was to compare the 16S rDNA sequences of *Streptococcus infantarius subsp. infantarius*, with those of completely sequenced genomes and analyze the different virulence patterns and their respective mobile genetic elements, targeting its potential in lactic acid fermentations.

MATERIALS AND METHODS

Obtaining the sequences of *Streptococcus infantarius subsp. infantarius*

The gene sequences of *Streptococcus infantarius subsp. infantarius* deposited by Medeiros et al. (2017) were obtained by accessing GenBank: KT990067; KT990068; KT990070; KT990071.

Aligning the genomic sequences of selected strains

The 16S rRNA region of the four strains of *S. infantarius subsp. infantarius* were submitted to the Basic Local Alignment Search Tool for nucleotide (BLASTn) of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast) in order to select complete genomes of *S. infantarius subsp. infantarius* of different origins and strains of species outside the SBSEC complex, to identify similarities and to verify the safety of strains for technological applications.

Determining the number of phages and Mobile Genetic Elements (MGEs) of selected genomes

The number of phages was quantified using an improved version of the search tool PHAGE (Search Tool Enhanced Release – PHASTER) under the following references: intact (score>90), questionable (score 70–90), and incomplete (score <70) (Arndt et al. 2016). The number MGEs (the protein transposases, integrases, and conjugative transponsons) for both chromosomes and the plasmid was quantified in a complete genome obtained from the GenBank.

Identifying Antibiotic Resistance Genes (ARG)

The Comprehensive Antibiotic Resistance Database (CARD) was used to predict the resistome under BLAST and RGI (under the criteria of perfect hit, rigorous hit alone, and perfect, and strict hit criteria), and to verify the position of the gene (chromosome and plasmid) in the complete genomes of *S. infantarius subsp. infantarius* (CJ18, ATCC BAA-102), *Streptococcus agalactiae* (SAG153, NJ1606) and *Streptococcus thermophilus* (ST106, CS18, IDCC2201, APC151). For the evaluation of antimicrobial resistance genes, ResFinger Server 3.0 was applied (Alcock et al. 2020).

Pan-Genome analysis and exploration (pan-X)

Pan-genome analysis & exploration (panX) was used to search for the presence or absence of specific genes in each species and to perform similarity analysis between *S. infantarius subsp. infantarius*, *S. thermophilus* and *S. agalactiae* (Ding et al. 2018).

Identifying pathogenesis islands between selected genomes

The IslandViewer 4 software was used to visualize the pathogenicity of genomic islands in *S. infantarius subsp. infantarius* CJ18, *S. thermophilus* ST106 and *S. agalactiae* SAG153 (Bertelli et al. 2017).

Identifying multiple genome alignment

Mauve software, with the DNASTAR extension, was used to perform sequence block synteny analyses, rearrangements and multiple genome alignments (default configuration) in *S. infantarius subsp. infantarius* (CJ18 and ATCC BAA-102), *S. thermophilus* ST106 and *S. agalactiae* SAG153.

RESULTS

Aligning the genomic sequences of selected strains

The 16S rRNA sequences of strains KT990067, KT990068, KT990070 and KT990071 (Fig. 1) submitted to BLAST/NCBI returned only a single, complete genome of *S. infantarius subsp. infantarius* (the CJ18 strain) and also other sequences homologous to 16S rRNA.

The CJ18 strain was then used for subsequent analyses as the representative of fermented dairy products due to its similarity with the studied strains of Coalho cheese from Paraíba-Brazil. Strains of *S. infantarius subsp. infantarius* of human origin (ATCC BAA-102), *Streptococcus agalactiae* (SAG153, NJ1606), considered a clinical pathogen, and *Streptococcus thermophilus* (ST106, CS18, IDCC2201, APC151), the only streptococcal species with GRAS "Generally Recognized As Safe" status, were used to identify similarities and to check that our native strains were safe for technological applications (Table I).

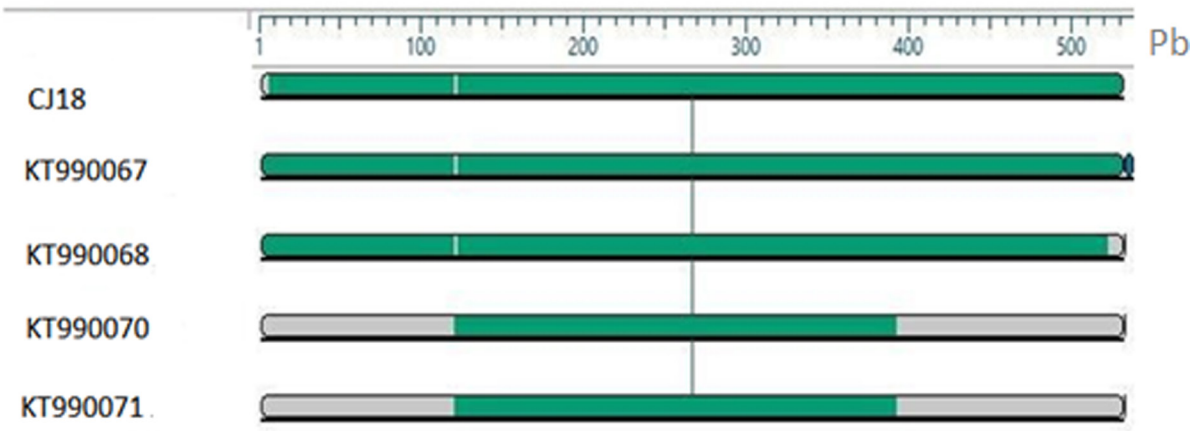

Figure 1. Similarity between 16S rDNA sequences *Streptococcus infantarius subsp. infantarius* (KT990067, KT990068, KT990070, KT990071) isolated from Coalho cheese from Paraíba and *Streptococcus infantarius subsp. infantarius* CJ18 from African fermented milk.

Table I. Strains of *Streptococcus infantarius subsp. infantarius, Streptococcus thermophilus* and *Streptococcus agalactiae* with complete genomes in GenBank and origins.

Species	*Strain*	*ID Genbank*	*Size genome*	*Plasmid*	*Country*	*Source*
S. infantarius subsp. infantarius	CJ18	CP003295.1	1988420 bp	1	Kenya	Fermented milk
	ATCC BAA-102	NZ_DS572694.1	233422 bp	-	United States	Human feces
S. thermophilus	ST 106	CP031881.1	1856083 bp	-	United States	Raw milk
	CS18	CP030928.1	1858890 bp	-	China	fermented milk
	IDCC 2201	CP035306.1	1794836 bp	-	South Korea	Cheese
	APC 151	CP019935.1	1839134 bp	-	Ireland	Intestine of fish
S. agalactiae	SAG153	CP036376.1	2174504 bp	-	China	Human
	NJ1606	CP026084.1	2136438 bp	-	China	Milk/ Cow

Determining the number of phages and Mobile Genetic Elements of selected genomes

The strain of biotechnological origin, *S. infantarius subsp. infantarius* CJ18, had a higher number of transposases and integrases compared to strain pathogenic origin, *S. infantarius subsp. infantarius* ATCC BAA-102 (Fig. 2).

In addition, CJ18 contains genes to encode conjugative transposon proteins, which are absent in ATCC BAA-102. However, the number of transposases and integrases of *S. thermophilus* strains was higher than those of the other species analyzed. With the exception of ATCC BAA-102, all strains had phages in their genomes. The CJ18 strain was infected by phages of bacteria of the genus *Streptococcus* spp., the strains of *S. thermophilus* (ST106, CS18, IDCC2201, APC151) by phages of *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *Lactococcus* spp. and *Oenococcus* spp., while strains of *S. agalactiae* (SAG153, NJ1606) were infected by phages of *Streptococcus* spp. and *Lactococcus* spp. (Fig. 3).

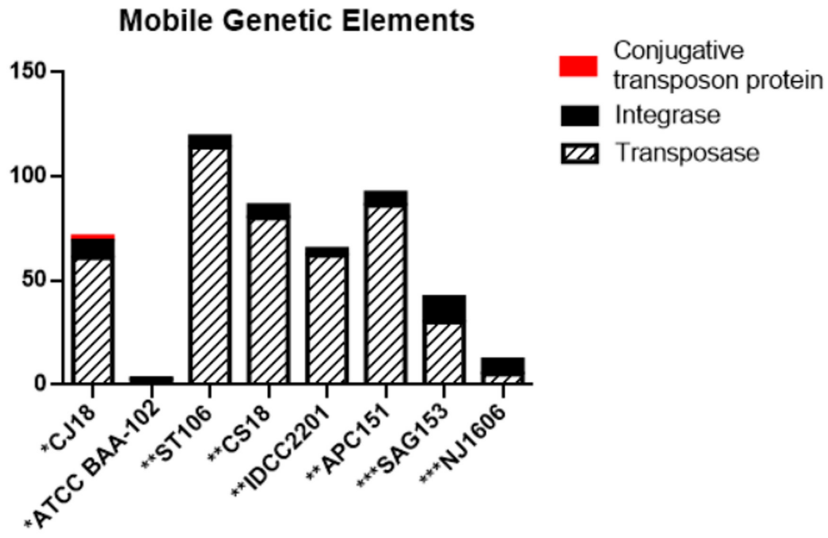

Figure 2. Variation of Mobile genetic elements in the chromosomes of *Streptococcus infantarius subsp. infantarius, Streptococcus thermophilus* and *Streptococcus agalactiae.*

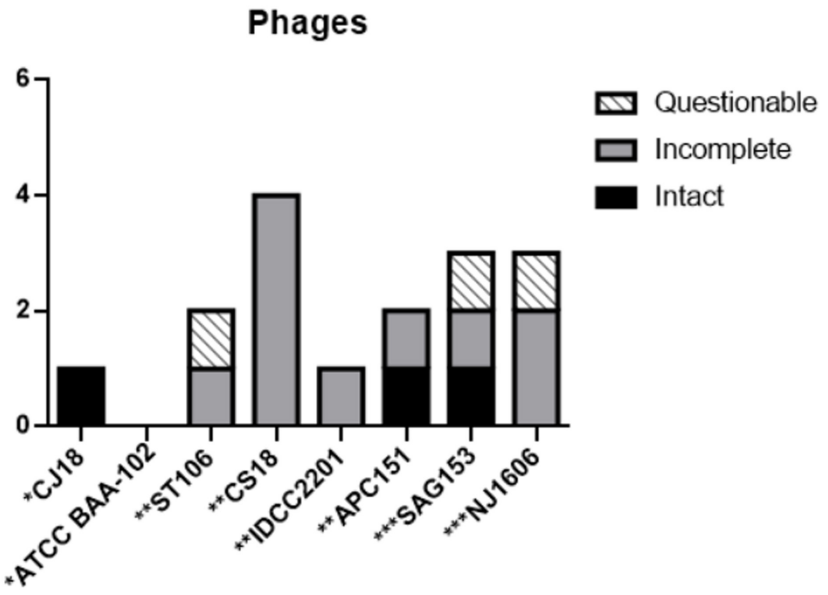

Figure 3. Presence of phages in the complete genomes *Streptococcus infantarius subsp. infantarius, Streptococcus thermophilus* and *Streptococcus agalactiae.*
** Streptococcus infantarius subsp. infantarius;*
*** Streptococcus thermophilus;*
**** Streptococcus agalactiae.*

Identifying Antibiotic Resistance Genes (ARG)

ARGs were not found in the complete genomes of *S. infantarius subsp. infantarius* (CJ18 and ATCC BAA -102) and *S. thermophilus* (ST106, CS18, IDCC2201, APC151). In contrast, resistance genes to tetracycline (*tet W/N/W* and *tetM*), to aminoglycosides (*APH (3')-IIIa* and *aad (6)*) and to defensins (*mprF*) were present in the two selected strains of *S. agalactiae* (SAG153, NJ1606). The *ermB* gene, which confers resistance to erythromycin, was only present in *S. agalactiae* SAG153 (Table II).

Pan-Genome analysis and exploration (pan-X)

Pan-genome analysis & exploration (panX) revealed the presence of bacteriocin, folate and riboflavin production genes (vitamins B9 and B2, respectively) for strains of *S. infantarius subsp. infantarius, S. agalactiae* and *S. thermophilus*. A strain of *S. agalactiae* also presented virulence genes for synthesis of adhesion proteins and cytolysin (Table III).

Table II. Antibiotic Resistance Genes evaluated in the chromosomes of ***Streptococcus infantarius subsp. infantarius*, *Streptococcus thermophilus*** and ***Streptococcus agalactiae.***

Genes	*S. infantarius subsp. infantarius*		*S. thermophilus*				*S. agalactiae*	
	CJ18	**ATCC BAA-102**	***ST106***	***CS18***	***IDCC 2201***	***APC151***	**Sag153**	**NJ1606**
APH (3 ') – IIIa	-	-	-	-	-	-	+	-
aad (6)	-	-	-	-	-	-	+	-
ErmB	-	-	-	-	-	-	+	-
tet (W/N/W)	-	-	-	-	-	-	+	-
tetM	-	-	-	-	-	-	-	+
mprF	-	-	-	-	-	-	+	+
vanA	-	-	-	-	-	-	-	-
vanB	-	-	-	-	-	-	-	-

+ Presence; - Absence.

Table III. Proteins evaluated with biotechnological or pathogenic potential found in ***Streptococcus infantarius subsp. infantarius, Streptococcus thermophilus*** and ***Streptococcus agalactiae.***

Protein	**Found on**	**Length**	***Duplicated***	***Diversity***
Folate biosynthesis protein folP	*S. thermophilus*	801 AA	NO	0.001
	S. agalactiae	804 AA	NO	0.003
	S. infantarius subsp. infantarius	-	-	-
Riboflavin biosynthesis protein RibF	*S. thermophilus*	917 AA	NO	0.005
	S. agalactiae	903 AA	NO	0.002
	S. infantarius subsp. infantarius	-	-	-
Bacteriocin biosyntesis protein	*S. thermophilus*	1056 AA	NO	0.001
	S. agalactiae	246 AA	NO	0.0
	S. infantarius subsp. infantarius	-	-	-
Adhesion protein	*S. agalactiae*	924 AA	NO	0.005
Cytolysin biosynthesis protein cylB	S. agalactiae	879 AA	NO	0.002

Identifying pathogenicity islands between selected genomes

IslandViewer 4 confirmed the presence of transposable elements and the absence of pathogenicity islands in *S. infantarius subsp. infantarius* CJ18 and *S. thermophilus* ST106, unlike *S. agalactiae* SAG153 which presented virulence and pathogenicity genes (Fig. 4).

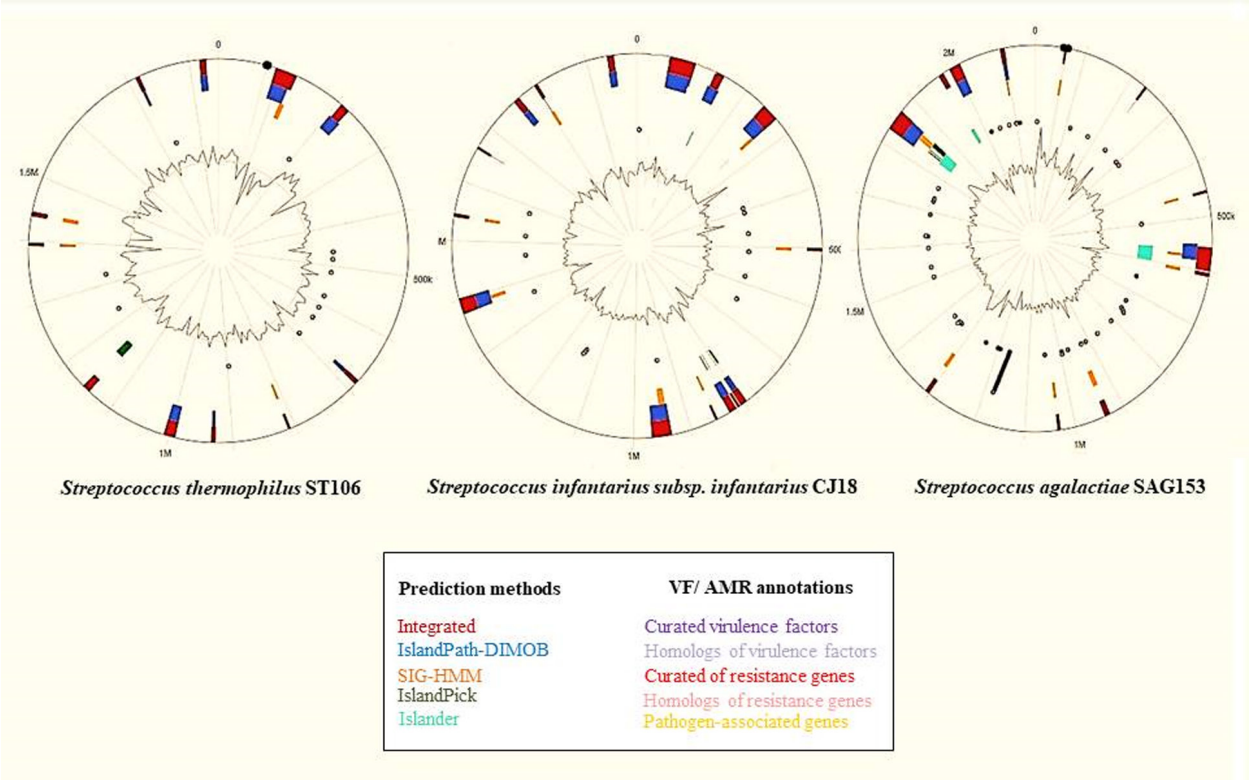

Figure 4. Patterns and diversity of genomic islands of *Streptococcus thermophilus* ST106, *Streptococcus infantarius subsp. infantarius* CJ18 and *Streptococcus agalactiae* SAG153.

Identifying multiple genome alignment

Alignment of the genomes of *S. infantarius subsp. infantarius* CJ18 and *S. infantarius subsp. infantarius* ATCC BAA-102 revealed the presence of similarity between the two strains. However, they presented different rearrangements due to gene positioning, insertions and deletions (Fig. 5). The collinear blocks of *S. thermophilus* ST106 and *S. agalactiae* SAG153 have inverted block groups when compared to the genome of *S. infantarius subsp. infantarius* CJ18 (Figs. 6 and 7), but it was possible to identify deletions of sequence blocks and small blocks unique (≃ 5.000 base pairs) while synteny is common for the three genomes (Figs. 5, 6 and 7).

DISCUSSION

The taxonomic relationship of *S. infantarius subsp. infantarius* with pathogenic members of the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) and its predominance and adaptation to dairy environments has been the subject of discussion (Jans et al. 2013, Santos et al. 2020a). In this scenario, comparative genomics analysis can elucidate questions of virulence or pathogenicity, biotechnological potential or even the probiotic profile of microorganisms.

Patterns of conserved similarities between our Coalho cheese strains and the African milk isolate CJ18 (Fig. 1), sequenced by Jans et al. (2013), indicate that the genomes are close, especially the KT990067 and KT990068. Jans et al. (2013) when comparing the genome of *S. infantarius subsp. infantarius* CJ18 with *S. infantarius subsp. infantarius* ATCC BAA-102 of human origin and *S. thermophilus* revealed that the pressure of natural selection promoted adaptations in CJ18 in the dairy environment, and similarly for *S. thermophilus*.

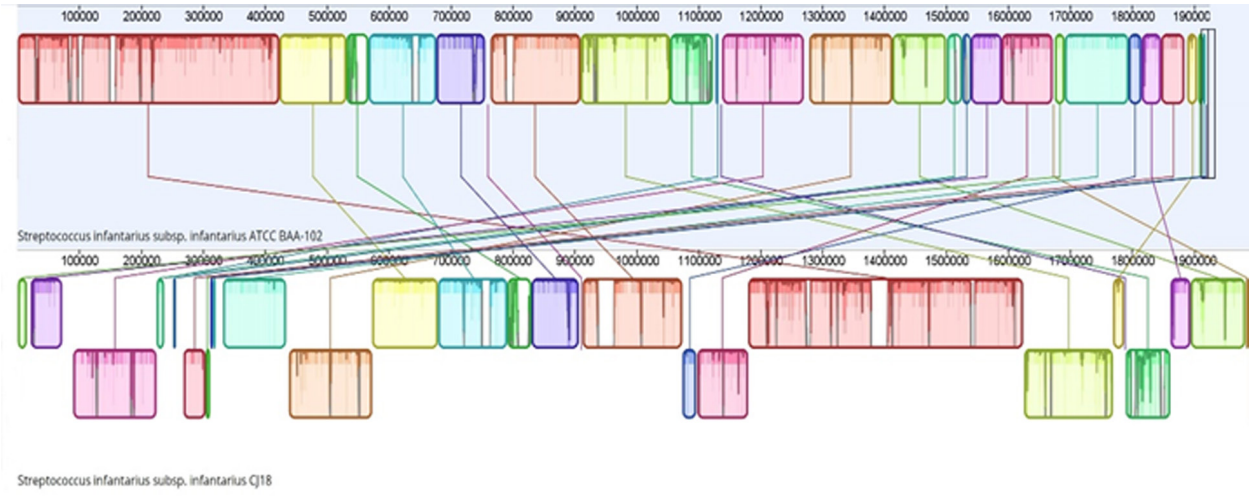

Figure 5. Synteny between *Streptococcus infantarius subsp. infantarius* ATCC BAA-102 and *Streptococcus infantarius subsp. infantarius* CJ18.

Genomic comparison analysis can reveal different virulence gene adaptations that may characterize the pathogenicity of microorganisms. Ghattargi et al. (2018) demonstrated that *Enterococcus faecium* 17OM39 does not have antibiotic resistance genes (ARGs) (vancomycin and tetracycline). Our analysis of the complete genome of CJ18 revealed the absence of antibiotic resistance genes, thus showing that the evolution of species generates populations with different genotypes that may or may not confer pathogenicity and therefore each population must be analyzed individually.

Recently, Tarrah et al. (2020) identified by conducting *in silico* genomic investigations that *Streptococcus macedonicus* 211MA isolated from Italian Malga cheese did not present adhesion virulence genes, when compared to other *S. macedonicus*, thus revealing that comparative genomic analyses are mechanisms that are efficient at elucidating questions of virulence among microorganisms. These adhesion properties can be interpreted as being beneficial or not beneficial for dairy technology or probiotics, what dependes on the genotype, environmental conditions, and the interaction with other bacterial species present in the specific ecological environment. Regardless of the origins and adaptive characteristics, MGEs such as transposases, integrases, conjugative transposons, phages and antibiotic resistance genes can modify the evolutionary dynamics in each microorganism (Table I and Figs. 2, 5, 6 and 7).

By analyzing the number of transposases, integrases and conjugative transposons of *S. infantarius subsp. infantarius* (CJ18, ATCC BAA-102), *S. thermophilus* (ST106, CS18, IDCC2201, APC151) and *S. agalactiae* (SAG153, NJ1606), it was found that the transposases were more abundant (Fig. 2), a fact that deserves further investigations for the SBSEC complex species, because MGEs are selfish genetic units that self-replicate and normally encode proteins that allow their proliferation in the genome and spread through hosts, thereby creating individual profiles (Jangam et al. 2017). These sequences can appear with specific characteristics and may present cellular function, thereby increasing or decreasing its expression.

Phage infections require the utmost attention in dairy technology as phages can transfer virulence or pathogenicity genes to fermenting bacteria. The analysis of the

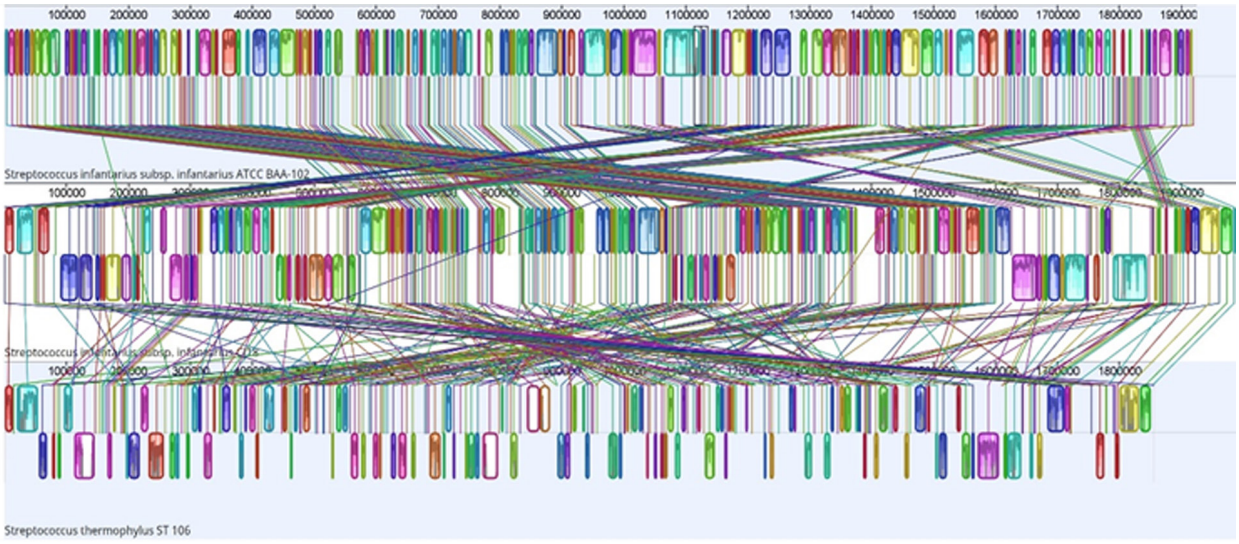

Figure 6. Synteny between *Streptococcus infantarius subsp. infantarius* ATCC BAA-102, *Streptococcus infantarius subsp. infantarius* CJ18 and *Streptococcus thermophilus* ST 106.

complete genome of the strains led to identifying that the African strain of *S. infantarius subsp. infantarius* CJ18 was infected by a phage of *Streptococcus* spp. (Fig. 3). Jans et al. (2013) report that this strain was not continuously exposed to phages for prolonged periods within a homogeneous environment of spontaneous fermentation. However, it is important to note that *S. infantarius subsp. infantarius* belongs to a complex of species from heterogeneous environments, which increase the chances of exposure of these bacteria to different types of phages, regardless of the exposure time, and that there are different defense mechanisms or incorporation of phages to plasmids or bacterial chromosomes that may be perpetuated across generations.

In the strains of *S. thermophilus* (ST106, CS18, IDCC2201, APC151) and *S. agalactiae* (SAG153, NJ1606) phages were also infected (Figures 3 and 4), a fact that requires attention, since, as described by Santos et al. (2020b) bacteriophages can package part of the host's genetic material, including ARGs, causing a rapid spread of resistance among bacteria.

The misuse of antibiotics and the rise of ARGs is a major public health threat (Olesen et al. 2020), and these genes can be transferred horizontally or vertically to other bacteria. ARGs were not found in *S. infantarius subsp. infantarius* (CJ18, ATCC BAA-102) and *S. thermophilus* (ST106, CS18, IDCC2201, APC151). However, pathogenic strains of *S. agalactiae* (SAG153, NJ1606) showed resistance genes to aminoglycosides, tetracyclines, erythromycins and defensins. Strains of *S. infantarius subsp. infantarius* isolated from bovine Coalho cheese in the State of Paraíba, Brazil, did not show antibiotic resistance genes (Table II), thus revealing the safety of these strains for biotechnological use in the dairy industry.

In contrast, the study by Santos et al. (2020a) found that *S. infantarius subsp. infantarius* K1-4 and K5-1 strains isolated from goat milk in the State of Ceará, Brazil, presented the *vanB* resistance gene (vancomycin resistance), which represents a safety concern about these strains, since vancomycin is a drug of last resort for the treatment of serious infections. Therefore, the analysis of ARGs in lactic acid bacteria (LAB) must be carried out continuously, because

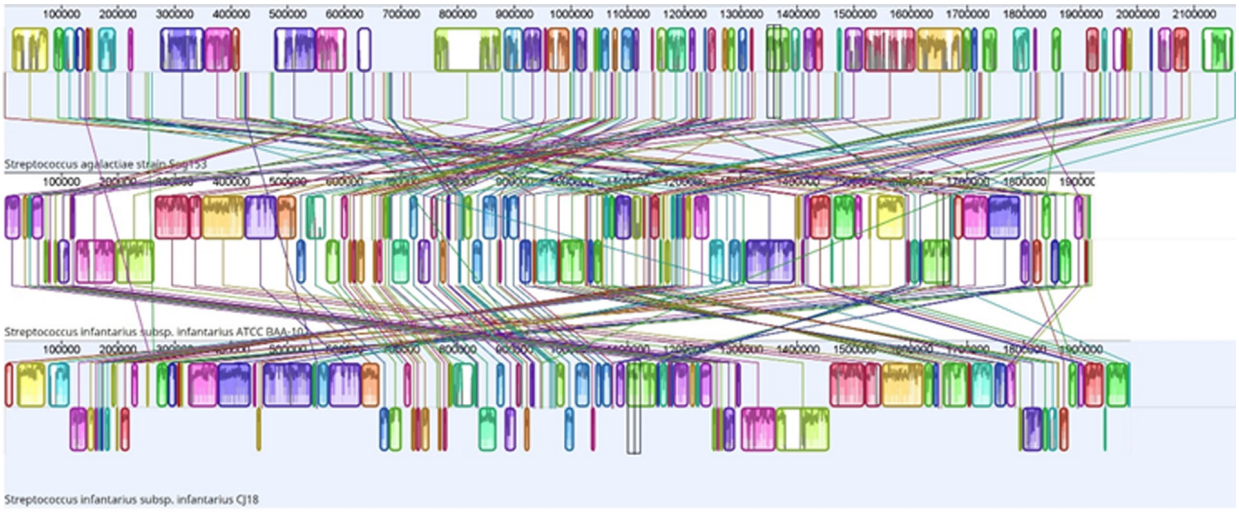

Figure 7. Synteny between *Streptococcus infantarius subsp. infantarius* ATCC BAA-102, *Streptococcus infantarius subsp. infantarius* CJ18 and *Streptococcus agalactiae* SAG 153.

depending on the ecological niche, bacteria can acquire or transfer ARGs through mobile genetic elements (Santos et al. 2020b). Furthermore, Dobrindt et al. (2015) explain that ARGs are often located in MGEs including pathogenicity islands common in pathogenic variants.

The pan-genomic analysis showed that all strains had genes for the production of bacteriocins and, as explained by Aspri et al. (2017), bacteriocins are active against *Staphylococcus aureus*, *Clostridium botulinum*, *Escherichia coli* and *Listeria monocytogenes*. In addition, genes for the synthesis of vitamins B9 and B2, which help cell metabolism, were also found, and, therefore, are essential for the health of microorganisms and animals (Table III). In contrast, pathogenicity islands containing genes for adhesion proteins were found in *S. agalactiae*, which individually do not characterize pathogenicity, but when these genes are present in strains that have additional virulence factors, such as ARGs, phages, toxin synthesis, the strain is now considered pathogenic, as is the case of *S. agalactiae*. The *cylB* gene encoding cytolysin is also present in *S. agalactiae* and as described by Hooven et al. (2018), cytolysin is an important cytotoxin implicated in facilitating the invasion of pathogens into the bloodstream of hosts.

Strains of *S. infantarius subsp. infantarius* K1-4 and K5-1, analyzed by Santos et al. (2020a), presented the genes *esp* (enterococcal surface protein), *gelE* (gelatinase), *efaA* (endocarditis antigen), *ace* (collagen protein adhesion) and *epfSTR* (extracellular factor). According to the authors, the K1-4 and K5-1 strains are not safe for the dairy industry, unlike the strains of *S. infantarius subsp. infantarius* isolated from Coalho cheese analyzed in the present work, which did not show adhesion protein genes.

The action of evolutionary processes can lead to the formation of gene blocks in different rearrangements, changing the genotypes of populations (Figs. 5, 6 and 7), which may have been built together with the genetic elements of their islands over time. Thus, the synteny between the genomes of the species allows us to understand how the order of genes has been changed over time, and as reported by Santos et al. (2020b), this can facilitate the initial understanding of adaptive patterns present.

Although the SBSEC complex has pathogenic species, the genomic analyses performed in the present study showed that pathogenicity is

absent in the African strain *S. infantarius subsp. infantarius* CJ18 which also belongs to the SBSEC complex, thus revealing that generalizing the genetic patterns for a species must not be made, but rather that the genome of each microorganism belonging to the species must be evaluated individually.

CONCLUSIONS

Genomic analyses of mobile genetic elements related to the pathogenicity of *S. infantarius subsp. infantarius* (CJ18, ATCC BAA-102), *Streptococcus agalactiae* (SAG153, NJ1606) and *Streptococcus thermophilus* (ST106, CS18, IDCC2201, APC151) revealed for the first time a dynamics of sequence block rearrangements among the strains of the species studied. Comparative genomics allowed us to identify that the isolated strains of Coalho cheese from Paraíba showed a genomic similarity with *S. infantarius subsp. infantarius* CJ18, and the analysis of mobile genetic elements showed a low number of transposases and integrases, infection by phage bacteria of the genus *Streptococcus* spp., the absence of antibiotic resistance genes and the presence of bacteriocin, folate and riboflavin production genes, thus indicating the potential of these strains for use in the dairy industry.

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Leandro Paes de Brito and Dayane da Silva Santos: were responsible for the *in silico* analysis of the genomes of the strains and construction of the graphics; Rosália Severo de Medeiros: collection, isolation and identification of strains; Nara Suzy Aguiar de Freitas and Paulo Roberto Eleutério de Souza: were responsible for orientation the *in silico* analysis. Maria Taciana Cavalcanti Vieira Soares and Ana Lúcia Figueiredo Porto were responsible for supervising and orientation the execution of the manuscript. All authors discussed the results and contributed to the writing of the manuscript.