Article

Characterization of *Bifidobacterium asteroides* Isolates

Alessandra Pino[1,2], Bachir Benkaddour[3], Rosanna Inturri[4,5*], Pietro Amico[5], Susanna Vaccaro[5], Nunziatina Russo[1,2], Amanda Vaccalluzzo[1], Gianluigi Agolino[1], Cinzia Caggia[1,2] Cinzia L Randazzo[1,2]

[1] Department of Agricultural, Food and Environment, University of Catania, Italy; alessandra.pino@unict.it; nunziatina.russo@unict.it; amanda.vaccalluzzo@unict.it; gianluigi.agolino@unict.it; ccaggia@unict.it; cinzia.randazzo@unict.it;
[2] ProBioEtna srl, Spin-off of University of Catania, Italy; alessandra.pino@unict.it; nunziatina.russo@unict.it; ccaggia@unict.it; cinzia.randazzo@unict.it
[3] Department of Biology, University of Biskra, Algeria; benkaddourbachir@gmail.com
[4] Department of Biomedical and Biotechnological Sciences, University of Catania, Italy; rinturri@unict.it
[5] R&D Unità locale Fidia Research Sud, Noto (SR), Italy; rinturri@fidiapharma.it; pamico@fidiapharma.it; svaccaro@fidiapharma.it

* Correspondence: rinturri@fidiapharma.it;

Abstract: Bifidobacteria have long been recognized as bacteria with probiotic and therapeutic features. The aim of this work is to characterize the *Bifidobacterium asteroides* BA15 and BA17 strains, isolated from honeybee gut. An in-depth assessment was carried out on safety properties (antibiotic resistance profiling, β-haemolytic, DNAse and gelatinase activities and virulence factor presence) and other properties (antimicrobial activity, auto-aggregation, co-aggregation and hydrophobicity). Based on phenotypic and genotypic characterization, both strains satisfied all the safety requirements. More specifically, genome analysis showed the absence of genes encoding for glycopeptide (*vanA*, *vanB*, *vanC*-1, *vanC*-2, *vanD*, *vanE*, *vanG*), resistance to tetracycline (*tet*-M, *tet*-L and *tetO*), and virulence genes (*asa1*, *gelE*, *cylA*, *esp*, *hyl*).

Keywords: Microbiological characterization, safety, *VanZ*, isolation, vancomycin resistant gene, genome, bee, honey.

1. Introduction

Bifidobacteria are Gram-positive, non-motile, and non-spore-forming bacteria with a curved and clubbed shape, often branched with Y and V forms. Their genome harbors a high G + C content. Increased interest in LAB and bifidobacteria has been registered in the last two decades [1]. Bifidobacteria are commonly isolated from the gastrointestinal tract of various animals such as mammals, birds, and insects. However, strains ascribed to *Bifidobacterium denticolens*, *Bifidobacterium dentium*, *Bifidobacterium inopinatum*, *Bifidobacterium lactis* species have been isolated from different ecological niches, such as the human oral cavity [2, 3, 4] and fermented milk products [5, 6]. *Bifidobacterium asteroides*, *Bidifobacterium coryneforme* and *Bifidobacterium indicum* are species that were isolated and characterized in the 1960s in pollinating insects, including honeybees *Apis mellifera*, *Apis cerana* and *Apis dorsata* [**2**, 7]. In the last decade, the new species of *Bifidobacterium bombi*, *Bifidobacterium actinocoloniiforme*, *Bifidobacterium bohemicum* and *Bifidobacterium commune* have been identified from *Bombus* spp. gut [8]. While gut microbiota in some insects is acquired from food and the environment, bifidobacterial populations in honey, wasps, and bumble bees are stable, and different molecules have been characterized as mediators of their *cross-talk* with the host [**8**]. In fact, similarly to humans, honeybees have a core in their gut microbiota that matures in the early stages of their life and remains stable throughout adult life. The gut of mammals, birds, and insects is known as an anaerobic environment, and bifidobacterial growth has been described as being inhibited by oxygen, which exerts toxic effects. However, the *Bifidobacterium asteroides* species has been found to tolerate oxygen levels higher than 20%, which represents the upper limit of tolerance

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for *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium boum*, B*ifidobacterium psychraerophilum* and *Bifidobacterium thermophilum* [**7**]. While the long history of safe use of some species, such as *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium longum*, *Bifidobacterium breve* and *Bifidobacterium bifidum*, has led to them being proposed for European Safety Authority (EFSA) Qualified Presumption of Safety (QPS) status, other species, such as *Bifidobacterium dentium*, cannot be used, because they are linked to cariogenic processes. Moreover, the current guidelines do not authorize the use in humans of strains of non-human origin [9].

Recently, researchers have turned their attention to different species of honeybees including *Apis mellifera*, on account of their elevated mortality rates. Honeybees and other pollinating species play a key role in production and global food security, which amounts to an estimated value of 150 billion euro. Many funding programs for insect pollinators have set up grants for the protection of specific species and their habitats. Decades-long investigation on insect physiology has made it possible to understand the single and combined effects of environmental and host stress factors such as pests, pathogens, toxins, and nutrient-limited food sources. Moreover, even if the vast majority of probiotic strain have been isolated from human or fermented foods, mainly dairy, the scientific research has been recently focused on the selection of new probiotics from *unconventional* sources such as meat, fruit, vegetables, cereals, honeybees and beehive products. In addition, the Internal Scientific Association for Probiotics and Prebiotics (ISAPP) recognized probiotic properties to strains belonging to *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Roseburia* spp. and *Eubacterium hallii*, not previously included in the QPS list [10, 11, 12, 13, 14].

Nowadays, studies on the gut microbiota of pollinators have clarified the role of the genera *Bifidobacterium* and *Lactobacillus* in the ability of honeybees to withstand environmental stressors and preserve their immunity and disease tolerance [15]. The core of the gut microbiota of honey bees, whatever their geographical origin, is composed of *Gilliamella apicola*, *Lactobacillus Firm-5* (*L. melliventris*, *L. kimbladii*, *L. kullabergensis*, *L. helsinborgensis*), *Lactobacillus Firm*-4 (*L. mellis*, *L. mellifer*), *Snodgrassella alvi* and *Bifidobacterium asteroides* [16]. The gut microbiota and its symbiotic bacteria exert a key role in the innate immunity system in bees producing antimicrobial peptides with a highly selective activity [**15**].The disruption of the microbial balance in the bee's gut is due to the perturbation of insect immunity through treatments with substances that show microbicidal or microbiostatic activity; the exposure to pesticides or herbicides at a sublethal concentration (called hidden treatment) leads to a decrease in *Firmicutes* and *Actinobacteria* and to an increase in *Gammaproteobacteria* such as *Gilliamella apicola* and *Escherichia coli*. Gut microbiota dysbiosis and the dysregulation of the innate immunity system expose bees to attacks by parasites like fungi (*Nosema* spp.) and trypanosomes (*Crithidia* and *Lotmaria* spp.). Moreover, honeybee gut plays a key role in their metabolic activity and nutrition status, especially impacting on vitamin biosynthesis.

Based on knowledge of human gut dysbiosis, two strategies have recently been used to control honeybee dysbiosis and dysregulation: a prebiotic strategy by diet supplementation with sucrose-based solutions or pollen-based feed and gut microbiota manipulation through supplementation with safe strains from the *Bifidobacterium* and *Lactobacillus* genera [**16**, 17].

There is a lack of studies in the literature reporting the selection of *Bifidobacterium* strains for the dietary supplementation of honeybees. However, several regulatory authorities have suggested that these strains could possess some important properties that make them suitable for human use. Studying the resistance genes is important for the confirmation of the isolates as non-resistant bacteria. Antibiotic resistance can be due to phenotypic or genotypic features, and the antibiotic resistance profiles of the *Bifidobacterium asteroids* species could be the result of the long-term exposure of honeybees to antibiotics [18].

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Recent studies have indicated horizontal antibiotic resistant gene transfer between bacteria residing in the gut of humans and animals. These genes are delivered by mobile genetic elements; the collection of the mobile elements of a microorganism has recently been defined as a mobilome and includes transposases, insertion elements, plasmids and prophages (bifidoprophages). The entire mobilome and resistome have recently been reconstructed. The most abundant antibiotic resistant gene in the *Bifidobacterium* resistome is the one conferring glycopeptide resistance (43%, 5999 putative enzymes), followed by the methyltransferase class (19%, 2618 putative enzymes), β-lactamase class (17%, 2437 putative enzymes), tetracycline class (16%, 2178 putative enzymes), sulfonamide class (4%), and metronidazole aminoglycosides classes (0.5%). Analysis of the putative mobile resistome of the *Bifidobacterium* genus reveals the presence of putative conjugative transposon that harbors the tetW gene responsible for protection from tetracycline activity; putative prophage-like elements which harbor the BacA gene responsible for protection from bacitracin; transposase that harbors a 23S rRNA methylase, which confers resistance towards erythromycin and clindamycin. Moreover, amongst the predicted transposase encoding genes, the presence of the *vanZ* gene has been observed which may confer low-level resistance to glycopeptide antibiotics [19].

One of the main selection criteria for defining the probiotic action of bifidobacterial and lactic acid bacteria is adhesion to human intestinal cells. Strains able to adhere can exert metabolic and immunomodulatory functions, stabilizing the intestinal mucus barrier and providing the competitive exclusion of pathogenic bacteria [20, 21, 22, 23; 24]. Exopolysaccharides can be involved in this capability to adhere to mucus [25]. The presence of genes and gene clusters encoding for *pilus-like* structures has recently been demonstrated in the genomes of bifidobacteria [26]. The degree of in vitro adhesion depends on many factors, such as the substrate used for the assay (abiotic or biotic surface) and growth medium composition.

The ability to tolerate oxygen allows these species to have other metabolic features. Genome analysis of strains belonging to the *Bifidobacterium asteroids* species demonstrated the presence of a "malolactate fermentation pathway", responsible for the conversion of malic acid to lactate, with the addition of the characteristic "fructose-6-phosphate pathway". The latter is uniquely responsible for fructose and glucose fermentation and contains fructose-6-phosphate phosphoketolase (F6PPK), but glucose-6-phosphate is absent [7] The *Bifidobacterium asteroids* species is reported to possess catalase [27].

In the present study, two *Bifidobacterium* strains isolated from honeybee gut were evaluated for safety and other properties.

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2. Materials and Methods

2.1 Reference strains and culture conditions

The hemolytic Gram-positive strains *Streptococcus pyogenes* ATCC 19615 and *Streptococcus pneumoniae* ATCC 6303 were cultured on Brain-Heart Infusion (BHI, Becton Dickinson GmbH, Germany) at 37 °C under 5% CO_2 conditions. *Escherichia coli* ATCC 25922, *E. coli* ATCC 9637, *Staphylococcus aureus* ATCC 6538, and ATCC 29213 were routinely cultured on Trypticase Soy Broth medium (Oxoid, Italy) at 37 °C under aerobic conditions. *Listeria monocytogenes* DSM 12464, *Salmonella enterica* serovar *typhimurium* ATCC 14028, and *Salmonella enterica* subsp. *enterica* serovar *enteritidis* ATCC 13076 were revitalized in BHI broth at 30 °C under aerobic conditions. The probiotic strain *Bifidobacterium animalis* BB12 (Christian Hansen AS, Hoersholm, Denmark), was grown in Bifidus Selective Medium Broth (BSM, Sigma Aldrich) at 37 °C, under anaerobic conditions, and included in every experiment for comparison.

2.2 Identification of isolates

Two bacterial isolates named BA15 and BA17, previously obtained from honeybee gut and subjected to morphological characterization (unpublished data), were analyzed for physiological and biochemical properties (catalase, oxidase, spore formation, gelatinase activities, production of indole, NH_3 from arginine and CO_2 from glucose) using the API rapid ID 32 A (BioMérieux, Italy). Based on the enzymatic profile, typical of the *Bifidobacterium* genus, both isolates were identified at species level. The total genomic DNA was extracted following the method previously described [28] and 16S rDNA was amplified using the primer pairs Bif164 and Bif662, according to Ruas-Madiedo et al. (2005) [29, 30] PCR products were purified using a Qiaquick PCR purification kit (Qiagen Hilden, Germany) and subjected to 16S rDNA sequencing. Comparison with sequences held in the BLAST database allowed both stains to be ascribed to the *Bifidobacterium asteroides* species. The accession numbers of the sequenced strains were as follows (code and identity percentage of isolates in parentheses): *Bifidobacterium asteroides* MG650026.1 (BA15, 99.60%) and *Bifidobacterium asteroides* CP017696.1 (BA17, 99.41%).

2.3 Safety assessment

2.3.1 Antimicrobial Susceptibility and MIC Determination

Antimicrobial susceptibility was determined according to the ISO 10932:2010 [31] broth microdilution procedure using eight antimicrobial agents (ampicillin sodium salt, chloramphenicol, clindamycin hydrochloride, erythromycin, gentamicin sulphate, kanamycin sulphate, rifampicin, streptomycin sulphate salt, tetracycline, vancomycin), all purchased from Sigma Aldrich (St. Louis, MO, USA). The Minimum Inhibitory Concentration (MIC), defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic-free control well, was determined by the microdilution method according to Russo et al. (2018) [32]. The experiments were conducted in triplicate.

The genomes of the BA15 and BA17 strains were analyzed for the presence of antibacterial resistant genes and other gene associations that can influence the safety profile of the strains. The analysis was performed using the Pathosystems Resource Integration Center (PATRIC) database [33].

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2.3.2 PCR Assay on Virulence Factors

The presence of virulence genes (*asa1*, *cylA*, *gelE*, *hyl*, *esp*) was evaluated by multiplex PCR following the method described by Vankerckhoven et al. (2004) [34] and using the primer pairs reported in Table 1. PCR reactions were performed in a final volume of 25 µl containing 1.0 µL of genomic DNA, 1.0 µL of each primer (100 mM) and 12.5 µL of 5-PRIME MasterMix including HotStarTaq DNA polymerase (Eppendorf, Italy). Amplification was carried out as follows: an initial activation step at 94 °C for 15 min, where DNA polymerase was activated; 30 amplification cycles of denaturation (94 °C for 1 min), annealing (56 °C for 1 min), and extension (72 °C for 1 min); followed by one final extension step consisting of 10 min at 72 °C. PCR products were analyzed by electrophoresis in a 1.5 % w/v of agarose gel for 1 h at 90 V in 1.0 × TAE buffer solution. After treatment with ethidium bromide solution, the amplicons were detected by UV light. The Lamba DNA/HindIII marker (Thermo Fisher Scientific, Italy) and the Φ174 DNA Marker Hae III Digest (Sigma Aldrich, Italy) were used as DNA ladders [35].

Gene	Virulence factor	Primer name	Oligonucleotide sequence (5′ to 3′)	Product size (bp)
asa1	aggregation substance	ASAfw	GCACGCTATTACGAACTATGA	375
		ASArw	TAAGAAAGAACATCACCACGA	
cylA	cytolysin	CYTfw	TATGACAATGCTTTTTGGGAT	213
		CYTrw	AGATGCACCCGAAATAATATA	
gelE	gelatinase	GELfw	ATAGACAATGCTTTTTGGGAT	213
		GELrw	AGATGCACCCGAAATAATATA	
hyl	hyaluronidase	HYLfw	ACAGAAGAGCTGCAGGAAATG	276
		HYLrw	GACTGACGTCCAAGTTTCCAA	
esp	surface protein	SPfw	AGATTTCATCTTTGATTCTTGG	688
		SPrw	AATTGATTCTTTAGCATCTGG	

Table 1. PCR primers used for the detection of virulence genes

2.3.3 Other assessments: hemolytic activity and DNAse and Gelatinase Activities

B. asteroides BA15 and BA17 strains, grown in BSM broth for 18–24 h at 37 °C under anaerobic conditions, were streaked onto blood agar plates containing sheep blood (Biolife, Milan, Italy), and anaerobically incubated at 37 °C for 24–48 h. After incubation, the plates were visually analyzed for the presence or absence of microbial hemolytic properties and distinguished as β-hemolysis, α-hemolysis, or γ-hemolysis, based on the appearance of a clear zone, green halo, or no zones around the colonies, respectively [**28**]. *S. pyogenes* ATCC 19615 and *S. pneumoniae* ATCC 6303 were used as positive controls. DNase and gelatinase activities were tested in triplicate, as suggested by Pino et al. (2019) [36].

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2.4 Hydrophobicity, Auto-Aggregation, and Co-Aggregation Abilities

Hydrophobicity (H%), Auto-aggregation (Auto-A%) and Co-aggregation (Co%) abilities were tested as described by Pino et al. (2021) [37]. *E. coli* ATCC 25922, *S. aureus* ATCC 6538, and *S. typhimurium* ATCC 14028 were used as the pathogenic strains in the co-aggregation assay.

2.5 Adhesion on abiotic surface

Adhesion capability was tested using overnight cell cultures grown in Man Rogosa and Sharpe (MRS, Oxoid, Italy) broth supplemented with 0.25 % L-cysteine (Sigma Aldrich, Italy) (MRSc) and incubated at 37 °C under anaerobic conditions (80 % N_2, 10 % CO_2 and 10 % H_2) using the AnaeroGen sachet (Oxoid, Italy). 200µL of a 1:100 dilution of each culture was transferred to a 96-well micro-ELISA plate (number of replicates: 32) and, after regular shaking, the absorbance was read at 600 nm (ELx808, BioTeK – software Gen5) and the plate was incubated. After incubation at 37 °C for 120 minutes under aerobic conditions, the plate was washed twice using sterile PBS to remove non-adherent bacteria and air-dried for 60 minutes at 60 °C. Then, 200 µL of a solution of 0.25 % crystal violet were added to each well and the plate was incubated at room temperature for 15 minutes. After incubation, the plate was rinsed twice using Milli-Q water (Millipore, Italy) to remove excess dye and 200 µL of a 98 % ethanol solution were added to each well. The absorbance was read at 570 nm. The adherence index was calculated as follow: Abs570/Abs600 [38].

2.6 Alignment for VanZ putative gene

The putative *VanZ* gene sequences for the *Bifidobacterium asteroides* BA15 and *Bifidobacterium asteroids* BA17 genomes were compared to those of reference strains *Bifidobacterium asteroides* DSM 20089 (CP017696.1:1959032-1960146) and *Bifidobacterium asteroides* PRL2011 (CP003325.1:10964-12078) using a multiple sequence alignment method with reduced time and space complexity (MUSCLE) and DNASTAR software [39].

2.7 Antagonistic activity against pathogens

2.7.1 Agar diffusion assay

Antagonistic activity was evaluated using *E. coli* ATCC 25922, E. coli ATCC 9637, *S. aureus* ATCC 6538, *S. aureus* ATCC 29213 and *S. typhimurium* ATCC 14028 as target bacteria. The assay was performed by the agar spot test [40], using the cell-free culture supernatants obtained as reported by Argyri et al. (2013) [41]. After incubation for 48 h, the appearance of inhibition zones was visually detected and, based on the diameter size, results were expressed as: (-) no inhibition zone; (+) inhibition zone < 5mm; (++) inhibition zone > 5mm [**21**].

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2.7.2 Killing activity

Antagonistic activity was evaluated using *E. coli* ATCC 9637 and *S. aureus* ATCC as models for Gram-negative and Gram-positive bacteria, respectively. The assay was performed using the broth microdilution method described by CLSI M7-A7 [42] for bacteria and modified by Inturri and et al (2019) [**24**], using the cell-free supernatants obtained as previously described. Incubation was performed under aerobic conditions at 37 °C, for 24 h. The absorbance was read at 630 nm (ELx808, BioTeK – software Gen5) after regular shaking with a frequency every 30 minutes. The killing curves were created by plotting OD values versus time, and bacterial growth kinetics were studied using Prisma 8 software. Each assay was performed three times in duplicate.

2.8 Statistical analysis

All data were expressed as a mean and standard deviation of three independent experiments. Data were subjected to one-way ANOVA followed by Tukey's Multiple Comparison Test and differences were considered statically significant at $p < 0.05$.

3. Results

3.1 Enzymatic profile

Table 2 shows the enzymatic profile of the tested *B. asteroides* BA15 and BA17 strains as well as the percentage of the positive reactions for the *Bifidobacterium* genus, carried out according to manufacturer's instructions. The results of the assay, analyzed by apiwebTM suggested an enzymatic profile for both strains that was characteristic of the *Bifidobacterium* genus (% ID 99.9). More precisely, the ID profile was 4537033705 for the BA15 strain, whereas the ID profile for the BA17 strain was 4517033505 (Table 2). Based on the fermentative profile (Table 3), both strains showed similar biochemical properties except for their ability to ferment L-Arabinose.

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Reaction/Enzyme	BA15	BA17	*Bifidobacterium* spp. (% of positive reaction)
Urease	-	-	0
Arginine dehydrolase	+	+	100
α-galactosidase	+	+	100
β-galactosidase	-	-	9
β-galactosidase-6-phosphate	+	+	100
α-glucosidase	+	+	91
β-glucosidase	+	-	45
α-arabinosidase	-	-	0
β-glucuronidase	+	+	64
N-acetyl-β-glucosaminidase	+	+	99
Mannose fermentation	+	+	93
Raffinose fermentation	-	-	0
Glutamic acid decarboxylase	-	-	0
α-fucosidase	-	-	9
Reduction of nitrates	-	-	1
Indole production	-	-	5
Alkaline phosphatase	+	+	100
Arginine arylamidase	+	+	99
Proline arylamidase	-	-	27
Leucyl glycine arylamidase	+	+	99
Phenylalanine arylamidase	+	+	91
Leucine arylamidase	-	-	9
Pyroglutamic acid arylamidase	+	+	99
Tyrosine arylamidase	+	-	64
Alanine arylamidase	+	+	99
Glycine arylamidase	+	+	91
Histidine arylamidase	-	-	1
Glutamyl Glutamic Acid Arylaminidase	+	+	91
Serine arylaminidase	-	-	0

Legend: positive reaction (+); negative reaction (-).

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Table 2. Enzymatic profile exhibited by the *Bifidobacterium asteroides* BA15 and BA17 strains.

Biochemical reactions	BA15	BA17
NH_3 from arginine	-	-
Gelatin liquefaction	-	-
Indole production	-	-
Glucosidase	+	+
Xylose	-	-
D-Fructose	+	+
D-Galactose	+	+
Maltose	-	-
Trehalose	-	-
D-Melibiose	+	+
Mannitol	-	-
Salicin	-	-
Sorbitol	-	-
L-Arabinose	-	+
Raffinose	-	-
D-Ribose	-	-
Lactose	+	+
Inulin	-	-
Cellobiose	-	-
Melezitose	-	-

Legend: positive reaction (+); negative reaction (-).

Table 3. Biochemical profile exhibited by the *Bifidobacterium asteroides* BA15 and BA17 strains.

3.2 Safety assessment

The antibiotic susceptibility profile was the same for both *B. asteroides* BA15 and B. *asteroides* BA17 strains. Based on EFSA criteria [43], the two strains were susceptible to the main tested antimicrobials, except for ampicillin, vancomycin and chloramphenicol (Table 4).

The PCR-based approach did not reveal the presence of genes encoding for gelatinase (*gelE*), hyaluronidase (*hyl*), aggregation substance (*asa1*), enterococcal surface protein (*esp*) and cytolysin (*cylA*).

The results obtained from the analysis of the bacterial genome using PATRIC bioinformatic services showed the absence of antibiotic resistant genes in the BA15 and BA17 genomes and the presence of a metabolic pathway responsible for vancomycin biosynthesis (Figure 1). The key enzyme was the dTDP-glucose 4,6-dehydratase (EC number 4.2.1.46), encoded by a gene located from nucleotide 1368973 to 1370001 for the BA15 strain, and from nucleotide 319105 to 320202 for the BA17 strain. The expression of this enzyme and

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production of vancomycin needs further investigation; however, this metabolic pathway could explain the resistance to vancomycin.

None of the tested *Bifidobacterium asteroides* strains showed the ability to produce DNase and gelatinase or to exert hemolytic activity.

Antagonistic activity against food spoilage and pathogenic bacteria shown by the BA15 and BA17 strains is reported in Table 7. Overall, both BA15 and BA17 strains showed antagonistic activity against all tested pathogens, with the exception of the BA15 strain, which did not show any antagonistic activity against *S. aureus* ATCC 6538 (Table 5).

	AMP(4)*	**VAN(2)***	**GEN(16)***	**STRE(32)***	**ERY(1)***	**CLI(1)***	**TET(8)***	**CHL(4)***
	Tested range (μg/mL)							
STRAINS	(0.5-16)	(0.5-16)	(4-128)	(8-256)	(0.25-8)	(0.25-8)	(2-64)	(1-64)
BB12	<0.5	0.5	128 R	128 R	0.25	<0.25	16 R	2
BA15	16R	>16R	4	8	0.25	0.25	2	64R
BA17	16R	>16R	<4	<8	<0.25	<0.25	<2	64R

* Microbiological cut-off according to the EFSA Journal, 2008. AMP: ampicillin; GEN: gentamicin; STRE: streptomycin; ERY: erythromycin; CLI: clindamycin; TET: tetracycline; CHL: chloramphenicol; R: resistant.

Table 4. Antibiotic resistance pattern of the Bifidobacterium asteroides BA15 and BA17 strains.

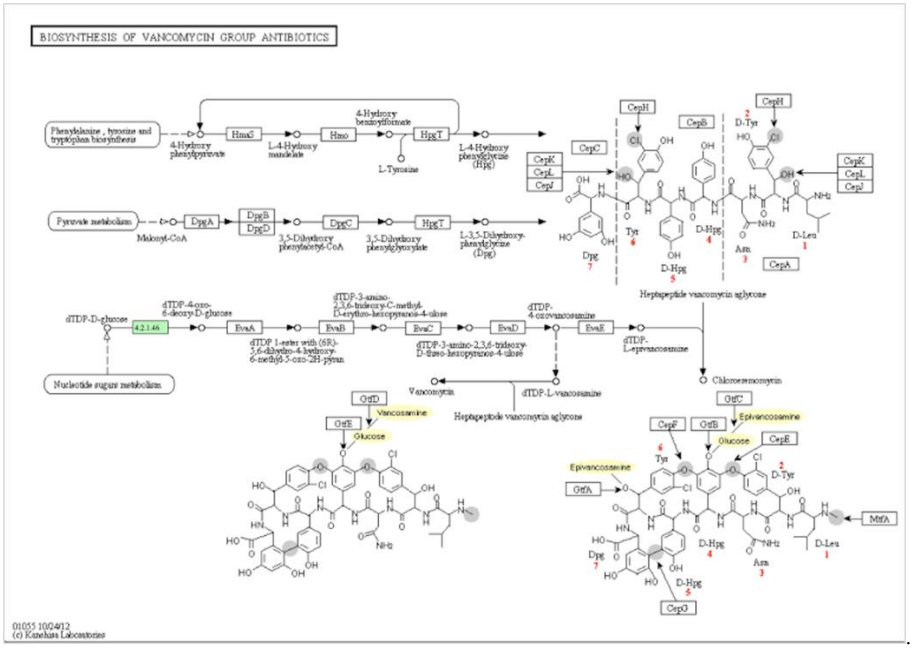

Figure 1. Metabolic pathway responsible for vancomycin biosynthesis. Analysis performed by Patric 3.6.9

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Strains	*E. coli* ATCC 25922	*E. coli* ATCC 9637	*S. typhimurium* ATCC 14028	*S. aureus* ATCC 6538	*S. aureus* ATCC 29213
BB12	++	++	++	++	++
BA15	++	+	++	-	+
BA17	++	+	++	++	+

(-) no inhibition zone; (+) inhibition zone <5mm; (++) inhibition zone >5mm.

Table 5. Antimicrobial activity against pathogenic bacteria

3.3 Adhesion to abiotic surfaces

Figure 2 shows the ability of *B. asteroides* BA15 and BA17 strains to adhere on abiotic surfaces (expressed as *adherence index*). Overall, both tested strains showed adhesion abilities and the highest adherence index was exhibited by the BA17 strain (4.00).

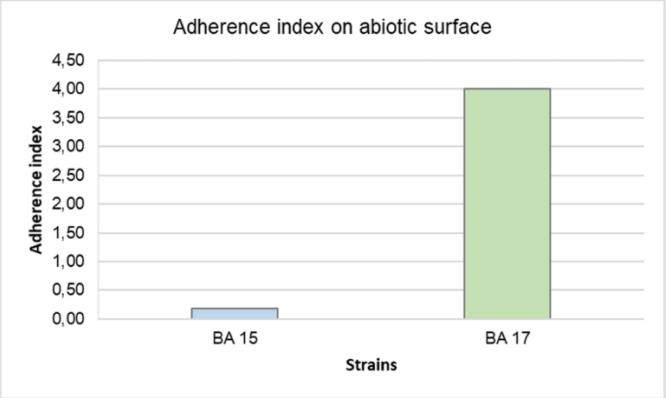

Figure 2. Adherence index of the *Bifidobacterium asteroides* BA15 and *Bifidobacterium asteroides* BA17 strains on an abiotic surface.

3.4 Alignment for VanZ putative gene

The *vanZ* gene is an orthologous gene belonging to the glycopeptide resistance protein family (*vanZ*-A, *vanZ*-F, *vanZ*-Pt and *vanZ*-1). Both tested stains showed the presence of a putative *vanZ* gene. More specifically, *vanZ* was detected in the genome of the *B. asteroides* BA15 strain in three different positions (Strain15_0001: 457-972; Strain15_0979: 1060104-1060616; Strain15_2441: 2576152-2577315) whereas it was only identified once in the *B. asteroides* BA17 genome (17_1679: 2161147-2162262). On the basis of *vanZ* nucleotide analysis using blastn, the *Strain15_0001*, *Strain15_0979*, *Strain15_2441* genes showed a high (100% identity) homology with the genes present in the *Lactobacillus plantarum* genomes. The gene 17_1679 showed a high (93.9 % identity) homology with the gene present in the *Bifidobacterium asteroides* genomes.

Figure 3 shows the putative proteins encoded by *VanZ* from *B. asteroides* BA15 and *B. asteroides* BA17. The number of amino acids of the putative protein encoded by the VanZ gene results as being 171 for gene *Strain15_0001*, 170 for gene *Strain15_0979*, 387 for gene *Strain15_2441*, and 371 for gene *Strain17_1679*, which is equal to those for the control strain PRL 2011. All proteins showed the conserved domain VanZ, which belongs to the *VanZ*-

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like family and contains several examples of the VanZ protein, as well as examples of phosphotransbutyrylases; however, they differ in amino acid length. Moreover, the putative protein encoded by *VanZ* from gene *Strain15_2441* also showed the RDD domain, that is a family of proteins which contains three highly conserved amino acids (one arginine and two aspartates). This region contains two predicted transmembrane regions: the arginine occurs at the N-terminus of the first helix and the first aspartate occurs in the middle of this helix. The molecular function of the RDD region is unknown, however this region may be involved in the transport of a set of ligands that are still unknown.

Table 6 shows the ratio between % identity and distance of the *VanZ* genes of BA15 and BA17 in comparison with the genes present in the genome of the reference strain PRL2011. The MUSCLE analysis algorithm showed a high homology (94 %) between the putative *VanZ* gene from *B. asteroides* BA17 and the reference strain *B. asteroides* PRL2011 (CP003325.1:10964-12078). Instead, a lower homology percentage (from 46.5 to 49.8 %) was shown by *VanZ* genes from the *Bifidobacterium asteroides* BA15 genome (Table 6).

Figure 4 (panel A) shows the unrooted phylogenetic tree relating the *VanZ* genes of BA15 and BA17 strains and their distance with the PRL2011 strains. Instead, panel B shows the nucleotide sequences of the putative *VanZ* gene of BA15 and BA17 strains and their distance with the DSM 20089 and PRL2011 strains, aligned using MUSCLE (MegaAlign Pro of DNAstar).

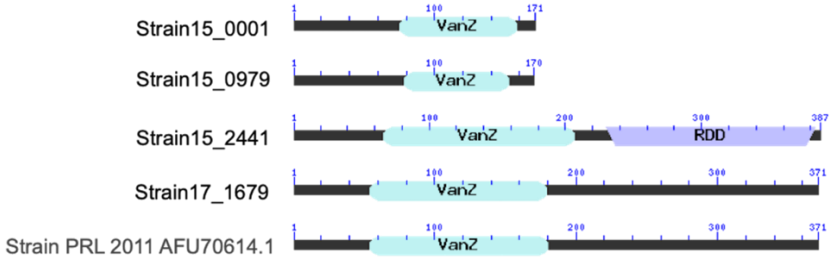

Figure 3. Domain of the putative protein encoded by the *VanZ* gene in the BA15 and BA17 genome, obtained by CDART (domain architectures) from the NCBI database.

	Strains	%identity	%Gaps	Identical	Gap Count	Gap Length	Score	Length
PRL2011	Strain15_0001	46.5	36.9	303	55	240	348	651
	Strain15_0979	49.0	32.8	329	62	220	379	671
	Strain15_2441	49.8	31.5	649	109	409	872	1297
	Strain17_1679	94.0	0.0	1048	0	0	4972	1115

Table 6. Results of single alignment (Pairwise) between nucleotides of the *VanZ* gene from reference strain PRL2011 (CP003325.1:10964-12078) vs Strain15_0001, Strain15_0979, Strain15_2441, Strain17_1679.

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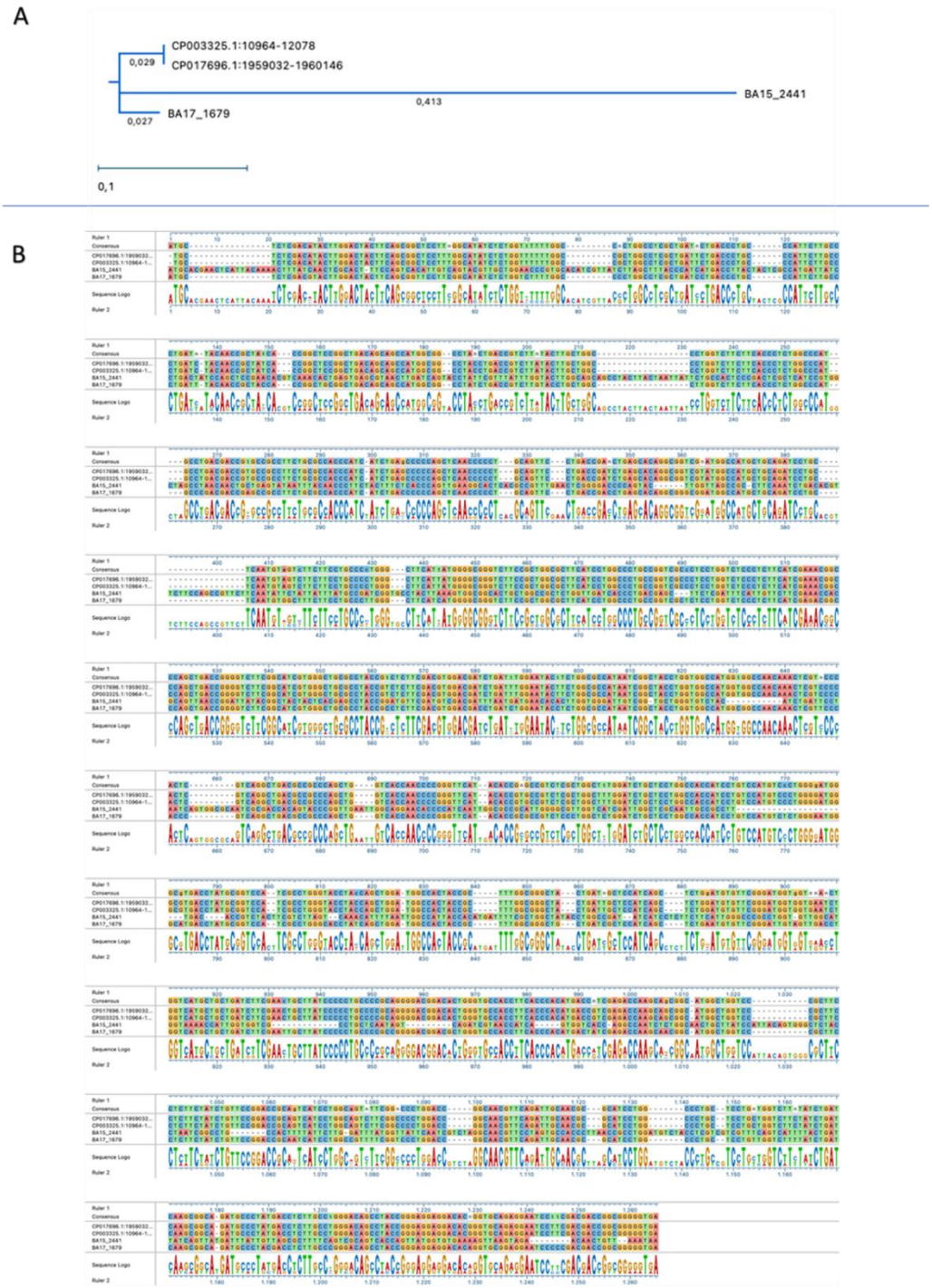

Figure 4. Unrooted phylogenetic tree (constructed by maximum likelihood: RAxML) relating the VanZ of BA15 and BA17 and their distance with the DSM 20089 and PRL2011 strains (panel A); nucleotide sequences of the putative VanZ gene of BA15 and BA17 strains and their distance with the DSM 20089 and PRL2011 strains aligned using MUSCLE (panel B).

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3.5 Killing activity

Figure 5 shows the killing and inhibitory activity of the BA15 and BA17 strains against *S. aureus* ATCC 29213, *E. coli* ATCC 9637. Both tested strains showed high activity against the Gram-positive *S. aureus* ATCC 29213, with a concentration range from 1:4 to 1:32 (Figure 5, panels A and C). Both of the tested supernatants resulted active towards the Gram-negative *E. coli* ATCC 9637 in a dilution range from 1:4 to 1:8 (Figure 5, panels B and D). Killing activity against *S. aureus* ATCC 29213 was exerted in the dilution range from 1:4 to 1:8 whereas, against *E. coli* ATCC 9637, it was only exerted at the higher tested concentration (dilution 1:4 of the wild supernatants). The supernatant obtained from BA15 and BA17 showed an inhibitory activity towards *S. aureus* ATCC 29213 when tested at the dilution range from 1:16 to 1:32, and towards *E. coli* ATCC 9637 when tested at a dilution of 1:8. In particular, both BA15 and BA17 appeared to reduce the growth of *S. aureus* ATCC 29213, *E. coli* ATCC 9637, which after 24 h of incubation reported an OD_{630} lower than the untreated control. The growth of *E. coli* ATCC 9637 was delayed by about 14 h from the time of incubation when treated with BA15 and by about 9 h (from the time of incubation) when treated with BA17, although after 24 h, the OD_{630} values were the same as those of the untreated control.

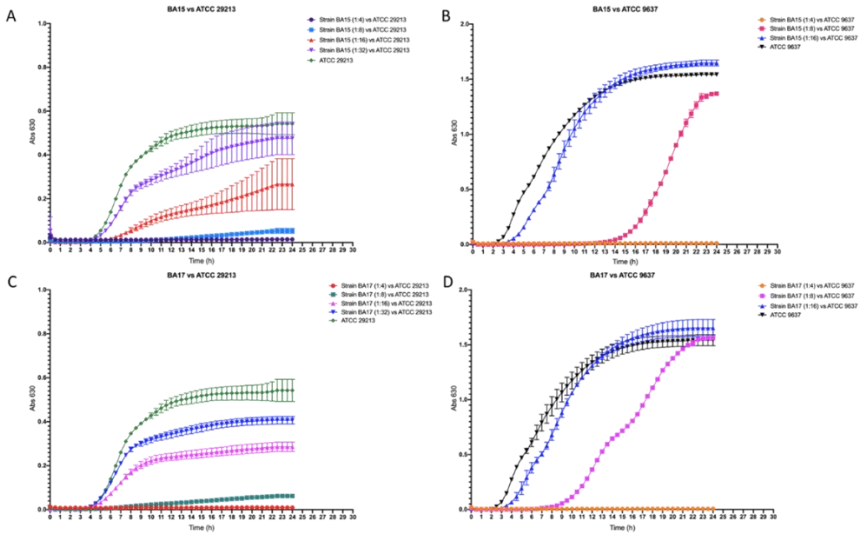

Figure 5. Killing curves of the supernatant obtained from 96h broth cultures of *Bifidobacterium asteroides* BA15 and *Bifidobacterium asteroides* BA17 strains at different concentrations (dilution 1:4, 1:8; 1:16; 1:32) against *Escherichia coli* ATCC 9637 and *Staphylococcus aureus* ATCC 29213 reference strains.

3.6 Auto-Aggregation, Co-Aggregation, and Hydrophobicity Abilities

Table 7 summarizes the surface characteristics (hydrophobicity, auto-aggregation, and co-aggregation) of the *B. asteroides* BA15 and BA17 tested strains, compared to those exhibited by the *Bifidobacterium animalis* BB12 strain. The BA15 and BA17 strains showed an auto-aggregation %, similar to that displayed by the BB12 reference strain. Both BA15 and BA17 strains also exhibited the ability to co-aggregate with the tested pathogens. In particular, both the *B. asteroides* BA15 and BA17 strain showed the highest Co-A% with *Listeria monocytogenes* ATCC 12466 (Table 7), when compared to the *B. animalis* BB12 strain. A variable degree of hydrophobicity was observed, with the highest percentage exhibited by the BB12 and Ba17 strains.

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Strains	Auto-A%	Co-A%					H%
		S. aureus ATCC 6538	*S. typhimurium* ATCC 14028	*E. coli* ATCC 25922	*S. enteritidis* ATCC 13076	*Listeria monocytogenes* ATCC 12466	
BB12	36.70±0.11^{a}	40.05±0.17^{b}	10.00±0.19^{a}	23.50±0.13^{b}	34.50±0.12^{c}	15.60±0.13^{a}	84.50±0.13^{c}
BA 15	34.13±0.13^{a}	14.22±0.12^{a}	19.25±0.17^{b}	15.97±0.12^{a}	15.97±0.15^{a}	19.18±0.15^{b}	59.67±0.14^{a}
BA 17	33.11±0.17^{a}	16.67±0.11^{a}	13.33±0.18^{a}	28.31±0.18^{b}	26.25±0.17^{b}	19.73±0.12^{b}	79.15±0.11^{b}

Results are expressed as average value and standard deviation of three separate experiments. The different letters in the same column indicate significant differences by one-way ANOVA test, followed by Tukey's post-hoc test ($P < 0.05$). Auto-A%: Auto-aggregation; CoA%: Co-aggregation; H%: Hydrophobicity.

Table 7. Auto-Aggregation, Co-Aggregation, and Hydrophobicity Abilities of the tested strains

4. Discussion

The gut microbiota of honeybees and insect pollinators is still an unexplored ecosystem. Recent findings on human gut microbiota have paved the way for a better understanding of its role in other living species on Earth. The evolutionary role of bifidobacteria among humans and animals seems to be related to their ability to ferment complex non-digestible carbohydrates and to modulate the host immune system through changes in innate and/or adaptive immune responses [44, 45]. The distribution of specific species of bifidobacteria across the human lifetime has been recently studied [46] and a high transfer level between family members demonstrated. A small number of bifidobacterial subspecies (*Bifidobacterium pseudolongum*, *Bifidobacterium adolescentis*, *Bifidobacterium pseudocatenulatum* and *Bifidobacterium bifidum*) have been recognized as cosmopolitan because they have been isolated from various animal and mammalian hosts, unlike other taxa which appear to be much less widely distributed [47]. A study of primate-associated bifidobacteria demonstrated the phylosymbiosis between the Hominidae family and bifidobacterial species isolated from humans, on the basis of observed bifidobacterial-host co-phylogeny [48]. Despite the social relevance of bees, their gut microbiota is still far from being completely understood. Recent ecological surveys on gut microbiota of insects have revealed that as mammals they rely on a mutualistic gut microbial community [49]. Differently from other insects, such as ants, whose microbiota is acquired from food and the environment, honeybees, similarly to humans, have a gut microbiota with a stable core that, after the early developmental stages, remains relatively stable through most of adult lifetime [50]. It has recently been discovered that the phyla that constitute the core of honeybee gut microbiota are three of the most important components of human gut microbiota (Firmicutes, Proteobacteria, Actinobacteria) [**15**]. Several studies using 16S rDNA surveys and metagenomic of the total DNA, highlighted that *Bifidobacterium asteroids*, along with *Lactobacillus* FIRM4 and *Lactobacillus* FIRM5, represent the so-called *core-bacteria*, being the most essential microorganism in the honeybee gut and these evidence could be related to a possible probiotic potential of *Bifidobacterium asteroids* strains [49, 50, 52]. The *Bifidobacterium asteroides* PRL2011 strain, isolated from the hindgut of *Apis mellifera*, represents the first reported case of the presence of a respiratory chain, which means that this strain may be able to growth in aerobic conditions. This species is phylogenetically distant from other bifidobacterial species and its ability to tolerate oxygen has been lost in bifidobacteria that inhabit the mammalian gut [51, 52]. The present study characterizes two strains of *Bifidobacterium asteroides* isolated from honeybees, with the aim of contributing to better understanding the properties of this species and their possible applications.

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The strains were typed and characterized by using both phenotypic and genotypic tests. In addition, a stepwise process, following the FAO/WHO working group [53] guidelines, was carried out to identify the strains at phenotypic/genotypic levels, including in vitro procedures, to investigate safety and other properties. The EFSA has suggested that more research on bacterial genomes should be carried out to provide an adequate characterization of new isolates [54]. Genome analysis can define the safety profile and is useful for the characterization of specific properties, such as the production of metabolites, polysaccharides, and compounds with antimicrobial activity [**7**, 55, 56, 57].

In accordance with former studies, our results confirm that the combination of genotypic and phenotypic methods is a powerful tool for strain discrimination [**30**, 58].

Safety concerns represent one of the main requirements that should be addressed for the selection of new functional strains. Other requirements are the absence of potential pathogenic traits (hemolytic, DNAse and gelatinase activities) and the study of the antibiotic resistance profile.

It is well known that strains able to transfer resistance to certain antibiotics are of great interest because they can be co-administrated with antibiotics, avoiding antibiotic side-effects [59, 60]. Therefore, in the present study, the *B. asteroides* strains were tested for antimicrobial resistance following the EFSA guidelines [**9**]. In accordance with previous studies, the phenotypic approach highlighted that both BA15 and BA17 stains were susceptible to gentamicin, streptomycin, erythromycin, clindamycin, and tetracycline [**60**, 61, 62, 63, 64]

Even though the tested *B. asteroides* BA15 and BA17 strains showed phenotypic resistance to ampicillin, vancomycin, and chloramphenicol, genome analysis discarded the risk of transferability to the host. Moreover, in the bifidobacterial genome the *VanZ* genes could be expected to confer low-level resistance to glycopeptide antibiotics, which act by preventing the incorporation of D-Alanine into peptidoglycan precursors. Specific strains of bifidobacteria contain a *VanZ* homolog flanked by a predicted transposase encoding gene (transposon family IS256) [**19**].

The *Bifidobacterium bifidum* Yakult strain YIT4007 is a mutant of *Bifidobacterium bifidum* Yakult strain YIT4001, showing enhanced resistance to neomycin, erythromycin and streptomycin, due to a chromosomal mutation on genes *rluD* and *rspL*, which increases the resistance to aminoglycosides. In silico analysis has revealed the presence of putative genes for β-lactamase resistance in the Bifidobacterium spp. genome, however laboratory-based investigations have demonstrated that non-representative strains are resistant to β-lactam antibiotics [65]. Based on collated data from worldwide sources, the European Committee on Antimicrobial Susceptibility testing [66] software displays the distribution of MIC-values (generated by methods calibrated to broth microdilution or agar dilution) and zone diameters (generated with EUCAST disk diffusion methodology), together with EUCAST epidemiological cut-off values (ECOFFs) and the species *Bifidobacterium asteroides* is not reported among those species subjected to surveillance, as like *B. adolescentis*, *B. angulatum*, *B. animalis*, *B. bifidum*, *B. breve*, *B. catenulatiom*, *B. dentium*, *B. longum*, *B. pseudocatenulatum*, *B. pseudolongum*, *B. ruminantium* and *B. thermophilum*. Thus, considering the generic indications for *Bifidobacterium* spp., it is reasonable surveilling the streptomycin and tetracycline resistance for these strains. The range of MIC susceptibility values for these two antibiotics (streptomycin and tetracycline) reported by EUCAST are from 4 to 512 μg/ml and from 0.025 to 512 μg/ml, respectively. These different from the range values reported by the EFSA (2012) (from 8 to 256 μg/ml for streptomycin and from 2 to 64 μg/ml for tetracycline). According to EUCAST, the highest MIC distribution percentage for streptomycin was 30.77%, with a MIC value of 64 μg/ml and for tetracycline the highest MIC distribution percentage was 25.27%, with a MIC value of 0.5 μg/ml (EUCAST 2019).

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According to these suggestions, the MIC value of 8 μg/ml shown by the *B. asteroides* BA15 strain for streptomycin is lower than the values reported by EUCAST, for most *Bifidobacterium* spp. (64 μg/ml); in fact, only 3.5% of *Bifidobacterium* spp. show a MIC value of 8 μg/ml using streptomycin. The MIC value of 2 μg/ml shown by the strain *B. asteroides* BA15 for tetracycline is higher than that reported by EUCAST for most *Bifidobacterium* spp. (0.5 μg/ml), in fact only 7.6% of Bifidobacterium spp. show a MIC value of 2 μg/ml using tetracycline.

The Actinobacteria phylum harbors antibiotic-producing bacteria and carries a large number of resistance genes. Bifidobacteria can harbor resistance genes to macrolide, lincosamide, streptogramin, ketolide, oxazolidinone (MLSKO), tetracycline (tetracycline resistance and efflux genes) and aminoglycosides (aminoglycosides resistance and efflux genes). As far as MLSKO resistance genes are concerned, *Bifidobacterium* spp., can harbor the *erm*(A), *erm*(X), *erm*(CD) and *erm*(Y) genes in their genomes; as regards tetracycline resistance genes, their genomes can include the *tet*(M), *tet*(S), *tet*(W), *tet*(O), *tet*(Q), *tet*(L) genes; among the aminoglycosides, they retain the *aph*(E) gene [67, 68]. Even though the tested *Bifidobacterium asteroides* BA 15 and BA 17 strains showed phenotypic resistance to ampicillin, vancomycin, and chloramphenicol, genome analysis discarded the risk of transferability to the host. Genome analysis reveals the absence of antibiotic genes associated to these antibiotics.

The informatic analysis performed on all putative proteins encoded by the *VanZ* gene showed a well-organized conserved domain. In particular, all putative proteins show the presence of the VanZ domain. The proteins encoded by VanZ 0001 from the *B. asteroides* BA15 genome is the only one showing two domains, VanZ and RDD. The VanZ proteins family may be involved in the transport of a still-unknown set of ligands, because it contains two predicted transmembrane regions, the arginine occurs at the N-terminus of the first helix and the first aspartate occurs in the middle of this helix [69].

The presence of a putative metabolic pathway for vancomycin synthesis in the genomes of both strains is noteworthy. Moreover, in the bifidobacterial genome, the *VanZ* genes could be expected to confer low-level resistance to glycopeptide antibiotic, which acts by preventing the incorporation of D-Alanine into peptidoglycan precursors. Specific strains of bifidobacteria contain a *VanZ* homolog flanked by a predicted transposase encoding gene (transposon family IS256) [**19**].

The ability to adhere to surfaces, hydrophobicity, and auto-aggregation [70], are considered a prerequisite for different applications, such as food for honeybees and antibiotic production. In the present study, in which xylene was chosen as an apolar solvent able to reflect cell surface hydrophobicity [71] the *B. asteroides* BA17 strains exhibited good adhesion to hydrocarbons. In addition, both tested strains showed an auto-aggregation ability similar to the one exhibited by the *B. animalis* BB12 reference strain. This feature is essential for epithelium cell colonization preventing elimination by peristalses [72]. Antimicrobial activity against pathogens has been the subject of numerous investigations [**59**, 73, 74]. In this study, a broad range of antagonistic activity was displayed by both BA17 and BA15 strains, confirming the ability to inhibit and displace pathogens, as previously reported [75, 76]. Several mechanisms have been suggested to explain this inhibitory activity of bifidobacteria towards both Gram-positive and Gram-negative pathogens [**74**, 77, 78], such as the decrease in local pH via the production of organic acids, as well as the production of bacteriocins or bacteriocin-like compounds [79].

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5. Conclusions

The present study provides evidence that honeybee gut can be considered a reservoir of bacteria with safety features. This suggests that honeybees could be exploited as an almost unexplored source of isolates for application in different fields, such as food for precious pollinator insects exposed to pesticides and toxic products at sublethal concentrations, and also for antibiotic production.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cunningham, M., Azcarate-Peril, M.A., Barnard, A., Benoit, V., Grimaldi, R., Guyonnet D., et al. (2021) Shaping the Future of Probiotics and Prebiotics. Trends Microbiol. 29(8):667-685. doi: 10.1016/j.tim.2021.01.003.

2. Biavati B., and Matterelli, P. (2006). The Family Bifidobacteriaceae Cap. 1.1.2 pp. 331-336. in The Prokaryotes, 3rd Edition, Springer, vol. 3, New York: Ed. by Dworkin M.

3. Okamoto, M., Benno, Y., Leung, K. P., & Maeda, N. (2008). Bifidobacterium tsurumiense sp. nov., from hamster dental plaque. International journal of systematic and evolutionary microbiology, 58(Pt 1), 144–148. https://doi.org/10.1099/ijs.0.65296-0

4. Ventura, M., Turroni, F., Zomer, A., Foroni, E., Giubellini, V., Bottacini, F., Canchaya, C., Claesson, M. J., He, F., Mantzourani, M., Mulas, L., Ferrarini, A., Gao, B., Delledonne, M., Henrissat, B., Coutinho, P., Oggioni, M., Gupta, R. S., Zhang, Z., Beighton, D., … van Sinderen, D. (2009). The Bifidobacterium dentium Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. PLoS genetics, 5(12), e1000785. https://doi.org/10.1371/journal.pgen.1000785

5. Watanabe, K., Makino, H., Sasamoto, M., Kudo, Y., Fujimoto, J., & Demberel, S. (2009). Bifidobacterium mongoliense sp. nov., from airag, a traditional fermented mare's milk product from Mongolia. International journal of systematic and evolutionary microbiology, 59(Pt 6), 1535–1540. https://doi.org/10.1099/ijs.0.006247-0

6. Arzamasov, A.A., van Sinderen, D., Rodionov D.A. (2018) Comparative Genomics Reveals the Regulatory Complexity of Bifidobacterial Arabinose and Arabino-Oligosaccharide Utilization. Frontiers in Microbiology 9, 2018, 776.

7. Bottacini, F., Milani, C., Turroni, F., Sánchez, B., Foroni, E., Duranti, S., et al. (2012). Bifidobacterium asteroides PRL2011 genome analysis reveals clues for colonization of the insect gut. PLoS One. 2012;7(9):e44229. doi: 10.1371/journal.pone.0044229.

8. Alberoni, D., Gaggìa, F., Baffoni, L., Modesto, M.M., Biavati, B., Di Gioia, D (2019). *Bifidobacterium xylocopae* sp. nov. and *Bifidobacterium aemilianum* sp. nov., from the carpenter bee (Xylocopa violacea) digestive tract. Syst Appl Microbiol. 42(2):205-216. doi: 10.1016/j.syapm.2018.11.005.

9. EFSA Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. The EFSA Journal (2007) 587, 1-16

10. Sornplang, P., & Piyadeatsoontorn, S. (2016). Probiotic isolates from unconventional sources: a review. Journal of animal science and technology, 58, 26. https://doi.org/10.1186/s40781-016-0108-2

11. Pennacchia, C., Vaughan, E. E., & Villani, F. (2006). Potential probiotic Lactobacillus strains from fermented sausages: Further investigations on their probiotic properties. Meat science, 73(1), 90–101. https://doi.org/10.1016/j.meatsci.2005.10.019

12. Chang, J. H., Shim, Y. Y., Cha, S. K., & Chee, K. M. (2010). Probiotic characteristics of lactic acid bacteria isolated from kimchi. Journal of applied microbiology, 109(1), 220–230. https://doi.org/10.1111/j.1365-2672.2009.04648.x

13. Vitali, B., Minervini, G., Rizzello, C. G., Spisni, E., Maccaferri, S., Brigidi, P., Gobbetti, M., & Di Cagno, R. (2012). Novel probiotic candidates for humans isolated from raw fruits and vegetables. Food microbiology, 31(1), 116–125. https://doi.org/10.1016/j.fm.2011.12.027

14. Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature reviews. Gastroenterology & hepatology, 11(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66

15. Daisley, B.A., Chmiel, J.A., Pitek, A.P., Thompson, G.J., Reid, G (2020). Missing Microbes in Bees: How Systematic Depletion of Key Symbionts Erodes Immunity. Trends Microbiol. 28(12):1010-1021. doi: 10.1016/j.tim.2020.06.006.

16. Bonilla-Rosso, G. and Engel, P. (2018). Functional roles and metabolic niches in the honey bee gut microbiota. Curr Opin Microbiol. 43:69-76. doi: 10.1016/j.mib.2017.12.009.

17. Website: https://www.efsa.europa.eu/en/science/scientific-committee-and-panels/feedap

18. Janashia, I., Carminati, D., Rossetti, L., Zago, M., Fronasari, M.E., Haertlé, T., Chanishvili, N., and Giraffa, G. (2016). Characterization of fructophilic lactic microbiota of Apis mellifera from the Caucasus Mountains. Ann Microbiol 66, 1387–1395.

19. Mancino, W., Duranti, S., Mancabelli, L., Longhi, G., Anzalone, R., Milani, C., Lugli, G.A., Carnevali, L., Statello, R., Sgoifo, A., van Sinderen, D., Ventura, M., Turroni, F (2019). Bifidobacterial Transfer from Mother to Child as Examined by an Animal Model. Microorganisms. 7(9):293. doi: 10.3390/microorganisms7090293.

20. Caggia, C., De Angelis, M., Pitino, I., Pino, A. & Randazzo, C. L. Probiotic features of Lactobacillus strains isolated from Ragusano and Pecorino Siciliano cheeses. Food Microbiol. 50, 109–117 (2015).

21. Inturri, R., Stivala, A. and Blandino, G. Microbiological characteristics of the probiotic strains B. longum BB536 and L. rhamnosus HN001 used in combination. Minerva Gastroenterol Dietol. 2015 Dec;61(4):191-7. PMID: 26657925.

22. Blandino, G., Fazio, D., Petronio, G.P., Inturri, R., Tempera, G., Furneri, P.M. Labeling quality and molecular characterization studies of products containing *Lactobacillus* spp. strains. Int J Immunopathol Pharmacol. 2016 Mar;29(1):121-8. doi: 10.1177/0394632015600534. Epub 2015 Dec 14. PMID: 26667227; PMCID: PMC5806746.

23. Inturri, R., Stivala, A., Furneri, P. M., & Blandino, G. (2016). Growth and adhesion to HT-29 cells inhibition of Gram-negatives by Bifidobacterium longum BB536 e Lactobacillus rhamnosus HN001 alone and in combination. European review for medical and pharmacological sciences, 20(23), 4943–4949.

24. Inturri. R., Trovato, L., Volti, G.L., Oliveri, S., Blandino, G. (2019). In vitro inhibitory activity of Bifidobacterium longum BB536 and Lactobacillus rhamnosus HN001 alone or in combination against bacterial and Candida reference strains and clinical isolates. Heliyon. 22;5(11):e02891. doi: 10.1016/j.heliyon.2019.e02891.

25. Ruas-Madiedo, P., Gueimonde, M., Margolles, A., de los Reyes-Gavilán, C.G., Salminen, S (2006). Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal mucus. J Food Prot. 69(8):2011-5. doi: 10.4315/0362-028x-69.8.2011.

26. Foroni, E., Serafini, F., Amidani, D., Turroni, F., He, F., Bottacini, F., O'Connell Motherway, M., Viappiani, A., Zhang, Z., Rivetti, C., van Sinderen, D., Ventura, M. (2011) Genetic analysis and morphological identification of pilus-like structures in members of the genus Bifidobacterium. Microb Cell Fact 10, S16 (2011). https://doi.org/10.1186/1475-2859-10-S1-S16

27. Killer, J., Kopecný, J., Mrázek, J., Rada, V., Dubná, S., Marounek, M (2010). Bifidobacteria in the digestive tract of bumblebees. Anaerobe. 16(2):165-70. doi: 10.1016/j.anaerobe.2009.07.007.

28. Pino, A., Bartolo, E., Caggia, C., Cianci, A., & Randazzo, C. L. (2019a). Detection of vaginal lactobacilli as probiotic candidates. Scientific reports, 9(1), 3355. https://doi.org/10.1038/s41598-019-40304-3

29 Ruas-Madiedo, P., Hernández-Barranco, A., Margolles, A., de los Reyes-Gavilán, C.G. (2005). A bile salt-resistant derivative of Bifidobacterium animalis has an altered fermentation pattern when grown on glucose and maltose. Appl Environ Microbiol. 71(11):6564-70. doi: 10.1128/AEM.71.11.6564-6570.2005.

30. Arboleya, S., Ruas-Madiedo, P., Margolles, A., Solís, G., Salminen, S., de Los Reyes-Gavilán, C.G., et al. (2011). Characterization and in vitro properties of potentially probiotic Bifidobacterium strains isolated from breast-milk. Int J Food Microbiol. 149(1):28-36. doi: 10.1016/j.ijfoodmicro.2010.10.036.

31. ISO: International Organization for Standardization. Milk and Milk Products—Determination of the Minimal Inhibitory Concentration (MIC) of Antibiotics Applicable to Bifidobacteria and Non-Enterococcal Lactic Acid Bacteria (LAB) ISO 10932/IDF 233 Standard 2010; ISO: International Organization for Standardization: Geneva, Switzerland, 2010.

32. Russo, N.; Caggia, N.; Pino, A.; Coque, T.M.; Arioli, S.; Randazzo, C.L. (2018). *Enterococcus* spp. in Ragusano PDO and Pecorino Siciliano cheese types: A snapshot of their antibiotic resistance distribution. Food Chem. Toxicol. 120, 277–286. doi: 10.1016/j.fct.2018.07.023.

33 Wattam, A.R., Abraham, D., Dalay, O., Disz, T.L., Driscoll, T., Gabbard, J.L., et al. (2014). PATRIC, the bacterial bioinformatics database and analysis resource. Nucleic Acids Res. 42(Database issue):D581-91. doi: 10.1093/nar/gkt1099.

34. Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., & Goossens, H. (2004). Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. Journal of clinical microbiology, 42(10), 4473–4479. https://doi.org/10.1128/JCM.42.10.4473-4479.2004

35. Depardieu, F., Perichon, B., and Courvalin, P (2004). Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. J Clin Microbiol 42(12): 5857-60.

36. Pino, A., Russo, N., Van Hoorde, K., De Angelis, M., Sferrazzo, G., Randazzo, C. L., & Caggia, C. (2019b). Piacentinu Ennese PDO Cheese as Reservoir of Promising Probiotic Bacteria. Microorganisms, 7(8), 254. https://doi.org/10.3390/microorganisms7080254

37. Pino, A., Rapisarda, A.M.C., Vitale, S.G., Cianci, S., Caggia, C., Randazzo, C.L., Cianci, A. (2021). A clinical pilot study on the effect of the probiotic Lacticaseibacillus rhamnosus TOM 22.8 strain in women with vaginal dysbiosis. Sci Rep. 28;11(1):2592. doi: 10.1038/s41598-021-81931-z.

38. Gross, M., Cramton, S.E., Götz, F., Peschel, A. (2001). Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. Infect Immun. 69(5):3423-6. doi: 10.1128/IAI.69.5.3423-3426.2001.

39. Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 19;5:113. doi: 10.1186/1471-2105-5-113.

40. Randazzo, C.L., Russo, N., Pino, A., Mazzaglia, A., Ferrante, M., Oliveri Conti, G., et al. (2018). Effects of selected bacterial cultures on safety and sensory traits of Nocellara Etnea olives produced at large factory scale. Food Chem. Toxicol 115, 491–498. doi: 10.1016/j.fct.2018.03.045.

41. Argyri, A.A:, Zoumpopoulou, G., Karatzas, K.AG, Tsakalidou, E., Nychas, GJ.E, Panagou, E.Z, Tassou,C.C.(2013) Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. Food Microbiology. 33(2):282-291.

42. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard Seventh Edition. CLSI document M7-A7 (ISBN 1-56238-587-9). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2006.

43. EFSA. Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. The G Journal (2008) 732, 1-15: output obsolete

44. Alessandri, G., Ossiprandi, M. C., MacSharry, J., van Sinderen, D., & Ventura, M. (2019). Bifidobacterial Dialogue With Its Human Host and Consequent Modulation of the Immune System. Frontiers in immunology, 10, 2348. https://doi.org/10.3389/fimmu.2019.02348

45. Pan, M., Nethery, M. A., Hidalgo-Cantabrana, C., & Barrangou, R. (2020). Comprehensive Mining and Characterization of CRISPR-Cas Systems in Bifidobacterium. Microorganisms, 8(5), 720. https://doi.org/10.3390/microorganisms8050720

46. Odamaki, T., Bottacini, F., Kato, K., Mitsuyama, E., Yoshida, K., Horigome, A., Xiao, J. Z., & van Sinderen, D. (2018). Genomic diversity and distribution of Bifidobacterium longum subsp. longum across the human lifespan. Scientific reports, 8(1), 85. https://doi.org/10.1038/s41598-017-18391-x

47. Turroni, F., Duranti, S., Milani, C., Lugli, G. A., van Sinderen, D., & Ventura, M. (2019). Bifidobacterium bifidum: A Key Member of the Early Human Gut Microbiota. Microorganisms, 7(11), 544. https://doi.org/10.3390/microorganisms7110544

48. Lugli, G. A., Alessandri, G., Milani, C., Mancabelli, L., Ruiz, L., Fontana, F., Borragán, S., González, A., Turroni, F., Ossiprandi, M. C., Margolles, A., van Sinderen, D., & Ventura, M. (2020). Evolutionary development and co-phylogeny of primate-associated bifidobacteria. Environmental microbiology, 22(8), 3375–3393. https://doi.org/10.1111/1462-2920.15108

49. Kešnerová, L., Emery, O., Troilo, M., Liberti, J., Erkosar, B., Engel, P. (2020). Gut microbiota structure differs between honeybees in winter and summer. ISME J. 14, 801–814. doi:10.1038/s41396-019-0568-8

50. Kwong, W.K., and Moran, N.A. (2016). Gut microbial communities of social bees. Nat. Rev. Microbiol. 14, 374–384. doi:10.1038/nrmicro.2016.43.

51. Leahy, S.C., Higgins, D.G., Fitzgerald, G.F., and Van Sinderen, D. (2005). Getting better with bifidobacteria. J. Appl. Microbiol. 98, 1303–1315. doi: 10.1111/j.1365-2672.2005.02600.x.

52. Nowak, A., Szczuka, D., Górczyńska, A., Motyl, I., Kręgiel, D. (2021). Characterization of Apis mellifera Gastrointestinal Microbiota and Lactic Acid Bacteria for Honeybee Protection—A Review. Cells 10, no. 3: 701. doi:10.3390/cells10030701.

53. FAO/WHO. Probiotics in food. Health and nutritional properties and guidelines for evaluation. Rome, 2006.ISBN 92-5-105513-0

54. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Guidance on the Assessment of Bacterial Susceptibility to Antimicrobials of Human and Veterinary Importance. EFSA J. 2012, 10, 2740.

55. Inturri, R., Ventura, M., Ruas-Madiedo, P., Lugli, G.A., Blandino, G. (2017a). Complete Genome sequence of Bifidobacterium longum W11 (LMG P-21586), Used as a Probiotic Strain. Genome Announc. 5(10):e01659-16. doi: 10.1128/genomeA.01659-16.

56. Inturri, R., Mangano, K., Santagati, M., Intrieri, M., Di Marco, R., Blandino, G. (2017b) Immunomodulatory Effects of Bifidobacterium longum W11 Produced Exopolysaccharide on Cytokine Production. Curr Pharm Biotechnol. 18(11):883-889. doi: 10.2174/1389201019666171226151551.

57. Marras, L., Caputo, M., Bisicchia, S., Soato, M., Bertolino, G., Vaccaro, S., & Inturri, R. (2021). The Role of Bifidobacteria in Predictive and Preventive Medicine: A Focus on Eczema and Hypercholesterolemia. Microorganisms, 9(4), 836. https://doi.org/10.3390/microorganisms9040836

58. Gueimonde, M., Delgado, S., Mayo, B., Ruas-Madiedo, P., Margolles, A., de los Reyes-Gavilan, C.G. (2004). Viability and diversity of probiotic Lactobacillus and Bifidobacterium populations included in commercial fermented milks. Food Res Intern. 37, 839-850. doi:10.1016/j.foodres.2004.04.006

59. Awasti, N., Tomar, S.K., Pophaly, S.D., Poonam, Lule V.K., Singh, T.P., Anand, S. (2016). Probiotic and functional characterization of bifidobacteria of Indian human origin. J Appl Microbiol. 120:1021-32. doi:10.1111/jam.13086.

60. Ku, S., Yang, S., Lee, H.H., Choe, D., Johnston, T.V., Ji, G.E., et al. (2020). Biosafety assessment of Bifidobacterium animalis subsp. lactis AD011 used for human consumption as a probiotic microorganism. Food Control. 117, 106985. doi:10.1016/j.foodcont.2019.106985

61 Gueimonde, M., Sánchez, B., de Los Reyes-Gavilán, C., Margolles, A. (2013). Antibiotic resistance in probiotic bacteria. Front Microbiol. 4:202. doi: 10.3389/fmicb.2013.00202.

62. Campedelli, I., Mathur, H., Salvetti, E., Clarke, S., Rea, M.C., Torriani, S., et al. (2018). Genus-wide assessment of antibiotic resistance in *Lactobacillus* spp. Appl. Environ. Microbiol. 85, e01738-18.

63. Kim, M. J., Ku, S., Kim, S. Y., Lee, H. H., Jin, H., Kang, S., Li, R., Johnston, T. V., Park, M. S., & Ji, G. E. (2018). Safety Evaluations of Bifidobacterium bifidum BGN4 and Bifidobacterium longum BORI. International journal of molecular sciences, 19(5), 1422. https://doi.org/10.3390/ijms19051422

64. Sirichoat, A., Flórez, A.B., Vázquez, L., Buppasiri, P., Panya, M., Lulitanond, V., et al. (2020). Antibiotic Susceptibility Profiles of Lactic Acid Bacteria from the Human Vagina and Genetic Basis of Acquired Resistances. Int. J. Mol. Sci. 2020, 21, 2594.

65. Fouhy, F., O'Connell Motherway, M., Fitzgerald, G.F., Ross, R.P., Stanton, C., van Sinderen, D., et al. (2013). In silico assigned resistance genes confer Bifidobacterium with partial resistance to aminoglycosides but not to β-lactams. PLoS One. 8(12):e82653. doi: 10.1371/journal.pone.0082653.

66. European Committee on Antimicrobial Susceptibility Testing, EUCAST 2019 http://www.eucast.org). The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0, 2019. http://www.eucast.org. Last accession MIC distributions for Bifidobacterium spp, 2021-06-02].

67. Gueimonde, M., Flórez, A. B., van Hoek, A. H., Stuer-Lauridsen, B., Strøman, P., de los Reyes-Gavilán, C. G., & Margolles, A. (2010). Genetic basis of tetracycline resistance in Bifidobacterium animalis subsp. lactis. Applied and environmental microbiology, 76(10), 3364–3369. https://doi.org/10.1128/AEM.03096-09

68. Fatahi-Bafghi M. (2019). Antibiotic resistance genes in the Actinobacteria phylum. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology, 38(9), 1599–1624. https://doi.org/10.1007/s10096-019-03580-5

69. Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C. J., Lu, S., Chitsaz, F., Derbyshire, M. K., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., Lu, F., Marchler, G. H., Song, J. S., Thanki, N., Wang, Z., Yamashita, R. A., Zhang, D., Zheng, C., … Bryant, S. H. (2017). CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic acids research, 45(D1), D200–D203. https://doi.org/10.1093/nar/gkw1129

70. Dixon, A., Robertson, K., Yung, A., Que, M., Randall, H., Wellalagodage, D., et al. (2020). Efficacy of Probiotics in Patients of Cardiovascular Disease Risk: A Systematic Review and Meta-analysis. Curr. Hypertens. Rep. 2020, 22, 1–27.

71. Collado, M.C., Meriluoto, J., Salminen, S. (2008). Adhesion and Aggregation Properties of Probiotic and Pathogen Strains. Eur. Food Res. Technol. 226:1065–1073.

72. Begum, S.B., Roobia, R.R., Karthikeyan, M., Murugappan, R. (2015). Validation of nutraceutical properties of honey and probiotic potential of its innate microflora. LWT. 60:743–750.

73. Rahman, M.M., Kim, WS., Kumura, H. Shimazaki, K. (2008). Autoaggregation and surface hydrophobicity of bifidobacteria. World J Microbiol Biotechnol 24, 1593–1598.

74. Kesen, M.A., Aiyegoro, O.A. (2018) Beneficial Characteristics and Evaluation Criteria of Probiotics. Int J Food Biosci Vol: 1, Issu: 1 (19-26).

75. Collado, M.C., Meriluoto, J., Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Lett Appl Microbiol. 45(4):454-60.

76. Monteagudo-Mera, A., Rastall, R. A., Gibson, G. R., Charalampopoulos, D., & Chatzifragkou, A. (2019). Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. Applied microbiology and biotechnology, 103(16), 6463–6472. https://doi.org/10.1007/s00253-019-09978-7

77. De Vuyst, L., Avonts, L., Makras, L., (2004). Probiotics, prebiotics and gut health. In: Remacle, C., Reusens, B. (Eds.), Functional Foods, Ageing and Degenerative Disease. Woodhead Publishing, Cambridge, pp. 416–482.

78. Cheikhyoussef, A., Pogori, N., Chen, W., Zhang, H. (2008). Antimicrobial proteinaceous compounds obtained from bifidobacteria: From production to their application. International Journal of Food Microbiology 125, 215–222.

79 . Igbafe, J., Kilonzo-Nthenge, A., Nahashon, S.N., Mafiz, A.I., Nzomo, M. (2020). Probiotics and Antimicrobial effect of Lactiplantibacillus plantarum, Saccharomyces cerevisiae, and Bifidobacterium longum against common foodborne pathogens in poultry. Agriculture. 10, 368.