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CICLO XXXVI

Dissecting the functionality of the human microbiota in different body districts

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Summary

The human microbiota is the collective term for the diverse and ever-changing community of microorganisms in many human body compartments, including the gastrointestinal (GIT), genitourinary, respiratory, and skin surfaces. Mainly, the gut and the vaginal microbiota are among the human-related microbial communities which have been the most investigated because of the high complexity and extreme heterogeneity of the microbial ecosystems that they retain. These ecosystems have co-evolved over decades to form a mutually beneficial relationship with the human host that benefits both parties. Since bacteria and human hosts have a constant molecular conversation that ultimately is responsible for the host's health, huge efforts have been placed into understanding the microbial composition and the functions of the GIT and vaginal microbiomes. Recently, it has been shown that "omics" technologies involving genomics and various functional genomics approaches are crucial when investigating the composition and activities exploited by bacteria, including those colonizing the human gut and the vaginal environments.

This Ph.D. thesis investigates the role of bacterial communities in the GIT and vaginal tract. Specifically, the main interest is in understanding the molecular mechanisms behind the health-promoting effects of *Lactobacillus crispatus* in the vaginal microbiota. Using omics approaches, this thesis aims to unravel the presence of *L. crispatus* in the human vaginal microbiomes and to assess its molecular interaction/s with other members, including various pathogenic microorganisms linked to vaginal infections. Additionally, this Ph.D. thesis explores the impact of amoxicillin-clavulanic acid (AMC), a commonly used antibiotic in pediatric care, on members of the *Bifidobacterium* genus, which represents one of the primary microbial colonizers of the human gut. Specifically, this thesis investigates AMC-resistant bifidobacteria and their role in preserving gut

microbiota diversity during antibiotic treatment. In investigating the molecular basis of this resistance, studies were carried out on extracellular structures, such as exopolysaccharides (EPS), present on the surface of AMC-resistant strains. We have molecularly characterized these EPS structures through omics approaches, providing evidence about their crucial role in shielding the bacterial cell against AMC antibiotic. In the final section of this Ph.D. thesis, we have identified prototype strains for various *Bifidobacterium* species using *in silico* methodology based on an ecological and phylogenomic-driven approach. These potential candidates were tested *in vitro* to evaluate their interactions with the human host and other gut microbiota members, aiming to preserve or restore gut microbiota homeostasis.

Chapter 1

General Introduction

A. The human microbiota

The human body is a complex ecosystem encompassing trillions of microorganisms, collectively known as the human microbiota [1]. This intricate community of bacteria, viruses, fungi, and other microbes resides on and within the human host, playing a pivotal role in the health and well-being of their host [2]. In this context, several bacteria can colonize different human body districts, such as the skin, mouth, respiratory tract, vaginal tract and, most prominently, the gut, where they are assembled [3] (Fig.1).

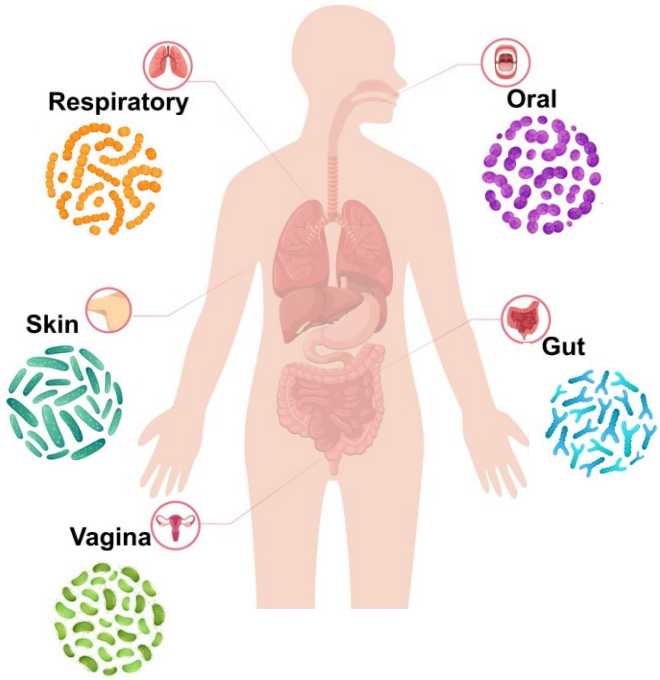

Figure 1. Schematic illustration of different microbiota colonizing the human body [modified by [4]].

Research on the human microbiota has surged in recent years, shedding light on its profound influence on human physiology, immunity, metabolism, and even mental

health [5]. Additionally, an imbalance in the composition of the microbiota, known as dysbiosis, has been associated with various health conditions, including autoimmune diseases, gastrointestinal infections, and metabolic disorders, such as obesity [6]. The microbial composition and diversity in the human body are highly variable between individuals and is influenced by factors like genetics, diet, environment, and lifestyle [7]. Millions of years of co-evolution between the intestinal bacterial communities and the human host have contributed to the establishment of multiple trophic interactions, including mutualistic relationships in which the host ensures nutrients and an ideal environment for the growth of the intestinal microbial population, while, in return, the latter performs multiple physiological and metabolic functions [8,9]. Specifically, the different chemical and physical conditions occurring in each human body district play a selection for the colonization of different bacterial species.

The most studied human microbial communities are those associated with the vaginal and the gut tract, revealing the crucial impact that they exploit on the promotion of health of the host.

B. The vaginal microbiota

The human vaginal microbiota is a complex ecosystem composed of a variety of microorganisms, primarily bacteria, colonizing the vaginal canal [10]. This microbiota plays a crucial role in maintaining the health of the female reproductive system. The composition of the vaginal microbiota can vary among individuals and may change over time due to various factors, such as hormonal fluctuations, sexual activity, and infections [11]. After birth, many bacteria colonize the vaginal tract, which is distinguished by an extremely low abundance of *Lactobacillus* species [12,13]. Following sexual maturation, lactobacilli become the dominant bacteria of the vaginal tract, accounting for up to 90% of the total amount of residing microorganisms in the healthy vaginal microbiota [10,13–18]. They help in maintaining a slightly acidic pH in the vagina through lactic acid production, which inhibits the growth of potential pathogens [19]. In a recent study, performed by Ravel *et al.*, a classification system for vaginal microbiota, based on their composition, was introduced, dividing them into five Community State Types (CST) [10]. Notably, four CSTs, namely CSTs I, II, III, and V, are characterized by the predominance of various *Lactobacillus* species. Specifically, CST I is primarily comprised of *Lactobacillus crispatus*, CST II is dominated by *Lactobacillus gasseri*, CST III features *Lactobacillus iners* as the dominant species, and CST V is composed mainly of *Lactobacillus jensenii* [10]. It's essential to note that CSTs I, II, and III are most associated with the vaginal microbiota of healthy women in their reproductive years [10]. Conversely, CST V is frequently established in menopausal women and individuals with urogenital dysfunction or pathologies [10,20,21]. Additionally, CST IV is distinct from the others, as it lacks a single predominant bacterial species and consists of various bacteria, including *Gardnerella vaginalis*, members of the Lachnospiraceae, Leptotrichiaceae, and Prevotellaceae families [22]. Moreover, CST

IV is characterized by low levels of *Lactobacillus* and a diverse microbial population, and it is associated with bacterial vaginosis, as well as bacterial, fungal, or viral infections [10,23,24] (Fig. 2). Furthermore, there is a correlation between the composition of the vaginal microbiota and the ethnicity of women [10]. Lactobacilli-dominant vaginal bacterial communities are more common in Asian and Caucasian women, while CST IV characterizes Hispanic and African females in their vaginal tract [10]. Moreover, a recent meta-analysis reveals the presence of three new CSTs, i.e., CST-G, CST-Vh, and CST-KK, respectively dominated by *Gardnerella vaginalis*, *Vibrio harveyi* and *Kocuria rosea*/*Klebsiella pneumoniae* [25]. Furthermore, *L. crispatus* has shown a strong correlation with a healthy vaginal environment, offering protection against pathogen attachment and bacterial infections [10,14,23,24]. This species is known to promote vaginal homeostasis and immune barrier functions [26,27]. Due to these intriguing features, *L. crispatus* is emerging as a promising health-promoting bacteria candidate for preventing urogenital tract infections and restoring a healthy vaginal environment [28–30].

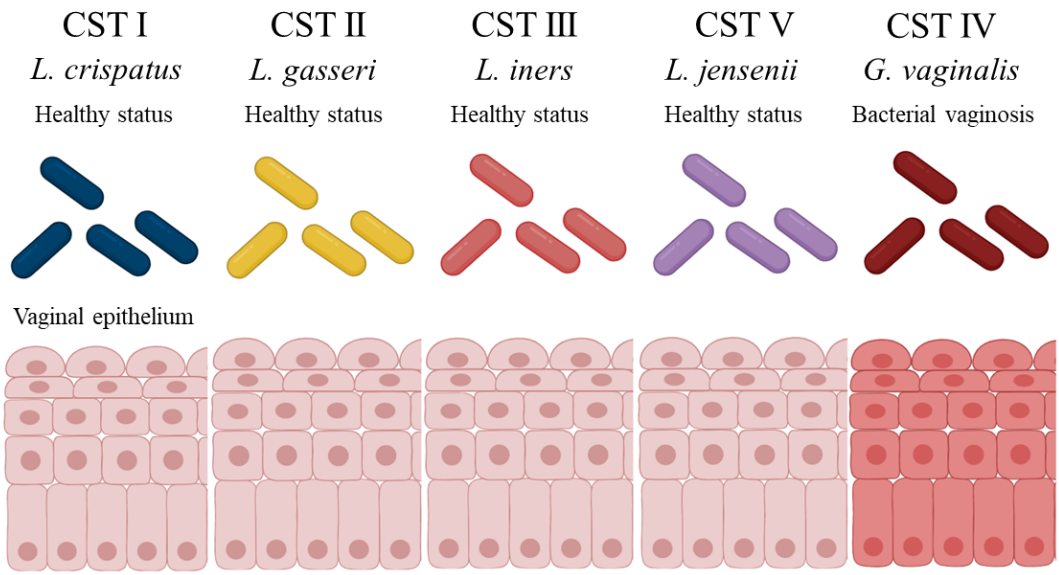

Figure 2. Classification of different vaginal Community State Types (CSTs) [created with Biorender].

C. The gut microbiota

The human gut microbiota is a complex microbial ecosystem residing within the human gastrointestinal tract (GIT). This microbial community plays a fundamental role in digestion, nutrient absorption, vitamin production, and interaction with the host's immune system [31,32]. In particular, the gut microbiota exploits many vital functions, including the digestion and fermentation of complex foods and the breakdown of carbohydrates that the human body alone cannot digest [33]. It is also involved in the protection against pathogens through competition with harmful microorganisms, preventing them from colonizing the gut [34]. Additionally, the gut microbiota plays a crucial role in the development and regulation of the immune system, helping to prevent excessive immune reactions [35]. Furthermore, some gut bacteria can synthesize vitamins such as vitamin K and certain B vitamins [36]. They also produce metabolites that influence overall health. Moreover, the gut microbiota is involved in the regulation of metabolism, including body weight and appetite control [37]. This intricated microbial community is highly complex and varies from individual to individual. Its composition can be influenced by various factors, including diet, lifestyle, age, and genetics. Regarding the composition of the human gut microbiota, it is considered sterile prior to birth and is colonized by several bacteria shortly after birth [38]. The microbiota community changes and becomes more complex after weaning, and with the introduction of solid food into the diet, and at three years of age, the gut microbiota reaches a composition like adults and remains more or less stable over time in adulthood [39]. Everyone has unique microbial profiling of several bacterial species that differs from that of other people [40]. Various studies have shown that four bacterial phyla accounting for approximately 97% of the adult microbial intestinal population, namely Bacteroidetes (20-25%), Firmicutes (60-65%), Proteobacteria (5-10%), and

Actinobacteria (1-3%) [41]. Interestingly, the Actinobacteria phylum, and particularly the *Bifidobacterium* genus, is numerically dominant in the infant gut [42]. Scientific investigations have shown that an imbalance in the gut microbiota, known as dysbiosis, can be associated with a range of health issues, including inflammatory bowel diseases, obesity, diabetes, allergies, and various other medical conditions, including colon cancer [43]. Research on the gut microbiota is continuously evolving, and scientists are gaining a deeper understanding of its importance for human health. Manipulation of the gut microbiota using probiotics or fecal transplants is a growing area of interest in medicine for treating certain medical conditions associated with the gut microbiota. [44].

D. Origin of the human intestinal microbiota

The development of the human intestinal microbiota is a process occurring after human delivery and is influenced by many perinatal factors such as the kind of birth and weaning strategy [9,45]. Furthermore, it is considered that elements not directly involved in the newborn's development during the first months of life, such as food, family genetic components, and environmental influences, may be relevant [46]. It was widely accepted by the scientific community that the uterine cavity is a fully microbially sterile environment during the human gestational period. However, some recent investigations have identified plausible evidence of fetal microbial colonization affected by the maternal microbiome [47]. It has previously been demonstrated that the delivery mode is the factor exerting the most influence on the establishment of the human gut microbiota [48,49]. Infants born naturally have a gut microbiota that is thought to be inherited maternally by vertical transmission through the birth canal [50]. Microorganisms from the maternal urogenital tract influence the gut microbiota of vaginally delivered newborns, with a high predominance of bacteria from the genera *Lactobacillus* and *Prevotella* [51,52]. Babies born via Caesarean section, on the other hand, are primarily colonized by bacteria derived from the external environment, and thus by microorganisms horizontally transmitted through the mother's skin and the hospital environment, with a prevalence of microorganisms belonging to the *Staphylococcus*, *Corynebacteria*, and *Propionibacterium* spp. taxa [53–57].

Diet is another major component influencing the composition of the baby's gut microbiota [58]. Breastfeeding in the first months of life is thought to play a crucial influence in the establishment of the newborn's gut microbiota [58]. Breastfed

newborns display high amounts of bifidobacteria, which are valuable microbial biomarkers indicative of a healthy human host. Additionally, breast milk is a crucial source of nutrition for newborns and contains various health-promoting components [46]. It is a fluid composed of lipids, vitamins, proteins, lactose, and oligosaccharides known as Human Milk Oligosaccharides (HMOs), as well as immune system components such as immunoglobulin A [46]. HMOs are the most prevalent carbohydrate component of the breast milk, along with lactose, and promote the colonization of members of the *Bifidobacterium* genus during the host's early life [59]. Notably, HMOs are not metabolized by mammalian enzymes, whereas few members of the *Bifidobacterium* genus are able to access them [60]. Infants weaned with formula milk, on the other hand, display a larger abundance of bacteria belonging to the genera *Bacteroides*, *Clostridium*, *Staphylococcus*, and members of the Enterobacteriaceae family [61–63]. As a result, the infant microbiota is more complex than that observed in breastfed infants and shares many similarities with that of adults. These changes may have significant ramifications for host physiology and immunology, contributing to disease vulnerability in adulthood, including asthma and atopic illnesses [64,65].

Subsequently, the first colonization of the human gut is subjected to major changes until it reaches maturation after the weaning. Noticeably, only a subset of microbes to which infants are initially exposed is expected to permanently colonize the human gut microbiota. After the third year of life, the human gut microbiota becomes relatively stable, assuming a certain complexity making it like that encountered in the large intestine of the adult [46,66]. It has been established that in the first months of life, the intestinal microbiota is mainly dominated by microorganisms belonging to Actinobacteria and Proteobacteria phyla, then over time it has been found an increase in colonization by bacteria of Bacteroidetes and Firmicutes phyla [67]. As a result, the first colonization of the human gut undergoes

significant changes until it reaches maturity after weaning. Notably, only a subset of the microbes to which infants are initially exposed is expected to colonize the human gut microbiota permanently.

E. The relationship between the composition of the human gut microbiota and health

As previously mentioned, the microbiota consists of microorganisms striving to maintain a harmonious balance with the host, referred to as eubiosis. Nevertheless, various factors, including the host's environment and lifestyle (diet and the use of antibiotics), can lead to alterations in the microbiota's composition, resulting in a condition termed dysbiosis [68]. The consequences of gut dysbiosis extend to the host's well-being, with enduring health implications and associations with various human diseases or disorders [46]. Dysbiosis conditions in the gut microbiota typically involve the depletion of health-associated microorganisms, particularly those producing short-chain fatty acids (SCFAs) [6]. Simultaneously, there is an upsurge in opportunistic pathogens such as mucolytic bacteria, *i.e.*, generators of hydrogen, methane, and hydrogen sulfide, Proteobacteria, and an elevation in LPS endotoxin levels [69]. These alterations can adversely affect the host, compromising intestinal mucosa integrity, inducing acute mucosal inflammation with bacterial translocation, and exploiting toxic effects on colonocytes. Additional consequences include oxidative damage, alterations in cytokine patterns, and other systemic effects [70–72] (Fig. 3).

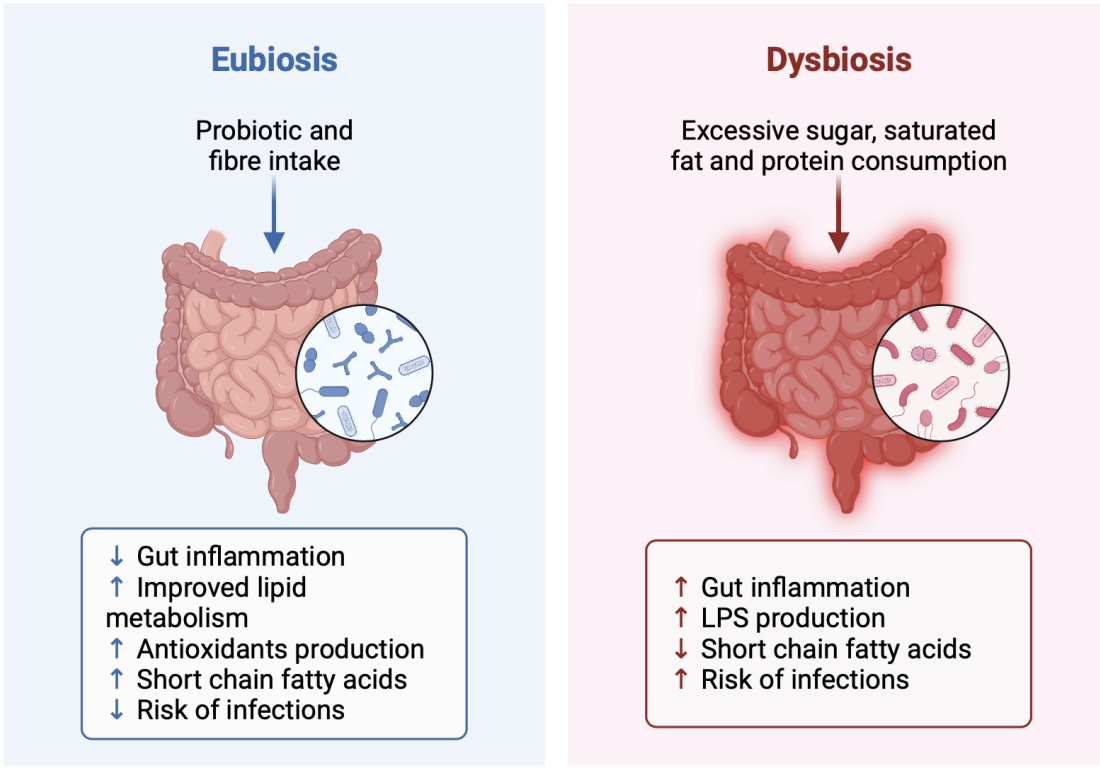

Figure 3. Schematic illustration of the factors causing shifts in human microbiota composition [created with Biorender].

Different studies focusing on the human gut microbiota have provided invaluable insights into the structure and functional potential of the microbial communities residing in the human intestine [73,74]. These investigations underscore substantial interindividual variability and apparent intraindividual stability. Furthermore, they facilitate the differentiation between eubiosis and dysbiosis. Specifically, dysbiotic microbiota profiles have been delineated in the context of gastrointestinal pathologies like Crohn's disease, ulcerative colitis, non-alcohol-related liver dysfunction, as well as disorders such as irritable bowel syndrome (IBS), obesity, and type 1 and 2 diabetes [75–78]. Furthermore, ongoing research is exploring potential connections between altered intestinal microbiota structure and other pathological

conditions, including autism spectrum disorders, multiple sclerosis, neurodegenerative diseases, and oncological disorders [79–81]. Recent meta-analyses, however, propose that intestinal microbial dysbiosis is dynamic over time, emphasizing the importance of understanding its evolution in formulating personalized therapeutic strategies [82,83]. The reduction in microbial diversity, assessed through microbial diversity analysis based on the quantitative evaluation of bacterial taxa in a community, serves as a crucial indicator of potential deviations from a balanced eubiotic state. In dysbiosis, lower microbial diversity is characterized by the decline of the most abundant commensal taxa [84,85]. Notably, a decrease in microbiota population richness is identified as a biomarker for metabolic disorders, although it is alone insufficient to confirm the presence of an ongoing pathological state [46].

F. Health-promoting bacteria and their influence on human health

The utilization of probiotic microorganisms for the modulation of the gut microbiota represents a promising strategy in the prevention of various diseases. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics in 2001, describing them as "live microorganisms that confer a health benefit on the host when administered in adequate amounts" [44,86]. Although the term "probiotics" was officially established in 2001, the concept traces back to 1908 when Metchnikoff observed the positive impact of consuming fermented foods on human health [87,88]. Probiotic bacteria, including those from *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, *Escherichia*, and *Saccharomyces* genera, play a widespread role in preventing various gastrointestinal disorders. They operate on multiple levels to regulate the gastrointestinal barrier, promoting the restoration of eubiosis status [89]. To be utilized in foods and in pharmaceutical supplements, a probiotic microorganism must meet specific criteria outlined by the National Ministry of Health, ensuring safety, functionality, and technological properties [90,91]. Safety criteria involve the absence of virulence factors and antibiotic resistance, while probiotic bacteria must demonstrate survival in diverse gastrointestinal conditions and adhere to the intestinal mucosa [88,92]. Additionally, a probiotic bacterium candidate should be human-derived, belonging to the autochthonous human microbiota. Probiotic bacteria are believed to promote health through various mechanisms, including modulation of the microbiota composition, enhancement of mucin production, reinforcement of the tight junctions of the intestinal mucosa, regulation of immune responses, and synthesis of beneficial compounds [93–95]. Given their role in

promoting health, probiotic products are commonly included in people's daily diets [95,96]. Ongoing research on human body ecosystems and genetic studies of microorganisms has led to the discovery of new probiotic bacteria with distinct characteristics and well-defined mechanisms of action, the so designated next generation probiotic bacteria. Next-generation probiotic bacteria are identified and characterized using advanced sequencing techniques and bioinformatics tools and offer new possibilities for preventing and treating gut microbiota dysbiosis [97,98]. Future Next-generation probiotics may include "classical" probiotic bacteria and other microbial groups from the human core gut microbiota [99,100]. In recent times, the significance of "omics" technologies in scrutinizing the composition and functions of commensal bacteria has been underscored [101]. Notably, the examination of potential probiotic candidates at the genomic level has given rise to a novel field known as Probiogenomics [102]. This discipline, coupled with functional genomic data, enhances the comprehension of the diversity, evolution, and positive effects of commensal bacteria. For this region, Probiogenomics was born with the study and characterization of strains belonging to the *Lactobacillus* and *Bifidobacterium* genus.

The *Bifidobacterium* genus

Bifidobacteria are commensal microorganisms belonging to the genus *Bifidobacterium*, which constitutes a numerous taxonomic genus of the Actinobacteria phylum [103]. Bifidobacteria are Gram-positive, non-motile, non-sporulating, non-gas-producing, anaerobic (except for some facultative aerobic species), catalase-negative, saccharolytic, and with a high Guanine + Cytosine (G+C) content [104]. Furthermore, depending on growth conditions, they can manifest two distinct morphologies, including a Y-shaped and a rod-shaped morphology, thus bifidobacteria have been demonstrated to exhibit pleomorphism on different growth conditions [105]. Bifidobacteria are mesophilic microorganisms with an optimal growth temperature range between 36 °C and 38 °C. Generally, however, it has been observed that bifidobacterial species isolated from animals require slightly higher optimal temperatures ranging between 41 °C and 43 °C c. Furthermore, bifidobacteria are moderately acid-tolerant microorganisms, requiring a pH range between 6.5 and 7.0 for optimal growth [106].

These human gut commensal bacteria were initially isolated in 1899 by Tissier from a fecal sample of a breastfed newborn. Since their discovery, bifidobacteria have been isolated from various ecological niches, including the gut of mammals and insects, oral cavities, wastewater, human blood, and fermented milk. Currently, the genus *Bifidobacterium* comprises 94 different bifidobacterial taxa, including 87 species and 7 subspecies [107]. Despite their wide ecological distribution, the ability of bifidobacteria to adapt to different ecological niches is species dependent. For instance, while *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium pseudolongum*, and *Bifidobacterium bifidum* can be considered cosmopolitan bifidobacterial species, others seem to have adapted to the intestinal ecosystem of specific animals, such as *B. angulatum* in cows, *B. cuniculi* in rabbits,

or *B. gallinarum* in chickens [103,108]. The differential ecological success of bifidobacteria in adapting to different environments is closely coupled to their genetic heritage. In this context, comparative genomic analyses have allowed the reconstruction of the core and pan-genome of bifidobacteria [109]. This has highlighted that most genes shared among different bifidobacterial species (core genome) encode functions involved in carbohydrate metabolism, suggesting that sugar metabolism is a fundamental function for the ecological success of bifidobacteria, which is also reinforced by their saccharolytic behavior. Conversely, pan-genome reconstruction has revealed the presence of unique genes, *i.e.*, genes traceable only in a specific bifidobacterial species. Although most identified unique genes cannot be attributed to a specific function, a fraction of these genes seems to be involved in host-microbe and microbe-microbe interactions, such as sortase dependent pili and exopolysaccharides structures [110]. The ability to degrade complex carbohydrates that cannot be digested by the host is one of the fundamental physiological characteristics of bifidobacterial [111,112]. These microorganisms are indeed capable of degrading complex sugars derived from the diet (*e.g.*, glucans, xylans, pectins, fructans, cellulose, or resistant starch) as well as host-derived glycans, including mucins, which are glycoproteins lining the intestinal epithelium and secreted by goblet cells of the GIT, in the form of O-linked and/or N-linked glycans, and oligosaccharides that may be present in breast milk, such as HMOs in the case of humans [111]. The genes involved in the degradation of these complex carbohydrates are generally organized in gene clusters comprising genes encoding glycoside hydrolases, ATP-dependent sugar-specific transporters, permeases, import pumps, and phosphoenolpyruvate phosphotransferase systems [110,111].

Interactions of bifidobacteria with the human gut

As previously mentioned, the human intestinal microbiota constitutes an intricate ecosystem with different metabolic and physiological activities. Recent research efforts have unveiled multiple mechanisms through which bifidobacteria interact with the host [8]. These mechanisms include the presence of genes that encode extracellular structures such as exopolysaccharides (EPS), serine protease inhibitors, and cell surface-encoding proteins known as pili [113–116] (Fig. 4). Pili or fimbriae, which are hair-like proteinaceous appendages protruding from the extracellular cell surface, are among the many functions that directly contribute to the successful colonization of a particular environment. These functions include adhesion to the intestinal epithelium, biofilm formation, and aggregation with other intestinal microorganisms [114,116]. Two distinct forms of pili, known as sortase-dependent pili and Tad pili, have been identified in bifidobacteria. These types of pili differ in terms of their genetic locus sequences and biogenesis systems [117,118]. In this connection, a case study of *B. bifidum* PRL2010 revealed that sortase-dependent pili play a pioneering role in encouraging bacterial cell aggregation in a heterogeneous population, hence boosting colonization of host intestinal mucosa [119]. Similarly, a related study found that sortase-dependent pili produced by *B. bifidum* PRL2010 can activate various signals in macrophages, inducing local high levels of TNF-cytokines but a reduced expression of other proinflammatory cytokines associated with systemic response, such as IL-12, favoring initial cross-talk between this bifidobacterial strain and host immune cells without causing a detrimental inflammatory cascade response [116]. Additionally, bifidobacterial Tad

pili may aid in the maturation of newborn epithelial cells, encouraging the formation of their thin intestinal mucosa and helping the host mucosal homeostasis [120].

The so-called exopolysaccharides (EPS), glycan layers that create an exterior envelope in a variety of bacteria, are another significant extracellular structure [121]. In addition to encouraging host colonization and persistence, EPS macromolecules provide EPS-producing bifidobacteria with defense against unfavorable circumstances including abrupt pH shifts or the presence of bile acids [122–126]. Furthermore, this extracellular structure is implicated in protecting bacterial cells against various environmental stresses, including the presence of pancreatin, acidity, and antimicrobial compounds such as antibiotics [127].

Furthermore, certain of these microbial biopolymers are gaining attention due to their role in enhancing human health [115,128,129]. In this regard, an *in vitro* experiment, aimed at evaluating the level of pro- and anti-inflammatory cytokine stimulation when EPS purified from 10 bifidobacterial strains were co-cultured with human peripheral blood mononuclear cells (PBMC), revealed that the differentiation of T cells toward T-helper(Th)1 (IL-12/IL-10), Th2 (IL-10/TNF-), and Th17 (IL-1/IL-12) effector cells is highly influenced. This data implies that there is a positive relationship between the content, structure, and size of an EPS polymer and the evoked immunological response [130].

Furthermore, some bifidobacterial species have evolved a defense mechanism against host proteases to survive the harsh and competitive GIT environment. This defense mechanism consists of two components that sense the environment and, when triggered, can cause the production of serine protein inhibitors, or serpins, which function as eukaryotic-type serine protease inhibitors [131,132]. Given that the release of serine proteases may occur because of intestinal inflammation caused by bacterial infection or intestinal tissue damage as seen in inflammatory bowel disease or ulcerative colitis, the production of serpins may elicit anti-inflammatory activity,

thus counteracting the negative effects of (high levels of) human serine proteases. Furthermore, self-produced serpins may help bifidobacteria defend against host-derived proteases and survive in a competitive environment [133,134].

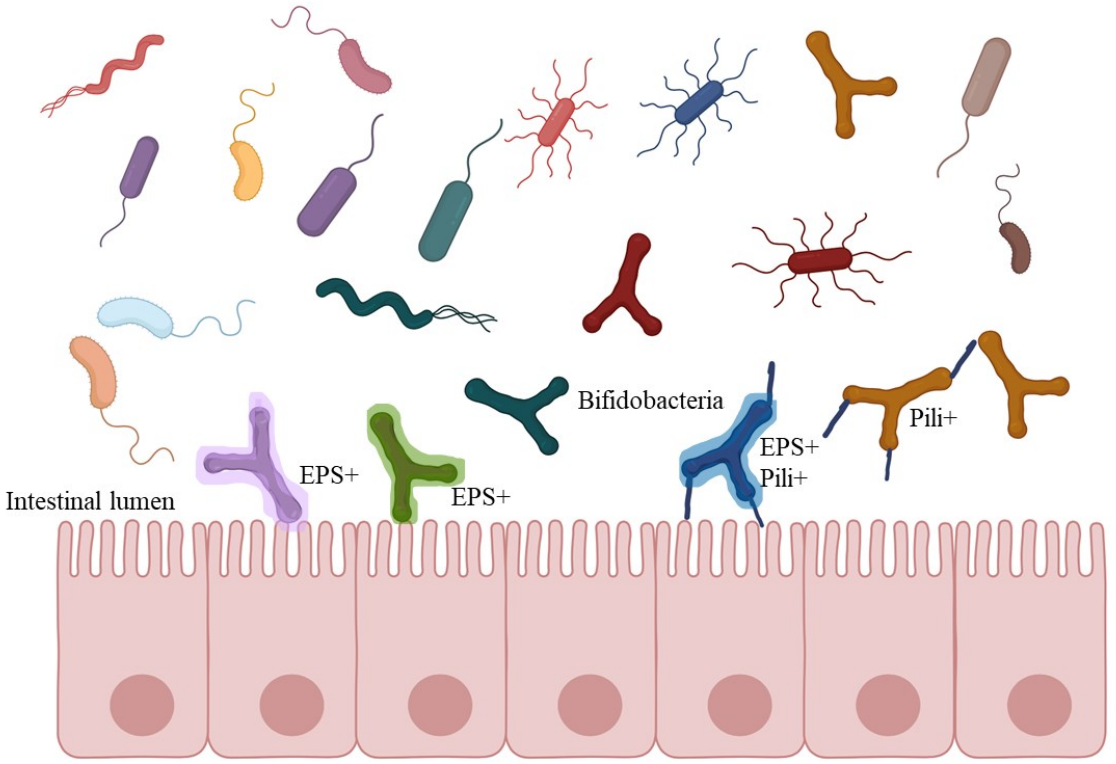

Figure 4. Bifidobacterial extracellular structures. Pili are depicted as blue appendages (Pili+) while exopolysaccharides structures (EPS+) are displayed as layers around the cells [modified by [8] and created with Biorender].

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Chapter 2

Outline of the thesis

This Ph.D. thesis aims to investigate the molecular functions of the human gut and vaginal microbiome, which are crucial for human health and disease prevention. The first section of the thesis focuses on the human vaginal microbiota, specifically examining the occurrence of *Lactobacillus*, particularly *Lactobacillus crispatus*, which is considered a valuable microbial biomarker for vaginal health. In the second section, the thesis explores the early colonization of specific *Bifidobacterium* species in the developing human gut, highlighting their beneficial effects on maintaining gut microbiota equilibrium during antibiotic treatment. The third section of this thesis discusses the importance of "omics" technologies, particularly in the emerging field of "Probiogenomics" [97,101,102]. This approach, integrating genomic and functional genomic data, enhances our understanding of commensal bacteria's diversity, evolution, and molecular interactions with the host. The overall goal of this research is to provide insights into the intricate relationships between microbial communities and human health.

Chapter 3 of this thesis examines the vaginal microbiota intra-species biodiversity of *Lactobacillus crispatus* through *in silico* analyses. Following this, we assessed the modulatory effects of *Lactobacillus* strains toward the vaginal microbiota. Special interest was given to evaluate the antibacterial activities of these members against various pathogenic microorganisms associated with vaginal infections.

Chapter 4 assesses single nucleotide differences in the genome sequences of protein-encoding genes shared among non-identical *L. crispatus* chromosomes. Additionally, *in vitro* experiments aim to explore the potential implications of the genetic differences identified, specifically in terms of intraspecies competition within the vaginal microbiota.

Chapters 5 and 6 present both *in vitro* and *in vivo* experiments utilizing an infant gut model and a rodent model, respectively. These experiments aim to assess the

potential benefits of amoxicillin and clavulanic acid (AMC)-resistant bifidobacterial strains in preserving gut microbiota diversity after exposure to AMC and how bifidobacterial exopolysaccharides (EPS) production could be a key factor in determining the resilience of the baby's gut flora after antibiotic exposure. We employed RNAseq experiments to track the molecular effects of antibiotics on the formation of EPS.

Chapter 7 identifies a prototype of *Bifidobacterium adolescentis*, which is recognized as a dominant bifidobacterial species in the human gut microbiota by using the gut metagenomic data of healthy individuals. Furthermore, the molecular cross talk of the identified *B. adolescentis* prototype strain, PRL2023, with the key members of the human gut microbiota was assessed by using metatranscriptomics analyses.

Chapter 8 illustrates the outcomes of a genome-based screening aimed at identifying an adult gut prototype for *Bifidobacterium breve*. The identified representative strain, designated as PRL2012, was assayed with various omics-based evaluations, aimed to unravel its abilities to interact with the human host as well as with the other members of the gut microbiota.

Chapter 3

Evaluation of modulatory activities of *Lactobacillus crispatus* strains in the context of the vaginal microbiota

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For Supplementary Materials see the article published in Microbiology Spectrum.

Abstract

It has been widely reported that members of the genus *Lactobacillus* dominate the vaginal microbiota, which are represented by the most prevalent species are *Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus gasseri* and *Lactobacillus iners*. *Lb. crispatus* is furthermore considered an important microbial biomarker due to its professed beneficial implications on vaginal health.

In order to identify molecular mechanisms responsible for health-promoting activities that are believed to be elicited by *Lb. crispatus*, we performed *in silico* investigations of the intra-species biodiversity of vaginal microbiomes followed by *in vitro* experiments involving various *Lb. crispatus* strains along with other vaginal *Lactobacillus* species mentioned above. Specifically, we assessed their antibacterial activities against a variety of pathogenic microorganisms which are associated with vaginal infections. Moreover, co-culture experiments of *Lb. crispatus* strains showing the most antibacterial activity against different pathogens, revealed distinct ecological fitness and competitive properties with regards to other microbial colonizers. Interestingly, we observed that even phylogenetically closely related *Lb. crispatus* strains possess unique features in terms of their antimicrobial activities and associated competitive abilities, which suggests that they exert marked competition and evolutionary pressure within their specific environmental niche.

Importance

The human vaginal microbiota includes all microorganisms that colonize the vaginal tract. In this context, a vaginal microbiota dominated by *Lactobacillus* and specifically by *Lactobacillus crispatus* is considered a hallmark of health. The role of *Lb. crispatus* in maintaining host health is linked to its modulatory activity towards other members of the vaginal ecosystem and towards the host. In this study, *in vitro* experiments followed by genetic analyses of the mechanisms used by *Lb.*

crispatus in colonizing the vaginal ecological niche, particularly in the production of different antimicrobial compounds were evaluated, highlighting some intriguing novel aspects concerning the genetic variability of this species. Our results indicate that this species has adapted to its niche and may still undergo adaptation to enhance its competitiveness for niche colonization.

Introduction

The human microbiota is the entire set of microorganisms that colonize the host (1-3). Among the latter, scientific interest in the vaginal microbiota (VM) has increased significantly in recent years due to its impact on female health. Several studies have highlighted the substantial diversity of the VM during the human lifespan, being heavily influenced by hormonal fluctuations (4-7). Immediately following birth, microorganisms originating from the mother and the environment commence colonization of the daughter's vaginal tract and, at the initial phase of development, the VM is characterized by low levels of lactobacilli and a high level of microbial diversity (8, 9). Subsequently, bacteria belonging to the genus *Lactobacillus* tend to dominate the VM, representing up to 95% of the VM community in healthy women (10, 11). However, both the prevalence and abundance of lactobacilli as well as the distribution of *Lactobacillus* species in the vaginal microbiota are subject to a considerable interindividual variability (11-13). Specifically, five distinct groups of vaginal microbial communities named vaginotypes or Vaginal Community State Types (VCSTs) have recently been proposed (11, 14), with vaginotypes I, II, III and V characterized by high abundance of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners* and *Lactobacillus jensenii*, respectively (11). It has been proposed that vaginotypes I, II and III are the most dominant in healthy women (11). Furthermore, VCST IV is characterized by a paucity of lactobacilli, which appear to be replaced by opportunistic bacteria often associated with bacterial vaginosis (15, 16). It seems that not all lactobacilli exert the same health-promoting activities and recent studies have highlighted that vaginotype I is more strongly associated with vaginal health than the other vaginotypes, thereby suggesting that *Lb. crispatus* can be considered a microbial biomarker of a healthy VM (17, 18). *Lb. crispatus* appears to be involved in maintaining the homeostasis of the vaginal environment, where it

supports the vaginal immune barriers without causing inflammation, while at the same time reducing pro-inflammatory cytokines, which typically increase during bacterial vaginosis (19, 20). Moreover, members belonging to this bacterial species produce various antimicrobial compounds (21, 22). The production of bacteriocins by lactic acid bacteria has been reported for several decades (23) and only recently the genetic determinants of bacteriocins produced by *Lb. crispatus* (LCB) have been characterized by *in silico* studies (24, 25). In the context of the latter studies, a total of six putative bacteriocin-encoding genes/loci, named LCB 1-6, were predicted to be harboured by eight genomes of *Lb. crispatus* strains isolated from vaginal swabs. In addition, two bacteriocin-encoding gene clusters, termed LCB 7-8, were found to be located on the genomes of seven *Lb. crispatus* which had been isolated from chicken fecal samples (24).

Another powerful antimicrobial compound produced by certain VM-associated lactobacilli, i.e., *Lb. crispatus* and *Lb. jensenii*, is hydrogen peroxide (26), which is believed to counteract colonization of pathogenic bacteria thereby exerting a protective role against bacterial infections of the human vagina. However, very little is known about the production of hydrogen peroxide in *Lb. crispatus* (27-29) and its potential role as a modulator of the VM. Lactic acid is another metabolic end product produced by *Lb. crispatus* with antimicrobial activity. Noticeably, *Lb. crispatus* represents a so-called homofermentative lactic acid bacterium, meaning that it is able to degrade sugars through the glycolytic pathway, leading to the formation of pyruvate. The latter is subsequently reduced to lactic acid thanks to the presence of lactate dehydrogenase (30). Moreover, previous studies have demonstrated that vaginal *Lb. crispatus* strains encode pullulanase activity, which is responsible for the degradation of glycogen, the main carbon source present in the vaginal lumen (31, 32). This property is believed to positively affect the

abundance of lactobacilli, in particular *Lb. crispatus*, as well as the overall composition of the vaginal microbiota (33)**.**

In the current work we describe the evaluation of the intra-species biodiversity of *Lb. crispatus* by *in silico* investigations of metagenomic datasets followed by assessment of *in vitro* modulatory effects of *Lb. crispatus* strains and other key members of the *Lactobacillus* genus toward the overall vaginal microbiota with a focus on their antibacterial activities against various pathogenic microorganisms associated with vaginal infections (28, 29, 34).

Results and Discussion

In silico investigation of the vaginal microbiota and *Lactobacillus* intra-species biodiversity in metagenomic datasets

The microbial biodiversity of the vaginal microbiota and the intra-species biodiversity of the most prevalent *Lactobacillus* taxa typically found in the vaginal environment, i.e. *Lb. crispatus*, *Lb. jensenii, Lb. iners* and *Lb. gasseri*, was investigated through k-mer profile-based analysis of metagenomic datasets obtained from healthy human vaginal microbiota samples. Specifically, we screened publicly available metagenomic data corresponding to the main recognized VCSTs. For each VCST, which is dominated by one of four above mentioned microbial species (10, 11, 18), twenty datasets (Supporting information Table S1) were processed by reconstruction of species-level taxonomic profiles and analysis of the intra-species biodiversity of dominating lactobacilli by comparison of k-mer profiles with that of a non-redundant database of microbial genomes. The latter was obtained by clustering of all publicly available microbial genomes of *Lb. crispatus*, *Lb. jensenii, Lb. iners* and *Lb. gasseri* followed by exclusion of genomes with a high Jaccard similarity coefficient (Supporting information Fig. S1).

Data retrieved revealed that dominance of *Lb. crispatus* is associated with lower biodiversity expressed as species richness (average of 2 species) when compared to *Lb. jensenii, Lb. iners* and *Lb. gasseri* (average species richness of 6.8, 7.1 and 11, respectively) (Supporting information Fig. S1). Moreover, *Lb. crispatus*, when present, is generally represented by a single dominant strain, with an average of 1.13 strains per biological sample of their corresponding VCSTs, while *Lb. jensenii, Lb. iners* and *Lb. gasseri* showed an average of 1.75, 2.1 and 1.7 strains. These differences in species and strain biodiversity observed for the four investigated *Lactobacillus* taxa suggest that *Lb. crispatus* is characterized by a more pronounced level of inter-species competition for niche colonization when compared to other

vaginal lactobacilli. These results are in accordance with recent scientific literature showing metabolic variability among *Lb. crispatus* strains, including production and/or degradation of biogenic amines, which can rise vaginal pH and favour opportunistic pathogen colonization (35), and phenotypic responses to host molecules such as estrogens (36). Genes commonly involved in the biogenic amine production pathway are the enzyme arginine decarboxylase (EC 4.1.1.19) which convert arginine into putrescine, lysine decarboxylase (EC 4.1.1.18) which decarboxylates lysine into cadaverine and tyrosine decarboxylase (EC 4.1.1.25), that converts tyrosine into tyramine (35). We performed a BLAST homology search employing a custom database encompassing all known arginine, lysine and tyrosine decarboxylase sequences available in public databases, which showed that the genes encoding arginine and lysine decarboxylases are conserved among all *Lb. crispatus* strains, whereas the gene encoding tyrosine decarboxylase appears to be absent (Supporting information Table S8). Moreover, almost all poultry strains and just one human strain, i.e., LB61, possess two lysine decarboxylase-encoding genes, a characteristic therefore that seems to be linked to the ecological niche.

Based on these findings, we decided to gain further insight into strain-level *Lb. crispatus* variability in terms of their interaction with other vaginal commensals and (opportunistic) pathogens.

In vitro* evaluation of antibacterial activities of different strains of *Lb. crispatus

In order to evaluate the antimicrobial activities of various members of the *Lb. crispatus* species against vaginal pathogens, the agar spot-diffusion method was employed. Fifteen *Lb. crispatus* strains isolated from human vaginal swabs or chicken fecal samples (27) were tested against 11 pathogens commonly involved in vaginal infections, such as bacterial vaginosis, vaginal candidiasis and urinary tract infections (Table 1), also including species that may have originated from fecal contamination (28, 29, 34). Furthermore, additional vaginal *Lactobacillus* species,

i.e., *Lb. jensenii, Lb. iners* and *Lb. gasseri*, were included in these analyses (Table 1). We decided to evaluate the antimicrobial activity of *Lb. crispatus* strains isolated from healthy vaginal swabs as well as from chicken fecal samples based on the high prevalence of this species in these environments in order to evaluate any phenotypic differences linked to the ecological origin (28, 37, 38).

Table 1. Bacteria used in this study.

Species	Strain[a]	Origin	Accession number of bacterial strains used in genomic analyses
Lb. crispatus	PRL2021	Vaginal swab	CP058996
Lb. crispatus	LB56	Vaginal swab	JACCPX000000000
Lb. crispatus	LB57	Vaginal swab	JACCPW000000000
Lb. crispatus	LB58	Vaginal swab	JACCPV000000000
Lb. crispatus	LB59	Vaginal swab	JACCPU000000000
Lb. crispatus	LB61	Vaginal swab	JACCPT000000000
Lb. crispatus	LB62	Vaginal swab	JACCPS000000000
Lb. crispatus	LB63	Vaginal swab	JACCPR000000000
Lb. crispatus	LB64	Chicken feces	JACCPQ000000000
Lb. crispatus	LB65	Chicken feces	JACCPP000000000
Lb. crispatus	LB66	Chicken feces	JACCPO000000000
Lb. crispatus	LB67	Chicken feces	JACCPN000000000
Lb. crispatus	LB68	Chicken feces	JACCPM000000000
Lb. crispatus	LB69	Chicken feces	JACCPL000000000
Lb. crispatus	LB70	Chicken feces	JACCPK000000000
Lb. gasseri	V105C	Vaginal swab	-
Lb. gasseri	ATCC9857	ATCC collection	-
Lb. jensenii	V79H	Vaginal swab	-
Lb. jensenii	V94G	Vaginal swab	-
Lb. iners	LMG 14328	LMG collection	-
Enterococcus faecalis	ATCC 19433	ATCC collection	-
Staphylococcus aureus	ATCC 43300	ATCC collection	-
Staphylococcus epidermidis	ATCC 35984	ATCC collection	-
Streptococcus agalactiae	ATCC 13813	ATCC collection	-
Escherichia coli	ATCC 11775	ATCC collection	-
Klebsiella pneumoniae	ATCC 13883	ATCC collection	-
Pseudomonas aeruginosa	ATCC 27853	ATCC collection	-
Gardnerella vaginalis	LMG 07832	LMG collection	-

Mobiluncus curtisii	LMG 07856	LMG collection	-
Prevotella bivia	LMG 06452	LMG collection	-
Candida albicans	ATCC 32032	ATCC collection	-
B. longum subsp. longum	ATCC 15707	ATCC collection	-
B. adolescentis	ATCC 15703	ATCC collection	-
Lb. paracasei ATCC 334	ATCC 334	ATCC collection	-
Bacteroides ovatus	PR2	Infant fecal sample	-
Escherichia coli	Nissle 1917	Probiotic product	-

[a] ATCC, American Type Culture Collection; BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms. V105C, ATCC 9857, V79H, V94G, LMG 14328, ATCC 19433, ATCC 43300, ATCC 35984, ATCC 13813, ATCC 11775, ATCC 13883, ATCC 27853, LMG 07832, LMG 07856, LMG 06452, ATCC 32032, ATCC 15707, ATCC 15703, ATCC 334, PR2, Nissle 1917 were not sequenced, because they were used only in the physiological experiments and not the genetic experiments.

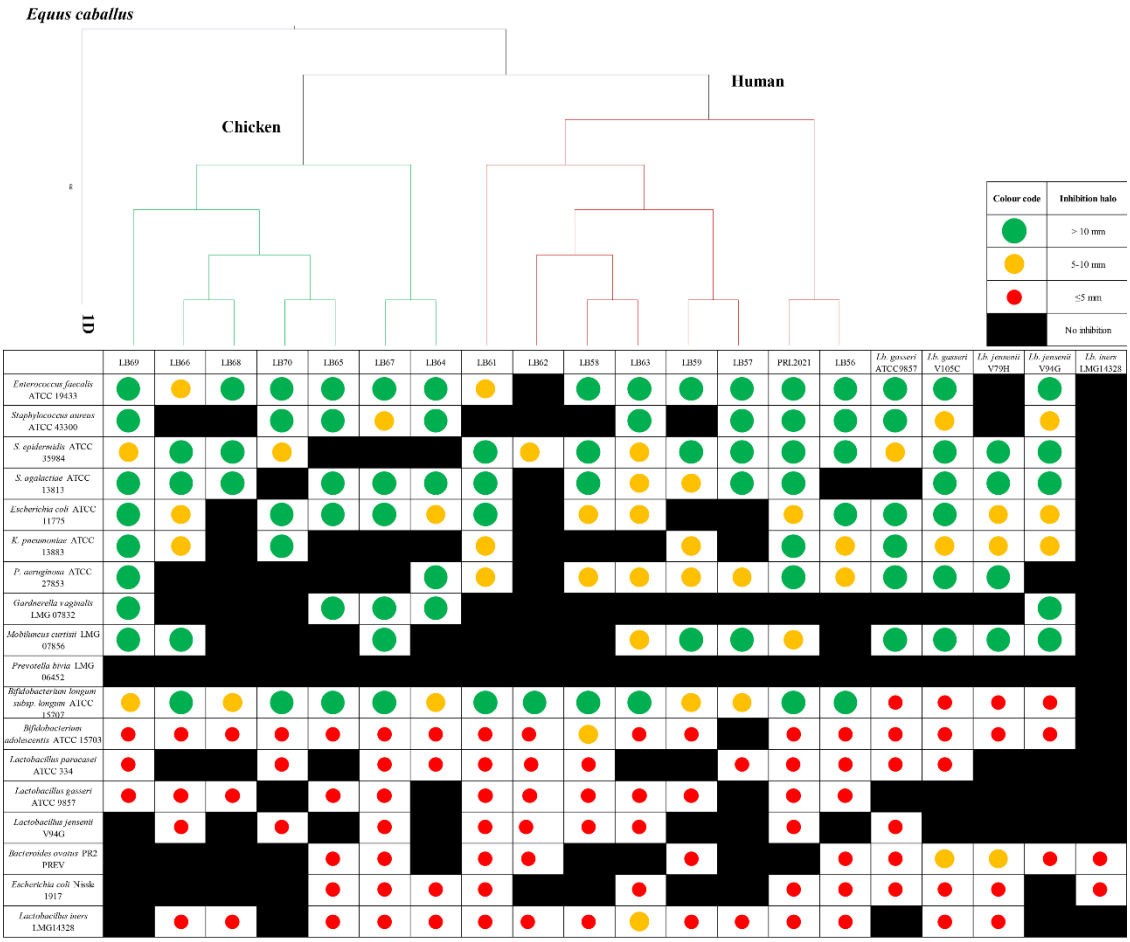
All tested *Lactobacillus* species, with the exception of *Lb. iners*, were shown to elicit antagonistic activity against the majority of pathogenic strains assayed, including opportunistic pathogens of intestinal origin. Notably, while species-specific differences were observed as expected, we also observed marked strain-specific differences among the *Lb. crispatus* strains in terms of inhibitory activity and inhibitory spectrum (Fig. 1; Table 2). Such results do not directly correlate with the phylogenetic relationship of these strains (Fig. 1). For example, *Staphylococcus epidermidis* is inhibited specifically by *Lb. crispatus* isolates of human origin, while *Gardnerella vaginalis* is inhibited exclusively by assessed strains of poultry origin, wherease *Enterococcus faecalis* is inhibited by the majority of tested *Lb. crispatus* strains from either of these ecological niches (Fig. 1) (Table 2).

Table 2. Antibacterial activity of *Lactobacillus* strains against different pathogens. Values are the mean of duplicate measurements of inhibition halo (mm).

	Firmicutes				**Proteobacteria**			**Actinobacteria**		**Bacteroidetes**	**Fungi**
	Enterococcus faecalis ATCC 19433	*Staphylococcus aureus* ATCC 43300	*Staphylococcus epidermidis* ATCC 35984	*Streptococcus agalactiae* ATCC 13813	*Escherichia coli* ATCC 11775	*Klebsiella pneumoniae* ATCC 13883	*Pseudomonas aeruginosa* ATCC 27853	*Gardnerella vaginalis* LMG 07832	*Mobiluncus curtisii* LMG 07856	*Prevotella bivia* LMG 06452	*Candida albicans* ATCC 32032
PRL2021	13	12	12	13	10	19	12	0	10	0	0
LB56	19	12	12	0	12	10	10	0	0	0	0
LB57	12	11	17	11	0	0	10	0	13	0	0
LB58	14	0	23	12	10	0	10	0	0	0	0
LB59	13	0	19	10	0	10	7	0	15	0	0
LB61	10	0	20	11	11	10	10	0	0	0	0
LB62	0	0	10	0	0	0	0	0	0	0	0
LB63	20	19	8	9	9	0	6	0	10	0	0
LB64	15	16	0	11	9	0	13	12	0	0	0
LB65	20	14	0	12	12	0	0	12	0	0	0
LB66	10	0	20	13	9	10	0	0	14	0	0
LB67	11	9	0	19	11	0	0	12	13	0	0
LB68	13	0	13	11	0	0	0	0	0	0	0
LB69	16	13	10	24	12	11	17	12	12	0	0
LB70	18	14	10	0	13	11	0	0	0	0	0
Lb. gasseri ATCC 9857	16	12	10	0	12	11	13	0	16	0	0
Lb. gasseri V105C	16	10	11	12	12	10	12	0	13	0	0

Lb. jenseni i V79H	0	0	11	31	10	10	15	0	12	0	0
Lb. jenseni i V94G	16	10	11	13	10	8	0	12	14	0	0
Lb. iners LMG14328	0	0	0	0	0	0	0	0	0	0	0

a)



b) ***Equus caballus***

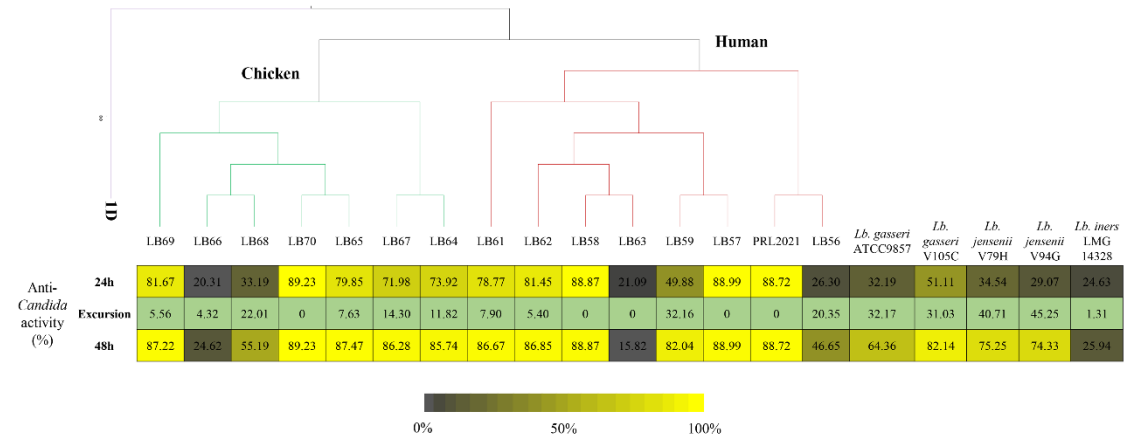


Figure 1. Antibacterial activity of *Lactobacillus* strains against different pathogens. Panel a) A phylogenetic tree of 17 *Lactobacillus crispatus* strains including eight isolated from humans, eight from chicken and one from *Equus caballus*, and its association with the diameter of the inhibition halos obtained for each *Lactobacillus* species grown with various (opportunistic) pathogens. Panel b) A phylogenetic tree of 17 *Lactobacillus crispatus* strains including eight isolated from humans, eight from chicken and one from *Equus caballus*, and its association with antimicrobial activity of *Lactobacillus* CFS toward *Candida albicans* ATCC 32032, tested following EUCAST guidelines. OD 530 nm values at 24h and 48h were normalized for positive controls and results were expressed as inhibition (%) of *Candida* growth. Excursion represents the difference between the inhibition (%) between 48h and 24h.

Noticeably, among the eight *Lactobacillus* strains isolated from vaginal samples, the *Lb. crispatus* PRL2021 strain was shown to be the most effective in inhibiting the growth of tested pathogens, being capable of antagonizing eight out of 11 tested pathogens (Fig. 1; Table 2), together with the poultry isolate *Lb. crispatus* LB69 that was shown to inhibit the growth of nine out of 11 tested pathogens. With the aim to evaluate anti-*Candida* activity, we performed the microdilution method, following the EUCAST guidelines (see Materials and Methods). Overall, CFS from most *Lb. crispatus* strains exhibit high levels of *Candida* growth inhibition (over 80%) (Fig. 1), except for five strains, i.e., LB56, LB59, LB63, LB66 and LB68, which showed a distinctly lower level of antifungal activity (under 50%) (Fig 1). These findings clearly confirm strain-specific differences of the *Lb. crispatus* species. In contrast, *Lb. gasseri*, *Lb. jensenii* and *Lb. iners* strains showed a comparatively low level of anti-*Candida* activity, which ranged from 20% to 50% (Fig. 1). These results suggest that lactobacilli display antimicrobial activities which are strain- and species-specific, though not correlated with the phylogenetic tree. This may be the result of evolutionary pressure in terms of competition for niche colonization.

In contrast, the inhibitory activities exerted by *Lb. crispatus* strains against other commensal *Lactobacillus* species was limited to a small number of strains and, when such activity was noted, it was presented as a small inhibition halo (Fig. 1; Table 3). In this regard, *Lb. crispatus* LB63 showed stronger antibacterial activity against *Lb. iners* LMG 14328, displaying a halo with a diameter of 7.5 mm (Fig. 1; Table 3). This might be explained by the fact that *Lb. gasseri*, *Lb. jensenii*, *Lb. paracasei* and *Lb. iners* strains naturally colonize the human vaginal tract alongside *Lb. crispatus* and thus may have adopted a co-habitation strategy rather than promoting competitive behaviour (11).

Altogether, the inhibition assays highlight a marked strain-level diversity among the tested lactobacilli in terms of their ability to inhibit growth of other (opportunistic) colonizers of the vaginal environment.

Based on these phenotypic data, we decided to investigate the mechanism/s responsible for the observed antibacterial activities of *Lb. crispatus* strains.

Table 3. Antibacterial activity of *Lactobacillus* strains against different bacterial species. Values are the mean of duplicate measurements of inhibition halo (mm).

	Actinobacteria		**Firmicutes**				**Bacteroidetes**	**Proteobacteria**
	Bifidobacterium longum subsp. longum ATCC 15707	*Bifidobacterium adolescentis* ATCC 15703	*Lactobacillus paracasei* ATCC 334	*Lactobacillus gasseri* ATCC 9857	*Lactobacillus jensenii* V94G	*Lactobacillus iners* LMG 14328	*Bacteroides ovatus* PR2	*Escherichia coli* Nissle 1917
PRL2021	14	4	2.5	3.5	1	1	0	1.5
LB56	12.5	4.5	3	3	0	1.5	1	1
LB57	5.5	0	2	-	0	2.5	0	0
LB58	15	7	2.5	3.5	1	3.5	0	0
LB59	6	3	0	1	0	4	1	0
LB61	14.5	3.5	2.5	3.5	1.5	7	1	1
LB62	14.5	4.5	1.5	2.5	2	3	1	0
LB63	10.5	3.5	0	2	1.5	7.5	0	1
LB64	8	3.5	1	-	0	3	0	1.5
LB65	11	5	0	2.5	0	4.5	2	2.5
LB66	10.5	4	0	2.5	1	4	0	0
LB67	16	4	1	5.5	2	4	1	3.5
LB68	7.5	3	0	5	0	0	0	0
LB69	6	4.5	1	2	0	0	0	0
LB70	17	4.5	1.5	4	1.5	0	0	0
Lb. gasseri ATCC9857	1	1	1	-	1	3	5	1
Lb. gasseri V105C	1.5	3.5	2	-	0	1	7.5	1
Lb. jensenii V79H	2	3	0	0	-	0	6.5	2
Lb. jensenii V94G	1	3	0	0	-	3	3.5	0
Lb. iners LMG14328	0	0	0	0	0	-	1	1

Bacteriocin prediction and pathogen inhibition

Bacteriocins are antimicrobial peptides produced by bacteria that are released into the surrounding environment in order to enhance competitiveness towards other microorganisms for niche colonization (39). Screening for the presence of genes associated with bacteriocin production in the genomes of *Lb. crispatus* species allowed the identification of eight putative bacteriocin genes/loci (LCBs) distributed across *Lb. crispatus* strains of both human and poultry origin, with the only exception of LCB 4 and 5, which were exclusively observed in isolates from human hosts, and LCBs 7 and 8 which were only identified in a strain of poultry origin, as previously reported (24, 40) (Fig. 2; Supporting information Table S2).

The presence of genes associated with bacteriocin production was statistically correlated with halo sizes obtained from *in vitro* experiments of antimicrobial activity (Fig. 1; Supporting information Table S2). However, due to a heterogeneous distribution of LCBs among analyzed strains, only LCBs 3, 4 and 5 could be included in the t-test statistical analysis (Supporting information Fig. S2; Table S2). In fact, LCBs 1, 2 and 6 are widespread among all analyzed strains, suggesting that these may have an ecological relevance for all members of the *Lb. crispatus* species. Furthermore, LCBs 7 and 8 were excluded since they were present in the chromosome of just a single poultry strain, therefore not allowing statistical investigations.

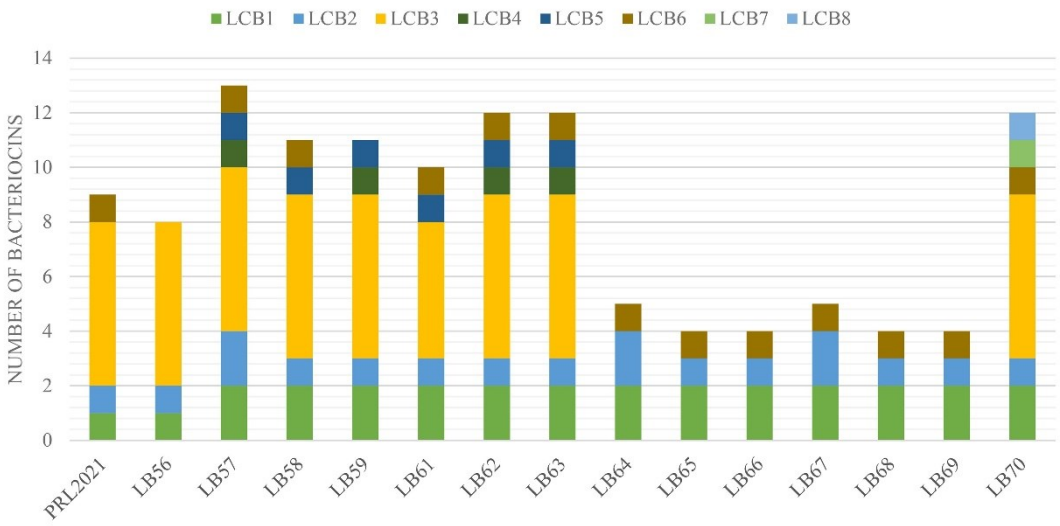
The antimicrobial data revealed that the presence of LCBs 3 and 5 is significantly correlated with a higher average diameter of the inhibition halo for *S. epidermidis* (Supporting information Fig. S2; Table S2), thus suggesting a possible target for the corresponding bacteriocins. Intriguingly, we also observed negative correlations, such as for LCBs 3 and 5, both associated with a reduced inhibition halo of *G. vaginalis*. Furthermore, LCB 3 was negatively correlated with *S. agalactiae* inhibition, while LCB 4 was negatively correlated with *Escherichia coli*.

Interestingly, LCB 3 is comprised of six genes which are predicted to encode a small peptide pheromone along with a baceteriocin immunity protein, normally transcribed together with the bacteriocin in order to protect the producer bacteria (41), a putative ABC transporter and a two-component regulatory system (24). Bioinformatic analysis suggests that this locus corresponds to the inhibition activity against *Streptococcus* species (41). Moreover, this locus is absent in *Lb. crispatus* species isolated from poultry, while it is found in all isolates from vaginal samples. In contrast, LCB 5 is widespread among various lactic acid bacteria, i.e., *Lactobacillus* spp., *Enteroccocus* spp., *Pediococccus* spp., *Leuconostoc* spp. and *Carnobacterium* spp. (24, 42-44).

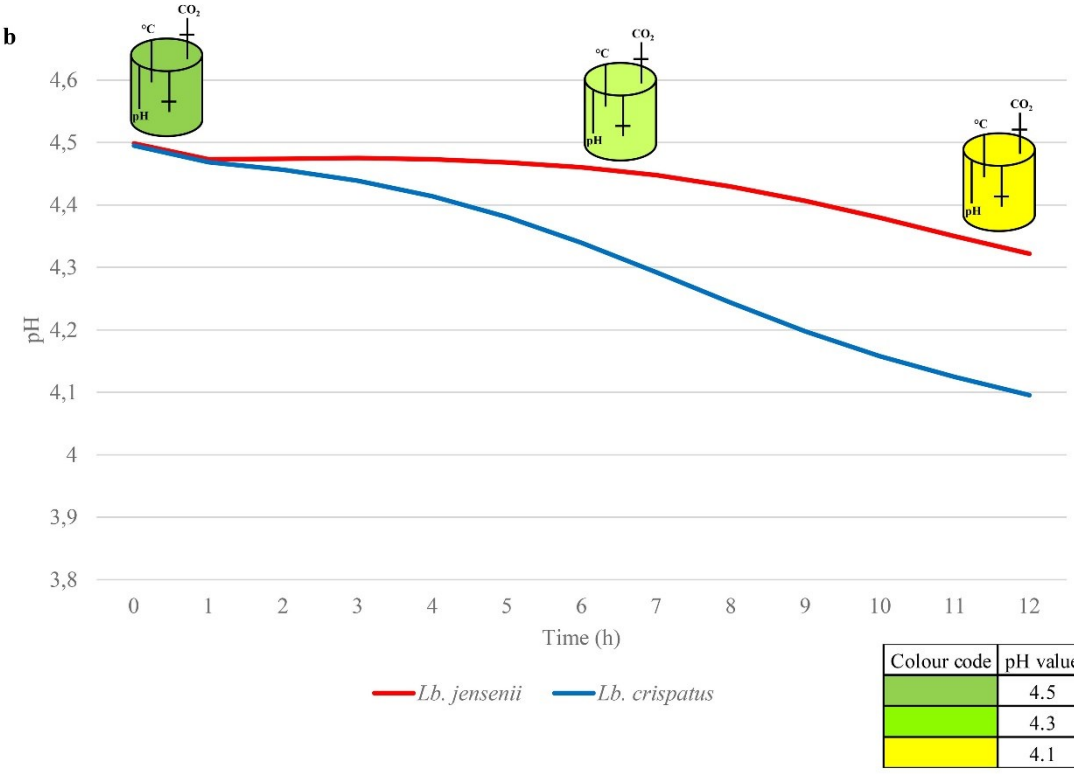
While these results do not provide definite indications regarding the microbial targets of such bacteriocins, probably also due to the counfonding effect of media acidification, these data do suggest a link between the phylogenetic relationship between strains and their ability to inhibit other bacterial taxa. Thus, we tested this notion by linking halo sizes and the presence/absence of bacteriocin families with a core gene-based phylogenetic positioning of the investigated strains (Fig. 4; Supporting information Fig. S2). A comparative genomic analysis was performed using 1034 identified *Lb. crispatus* core genes based on the strains assessed in this study to investigate the phylogenetic relationship. Notably, this approach for phylogenetic reconstruction, based on alignment of all the genes shared by a set of genomes, has been proven to provide high resolution, as described previously (45, 46). Intriguingly, while the presence of bacteriocin families correlates with the phylogenetic clusters observed in the core-gene supertree, the latter does not correlate with the inhibition halo data. Remarkably, these observations support the notion that vaginal lactobacilli still undergo significant evolutionary development to improve their competitiveness with co-colonizer species, which cannot be directly correlated with acquisition or loss of genes, thus suggesting a role of

divergent evolution of specific genes in terms of sequence and expression. No significant correlation was observed between the quantity of lactic acid and halo sizes obtained from *in vitro* experiments of each antimicrobial activity test (Fig. 1).

a



b

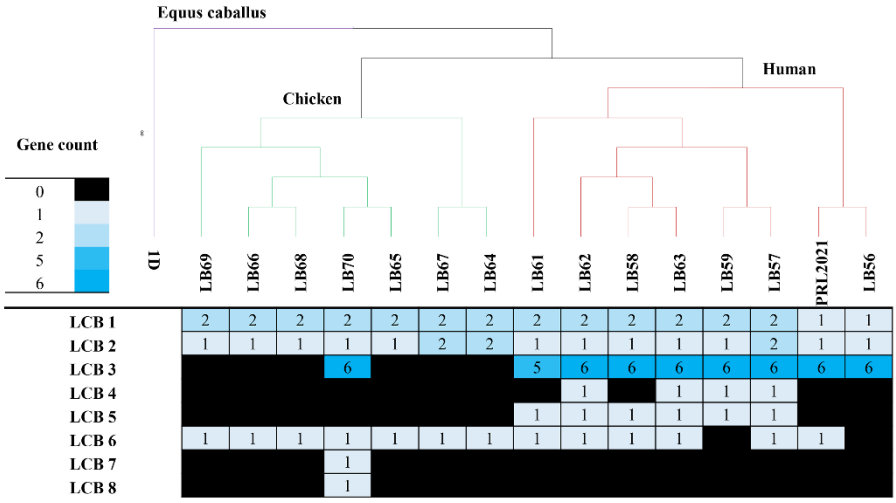


Colour code	pH value
	4.5
	4.3
	4.1

Figure 2. Evaluation of antimicrobial compounds in *Lb. crispatus* strains.

Panel a) depicts a graphical count of bacteriocin-associated loci identified in Lb. crispatus and represented as a bar plot. Panel b) shows the pH decrease (due to lactic acid production) during fermentation in Simulated Vaginal Fluid (SVF). The variation of pH is reported as a function of time.

a)



b)

Strain Tested	Sig. (2-tailed) LCB 3	LCB 4	LCB 5
Enterococcus faecalis ATCC19433	0.716	0.291	0.192
Escherichia coli ATCC 11775	0.616	0.003	0.102
K. pneumoniae ATCC 13883	0.293	0.252	0.448
P. aeruginosa ATCC 27853	0.391	0.725	0.501
S. agalactiae ATCC 13813	0.022	0.249	0.520
S. aureus ATCC43300	0.860	0.813	0.267
S. epidermidis ATCC 35984	0.037	0.671	0.027
Gardnerella vaginalis LMG07832	0.001	0.207	0.044
Mobiluncus curtisii LMG07856	0.628	0.154	0.996

c)

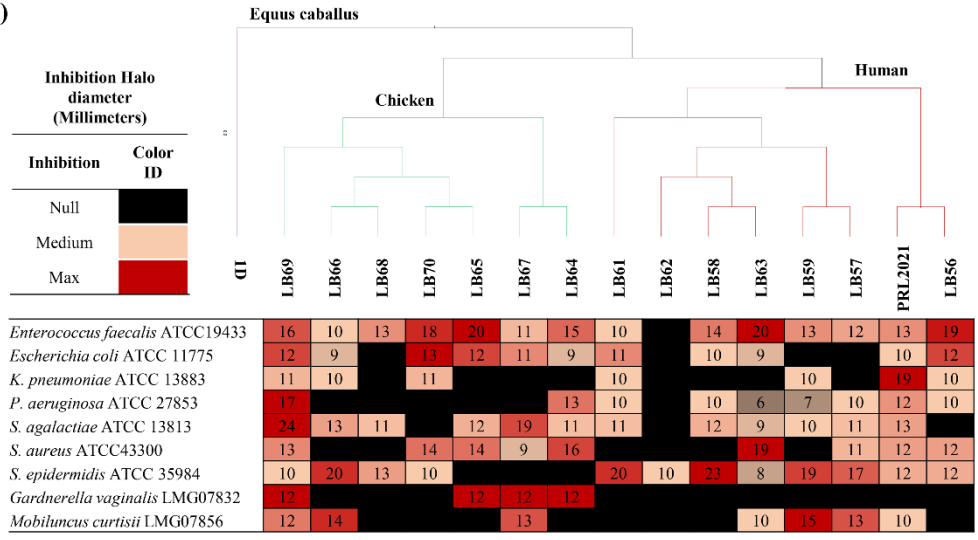


Figure 4. Phylogenetic tree of *Lb. crispatus* and related inhibition data.

A phylogenetic tree of 17 *Lactobacillus crispatus* strains, eight isolated from human, eight from chicken and one from *Equus caballus*, is related in panel a) to bacteriocin distribution with relative gene count and in panel c) to inhibition halo, expressed in millimeters, of nine different species used as control test. Panel b) reports the statistical analysis relative to impact of bacteriocin LCB 3, 4 and 5 versus the nine species tested.

Evaluation of lactic acid production by vaginal lactobacilli

It is widely accepted that the production of organic acids, especially lactic acid, contributes to the lowering of the vaginal pH and therefore making this environment unfavorable for growth of pathogenic microorganisms (47). In order to evaluate the metabolic performances of the lactobacilli isolated from the vaginal tract in terms of lactic acid production, we applied two distinct experimental sets, both involving a bioreactor model inoculated by i) a synthetic growth medium, i.e., De Man Rogosa and Sharpe (MRS) medium, or ii) a medium simulating the vaginal fluid (Simulated Vaginal Fluid, SVF) (48).

During the first three hours, the pH remained constant for all *Lactobacillus* species tested (Fig. 2; Supporting information Fig. S3; Supporting information Table S3), after which it rapidly decreased to 4.0. In this context, *Lb. iners*, lowered the pH to 4.0 in about 20 hours (Fig. 2; Supporting information Fig. S3; Supporting information Table S3), thus representing the species with the slowest acidification times, while the two *Lb. jensenii* strains caused acidification of the medium to pH 4.0 in just four hours, thus being the fastest acidifiers. Notably *Lb. jensenii*, unlike the other *Lactobacillus* species tested, is a facultative heterofermentative, therefore its fast acidification may be due to the formation of other types of acids besides lactic acid (49). Remarkably, the *Lb. crispatus* strains tested showed variable acidification times that ranged from 5 hours to 9 hours (Fig. 2; Supporting information Fig. S3; Supporting information Table S3). Moreover, for all tested *Lactobacillus* strains the steady-state condition was reached after around 13 h of incubation with a pH value of 3.8, at which point cells are no longer able to grow probably due to the high toxicity of lactic acid (plateau phase, OD_{600nm} ~ 3.0) (50) (Supporting information Fig. S3; Supporting information Table S3). Although lactobacilli are known to survive well at low pH condition, we observed that each tested strain showed a specific performance with regards to its acidification rate,

perceptible also among *Lb. crispatus* species, a feature that has been previously correlated to increased ecological fitness and colonization performance (Fig. 2; Supporting information Fig. S3; Supporting information Table S3) (51-53). All tested strains produced lactic acid in the range 5 mM to 8.82 mM (Table 4 and Table S4).

The more powerful pH-lowering capabilities, lactic acid production, higher number of bacteriocin clusters and larger halos of *Lb. crispatus* and *Lb. jensenii* when compared to *Lb. gasseri* and *Lb. iners* as observed in MRS (Table 4 and Supporting information Table S4) were also validated through fermentation of one strain per species in SVF. Interestingly, the tested lactobacilli were shown to cause a pH decrease from 4.5 to 4.0 in 12 hours, except for *Lb. jensenii* V94G, which reduced the pH to 4.0 in 24 hours (Supporting information Fig. S3; Supporting information Table S3). Interestingly, fermentation on SVF resulted in an even stronger pH-lowering performance of *Lb. crispatus* when compared to fermentation on MRS, highlighting their superior pH-lowering ability when compared to *Lb. jensenii* (Fig. 2). These findings suggest that *Lb. crispatus* has adapted specifically to the vaginal environment, which could give this species greater ecological fitness in this specific ecological niche.

Table 4. Production of lactic acid in cultures of lactobacilli.

Species	No. of strains	Conc lactic acid (mM)/10^8 cells	
		Mean	SD
Lb. crispatus	8	8.30	0.52
Lb. gasseri	2	7.90	0.03
Lb. jensenii	2	8.51	0.08
Lb. iners	1	5.06	3.58

Genomic investigation of lactate-producing genes

Production of lactic acid, present as L- and D-isomers, is considered the main reason for pH reduction by homofermentative *Lactobacillus* species (54). Thus, in order to investigate the genetic basis for the varying acidification performances observed for *Lb. crispatus* strains included in this study, we performed a BLAST homology search employing a custom database encompassing all known lactate dehydrogenase sequences available in public databases. Results showed that all *Lb. crispatus* strains isolated from the human vagina possess two L-lactate dehydrogenase-encoding genes (DH_1 and DH_2) and a single D-lactate dehydrogenase-encoding gene (DH_3), showing therefore the same genetic repertoire for the production of D/L-lactic acid.

Among the eight analyzed strains of human-derived *Lb*. *crispatus*, we found that the DNA sequences of the three lactate dehydrogenase-encoding genes are highly conserved, with the presence of only five Single-Nucleotide Polymorphism (SNPs) in DH_1 and only one SNP in DH_3 (Supporting information Fig. S4). Besides, only one SNP present in DH_1 was a non-synonymous SNP affecting the protein sequence. However, no correlation was found between SNP and pH data, indicating that this mutation is not relevant to the functionality of the encoded protein (Supporting information Fig. S4). This may therefore be an indication of the key biological relevance of these three genes for *Lb. crispatus* strains. Nevertheless, future investigation of their transcriptional profiles may provide additional clues regarding the different performances of acidification rates observed for the investigated *Lb. crispatus* strains (Supporting information Table S4).

Co-culture experiments to evaluate competitive behaviour on glycogen

In order to define the cut-off value of glycogen as a growth-limiting factor, we evaluated growth performances of the different *Lactobacillus* strains, used in co-

culture experiments, on different concentrations of glycogen. We observed that each strain has different needs, ranging from 0.25% to 2% (OD>0.5), therefore we decide to use a moderate level of 0.5% glycogen in the co-culture experiments (Supporting information Table S5). In this context, results confirmed that *Lb. crispatus* is a species that is well adapted to growth on glycogen, since all tested strains are able to exhibit superior growth on this carbon source (OD>0.5), when compared to other vaginal lactobacilli such as *Lb. jensenii* and *Lb. gasseri*, thus confirming previous studies (27, 32) (Supporting information Table S5). These findings may explain the higher colonization and acidification performances of the *Lb. crispatus* species in the vaginal environment since glycogen is a complex carbohydrate commonly present in human vaginal fluid (32). To evaluate glycogen breakdown capabilities of the various *Lactobacillus* species that colonize the human vagina, we decided to perform nine different co-cultivation experiments where two strains of *Lb. crispatus*, i.e., PRL2021 and LB57, were co-inoculated with other representative microorganisms of the VM, i.e., *Lb. jensenii*, *Lb. iners*, *Lb. gasseri* and *G. vaginalis* with glycogen as the sole carbon source. Strains PRL2021 and LB57 were selected as representatives of the *Lb. crispatus* species since they showed antibacterial activity against different pathogens (Table 2), while they also appear to effectively decrease the pH of the simulated vaginal fluid (Fig. S3). A qPCR approach was employed to quantify the bacterial DNA of each species relative to the total DNA extracted from co-culture cultivation experiments.

The qPCR analysis revealed diverging performances of the two *Lb. crispatus* strains. In detail, *Lb. crispatus* PRL2021 appears to grow well in co-culture with *Lb. gasseri* V105C or *Lb. iners* LMG 14328 showing no sign of competitive exclusion or antagonistic behaviour, whereas *Lb. crispatus* LB57 seemed to strongly compete with or antagonize growth of the latter species (Fig. 3). On the other hand, both *Lb. crispatus* strains were shown to grow together with *Lb. jensenii*

V94G without noticeable effect on their growth behaviour (Fig. 3). As mentioned above, these data confirm that even phylogenetically related *Lb. crispatus* strains isolated from the same ecological niche display diverging phenotypes that may be associated with ecological fitness and competition with other microbial colonizers (55). In the case of the co-culture between *G. vaginalis* LMG 7832 and vaginal lactobacilli used in this experiment, we observed that the included *Lactobacillus* species are able to cohabit without apparent competition (Fig. 3). This finding may be explained by the fact that these bacteria share the same ecological niche and thus may have evolved co-habitation strategies, corroborated by the observation that *Lb. gasseri* and *Lb. jensenii* struggle to grow on a medium containing glycogen as the sole carbon source. Instead, in co-cultures with *Lb. crispatus* they exhibited increased growth (Fig. 3), indicative of cross-feeding behavior. This possibility seems to be confirmed by the glycogen consumption pattern, as in co-cultures after 30h of growth, glycogen was completely consumed in contrast to the mono-associations. It could be assumed that *Lb. crispatus* metabolizes glycogen early, providing simpler structures for the growth of other *Lactobacillus* species (Supporting information Table S6). These data corroborate the notion that all *Lactobacillus* species typically found in the vaginal environment are genetically adapted to grow on glycogen as the main shared carbon source. The glycogen breakdown capabilities of *Lb. crispatus* strains used, i.e. LB57 and PRL2021, is further confirmed by the presence of a conserved gene cluster (Supporting information Fig. S5). This conserved set of genes encompasses a gene encoding a predicted amylase (GH13) followed by genes that are predicted to specify a maltose phosphorylase, a beta-phosphoglucomutase and an ABC (ATP-binding cassette) system for carbohydrate uptake (Supporting information Fig. S5), all of which are implicated in glycogen degradation (33). The specific adaptation of these *Lactobacillus* species to the vaginal environment is also confirmed by the

observation that the cell number of *G. vaginalis* appears to decrease over time when various *Lactobacillus* species typical of the VM are co-cultivated together, underscoring the ability of lactobacilli toward collaborative utilization of this carbon source and concomitant inhibition of other microbial taxa. Future studies will therefore involve co-cultivation experiments between the two *Lb crispatus* representatives, i.e. PLR2021 and LB57, in order to perform an in depth evaluation of possible competition mechanisms between them in the complex vaginal environment.

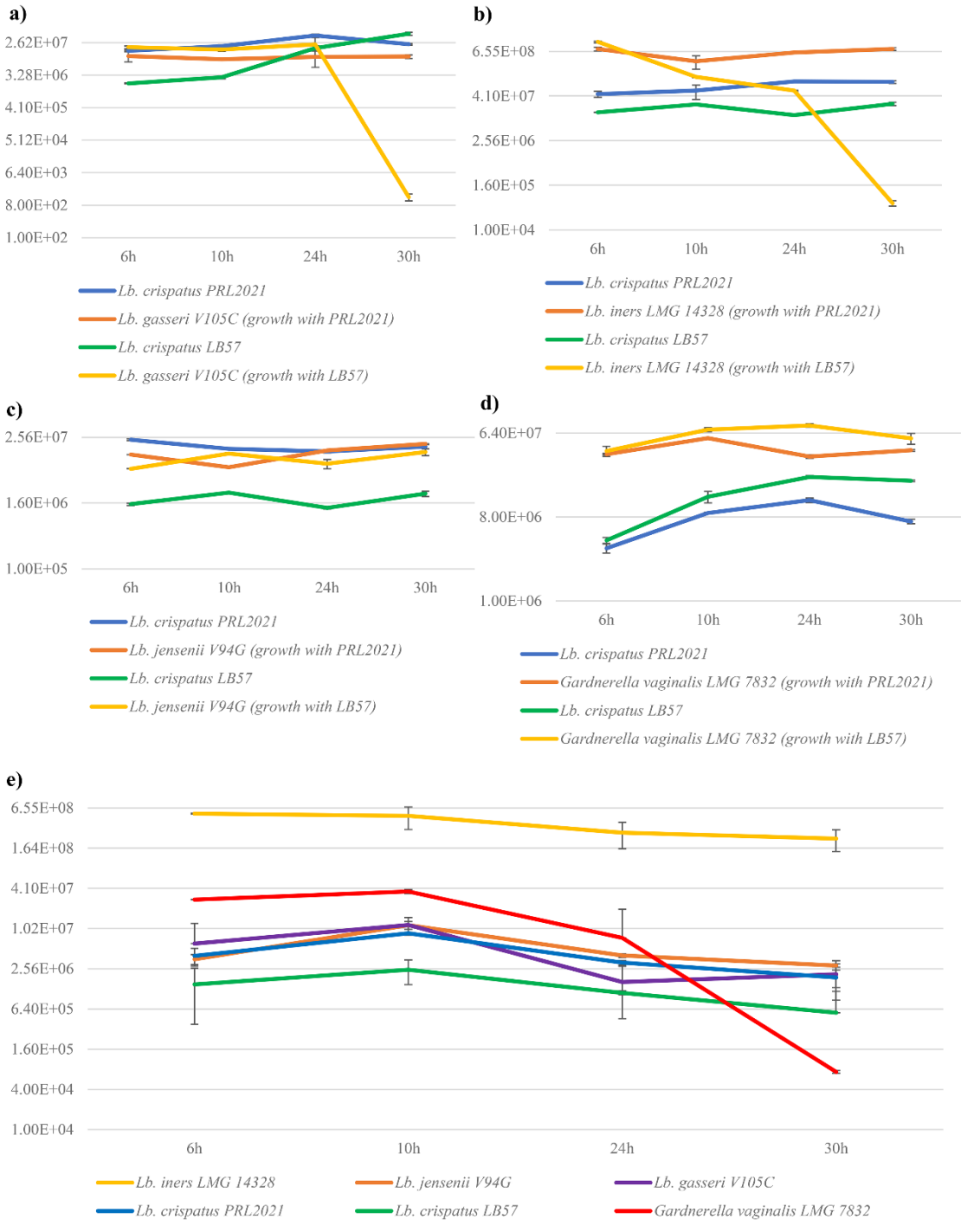

a)
2.62E+07
3.28E+06
4.10E+05
5.12E+04
6.40E+03
8.00E+02
1.00E+02
6h
10h
24h
30h
Lb. crispatus PRL2021
Lb. gasseri V105C (growth with PRL2021)
Lb. crispatus LB57
Lb. gasseri V105C (growth with LB57)
b)
6.55E+08
4.10E+07
2.56E+06
1.60E+05
1.00E+04
6h
10h
24h
30h
Lb. crispatus PRL2021
Lb. iners LMG 14328 (growth with PRL2021)
Lb. crispatus LB57
Lb. iners LMG 14328 (growth with LB57)
c)
2.56E+07
1.60E+06
1.00E+05
6h
10h
24h
30h
Lb. crispatus PRL2021
Lb. jensenii V94G (growth with PRL2021)
Lb. crispatus LB57
Lb. jensenii V94G (growth with LB57)
d)
6.40E+07
8.00E+06
1.00E+06
6h
10h
24h
30h
Lb. crispatus PRL2021
Gardnerella vaginalis LMG 7832 (growth with PRL2021)
Lb. crispatus LB57
Gardnerella vaginalis LMG 7832 (growth with LB57)
e)
6.55E+08
1.64E+08
4.10E+07
1.02E+07
2.56E+06
6.40E+05
1.60E+05
4.00E+04
1.00E+04
6h
10h
24h
30h
Lb. iners LMG 14328
Lb. jensenii V94G
Lb. gasseri V105C
Lb. crispatus PRL2021
Lb. crispatus LB57
Gardnerella vaginalis LMG 7832

Figure 3. Evaluation of *Lactobacillus* load in co-culture experiments.

Quantitative PCR evaluation of the relative number of *Lactobacillus* and *Gardnerella* strains in co-culture experiments. The graph highlights the average abundance observed through qPCR at 6h, 10h, 24h and 30h. Panel a) represents the co-colture results of two different experiments in which PRL2021 or LB57 *Lb. crispatus* strains were grown together with *Lb. gasseri* V105C. Panel b) similarly shows the relative load of *Lb. crispatus* strains (PRL2021 or LB57) with *Lb. iners* LMG 14328. Panel c) displays the relative number of *Lb. crispatus* strains (PRL2021 or LB57) with *Lb. jensenii* V94G. Panel d) represents the qPCR evaluation between *Lb. crispatus* strains (PRL2021 or LB57) with *Gardnerella vaginalis* LMG 7832. Panel e) shows the relative load of all microorganisms used in the co-cultivation experiments when co-cultivated together.

Conclusions

The human VM includes all microorganisms that colonize the vaginal tract. Among these, *Lb. crispatus* is reported to exert a key role in maintaining host health. In the current study, we performed *in vitro* experiments in order to evaluate the possible modulatory effects of *Lb. crispatus* strains by maintaining the host VM homeostasis through the production of different antimicrobial compounds, such as bacteriocins and lactic acid. Furthermore, the ability of this species to interact with other members of this ecological niche was evaluated in carbohydrate competition experiments.

Our findings revealed that lactobacilli typically found in the vaginal environment appeared to have adapted to this niche and perhaps are still undergoing adaptation in terms of improved competition for ecological niche colonization. From a species-level perspective, *Lb. crispatus* showed the most advanced colonization performance, while various strain-specific characteristics were observed even in phylogenetically closely related strains, i.e., differences in gene content and sequence divergence, as well as phenotypic characteristics. Particularly in this work we highlighted some intriguing novel aspects about high genetic variability of *Lb. crispatus* in terms of antibacterial activity against human vaginal pathogens as well as the production of bacteriocins and other antimicrobial compounds such as lactic acid. All together these findings clearly support the notion of *Lb. crispatus* as an important driver of the vaginal microbiota. In this regard, further investigations encompassing a large collection of *Lb. crispatus* strains and additional multiomics approaches will be pivotal to elucidate the evolutionary mechanisms that drive adaptation to the vaginal environment.

Materials and Methods

Taxonomic profiling and strain tracking

We selected 80 different vaginal samples from four different BioProjects: PRJNA48479, PRJNA275349, PRJNA576566 and PRJEB38528 (Supporting information TableS1) aimed at analyzing the taxonomic profiles. METAnnotatorX2 (56) was used with default settings, 50k input reads and human read filtering. We retrieved from the public dataset a representative complete subset of data sequenced on an Illumina MiSeq platform (57). The strain tracking analysis was instead carried out using all publicly available genomes of *Lb. crispatus, Lb. iners, Lb. gasseri* and *Lb. jensenii* through the StrainGE software (https://github.com/broadinstitute/StrainGE), with default parameters, against 80 publicly available vaginal shotgun sequencing samples (selected based on its high depth sequencing) and downloaded from the Sequence Reads Archivie (SRA) of NCBI (Supporting information Table S1).

Strains and culture conditions

Lactobacillus strains used in this study are shown in Table 1 (27). The strains used were isolated as part of previous work, in which written informed consent from each donor was obtained prior to inclusion in the study. Lactobacilli were grown anaerobically in De Man, Rogosa, Sharpe (MRS) medium (Scharlau) supplemented with 0.05 % L-cysteine-HCl and incubated at 37°C for 24 h. Anaerobic conditions were achieved by the use of an anaerobic cabinet (Ruskin), in which the atmosphere consisted of 17 % CO_2, 80 % N_2, and 2.99 % H_2. Pathogenic microorganisms and other microorganisms used in this study are listed in Table 2. Indicator microorganisms for antimicrobial activity were grown aerobically on Brain Heart Infusion (BHI) broth at 37°C, for 24h. The strains *Gardnerella vaginalis* LMG 07832, *Mobiluncus curtisii* LMG 7856 and *Prevotella bivia* LMG 06452, were obtained from Belgian Co-ordinated Collections of Microorganisms. These

microorganisms were cultivated anaerobically on Brain Heart Infusion (BHI) supplemented with 5 % of defibrinated horse blood (Thermo Scientific Oxoid) at 37°C, for 48h. Bifidobacteria were grown anaerobically in De Man, Rogosa, Sharpe (MRS) medium (Scharlau) supplemented with 0.05 % L-cysteine-HCl and incubated at 37°C for 24 h. Anaerobic conditions were achieved by the use of an anaerobic cabinet (Ruskin), in which the atmosphere consisted of 17 % CO_2, 80 % N_2, and 2.99 % H_2. *Bacteroides ovatus* PR 2 was grown anaerobically on Brain Heart Infusion (BHI) broth at 37°C, for 24h. *Escherichia coli* Nissle 1917 was grown aerobically on Luria-Bertani borth (LB) at 37°C, for 24h.

Experimental design of the *in vitro* trial

The antibacterial activity of the selected *Lactobacillus* spp. isolates was tested by agar spot-diffusion test (28, 29, 58, 59) against 13 microorganisms, comprising both pathogens and commensals, i.e., *Enterococcus faecalis* ATCC19433, *Staphylococcus aureus* ATCC43300, *Staphylococcus epidermidis* ATCC 35984, *Streptococcus agalactiae* ATCC 13813, *Escherichia coli* ATCC 11775, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Gardnerella vaginalis* LMG 07832, *Mobiluncus curtisii* LMG07856, *Prevotella bivia* LMG 06452, *Candida albicans* ATCC 3203, *Bacteroides ovatus* PR2 and *Escherichia coli* Nissle 1917. These cultures were spread onto BHI agar medium plates. Furthermore, the antibacterial activity of *Lactobacillus* spp. was tested against *Bifidobacterium longum* subsp. *longum* ATCC 15707, *Bifidobacterium adolescentis* ATCC 15703, *Lactobacillus paracasei* ATCC 334, *Lactobacillus gasseri* ATCC 9857, *Lactobacillus jensenii* V94G and *Lactobacillus iners* LMG 14328. In this case, cultures were spread onto MRS agar medium plates. Specifically, overnight cultures of microorganisms were diluted in order to obtain a final inoculum with an OD_{600nm} of 1.0 (bacterial cell counts standardized to 2.25

$\times 10^8$ CFU/mL). The different *Lactobacillus* strains were spotted (10 µL) on the medium containing the competing microorganism, two spots per plate.

Then, plates were incubated for 48 hours at 37°C in aerobic or anaerobic atmosphere depending on pathogenic and commensal strains used (see above).

The antimicrobial activity was assessed by the diameter size of the inhibition halo, i.e., a inhibition halo with a diameter smaller than 5 mm was considered as low inhibition, a halo with a diameter between 5 and 10 mm was perceived as medium inhibition and a halo with a diameter larger than 10 mm was considered as a high-level of inhibition as previously described (29).

Anti-*Candida* activity of *Lactobacillus* Cell-Free Supernatants

Anti-*Candida* activity of Cell-Free Supernatants (CFS) was tested by microdiluition assay, following EUCAST guidelines (60, 61). For this purpose, lactobacilli were first grown anaerobically in MRS medium supplemented with 0.05 % L-cysteine-HCl and incubated at 37°C for 72 h. Cultures were then harvested by centrifugation (2,750 × g, 10 min) and filtered through a 0.2-µm filtropur to obtain CFS. CFS obtained were stored at −20°C until their use. Subsequently, stock *Candida albicans* ATCC 32032 suspensions prepared in sterile water at 0.5 Macfarland was diluted appropriately in RPMI 1640 medium (Gibco™, ThermoFisher Scientific, USA) buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid buffer) (Merck, USA) and supplemented with 2 % glucose. Final yeast suspensions, corresponding to 10^5 cfu/ml, were inoculated in flat-bottomed 96-well plates (0.1 ml per well) and added with the same volume of each *Lactobacillus* CFS. Positive growth control wells contained 0.1 ml of *Candida* suspension added with the same volume of sterile MRS medium. The plates were incubated at 37°C for 24 h and 48 h, and *Candida* growth was evaluated by reading the absorbance at 530 nm with a VICTOR® Nivo™ Multimode Microplate Reader. *Candida* growth inhibition was

calculated relative to the absorbance of the corresponding positive controls as previously reported (62).

Evaluation of lactic acid production by vaginal lactobacilli

Lactobacilli were grown anaerobically in Simulated Vaginal Fluid (SVF) (48) or in MRS, in order to adapt the microorganisms to the medium at 37°C for 24 h. We decided to use these two media because MRS is a synthetic medium of choice for lactobacilli (63), while SVF is a medium that simulates the vaginal environment. Anaerobic conditions were achieved by the use of an anaerobic cabinet (Ruskin), in which the atmosphere consisted of 17 % CO_2, 80 % N_2, and 2.99 % H_2. Revitalized cells at exponential phase (OD_{600nm} between 0.6 and 0.8) were inoculated under anaerobic conditions in 500 mL of SVF or MRS medium and cultivated in a bioreactor system (Solaris Biotech Solutions, Italy). Strains were cultivated for 24h at 37°C with a mechanical agitation at 180 rpm and pH variation was recorded every 45 seconds through a pH meter. At the end of the homofermentative process, the production of lactic acid was measured enzymatically, using D/L-lactic acid (D/L-lactate) (Rapid) assay kit (Megazyme, Bray, Ireland).

Enzymatic identification of lactic acid

A D/L-lactic acid (D/L-lactate) (Rapid) assay kit (Megazyme, Bray, Ireland) was used to assess the quantity of each optically active forms of lactic acid produced by the fermentation of the microorganism. The assay was performed following the manufacturer's instructions. Briefly, an aliquot of the fermentation of the single strain was taken for the enzymatic assay. Then, 5 mL of this aliquot was centrifugated at 10,000 rpm for five minutes at 4°C and the supernatant was filtered through a 0.2 µm filter. Next, 50 µL of the supernatants filtered were submitted for the analysis. Later, the 750 µL of sterile distilled water was added to each supernatant, as well as the solutions included in the test kit, such as a buffer (250 µL), NAD+ solution (50 µL) and D-GTP (10 µL). After 3 min of incubation, the

optical density was measured (OD) and the D-LDH or L-LDH (10 µL) was added and incubated. The collected OD values were used for the calculation of the total quantity of lactic acid following the manufacturer's instructions.

Genomic analyses

With the aim to build a phylogenetic tree on bacteriocin gene production of the different *Lb. crispatus* strain, a pangenome calculation was performed using the pan-genome analysis pipeline PGAP, including each *Lb. crispatus* genome included in this study (Supporting information Table S7). Each predicted proteome of a given *Lb. crispatus* strain was screened for orthologues against the proteome of every other assessed *Lb. crispatus* strain by means of BLAST analysis (cutoff, E value of $<1 \times 10^{-4}$ and 50 % identity over at least 80 % of both protein sequences). The resulting output was then clustered into protein families by means of MCL (graph theory-based Markov clustering algorithm), using the gene family method. A pangenome profile was built using all possible BLAST combinations for each genome being sequentially added. Using this approach, unique protein families encoded by the analyzed *Lb. crispatus* genomes were also identified, ranging from 29 for *Lb. crispatus* PRL2021 to 164 for *Lb. crispatus* LB58. Protein families shared between analyzed genomes allowed us to identify the core genome, which encompasses 1037 genes, of the *Lb. crispatus* species. Each set of orthologous proteins, belonging to the core genome, was aligned using Mafft software, and phylogenetic trees were constructed using ClustalW. Based on these comparative analyses, a *Lb. crispatus* supertree was constructed and visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). The presence of potential genes for bacteriocins were evaluated using the Bagel4 software for each single genome of *Lb. crispatus*, then removing all those sequences that turned out to be incomplete hits.

Statistical analysis

Statistical analyzes such as the independent t-test were carried out using SPSS software, while covariance and hierarchical clustering (HCL) analyzes were carried out using Origin pro 2021b. Statistical analyses always included the inferential Levene test for the equality of variances.

Glycogen growth assays

Lb. crispatus PRL2021, LB57, *Lb. jensenii* V94G, *Lb. gasseri* V105C and *Lb. iners* LMG 14328 were cultivated on semisynthetic MRS medium without glucose supplemented with different concentrations of glycogen, i.e., 2%, 1.5%, 1%, 0.75%, 0.5%, 0.25%, 0.1% and 0.08%. The optical densities (measured at a wawelenght of 600 nm) were recorded using a plate reader (BioTek, Winooski, VT, USA). OD was read in intermittent mode, with absorbance readings performed at 3-min intervals for three times after 48 h of growth, where each reading was ahead of 30 s of shaking at medium speed. Cultures were grown in biologically independent triplicates, and the resulting growth data were expressed as the means of these replicates. Glycogen was purchased from Fisher Scientific, ACROS Organics™ (US).

Growth on glycogen as a selection factor for *Lb. crispatus*

For the co-culture experiments, MRS medium was used without the presence of glucose, yet supplemented with 0.5% of glycogen from beef liver (Fisher Scientific, ACROS Orgaincs™, US). For growth experiments, overnight *Lactobacillus* cultures were diluted to an OD value of 1.0 and overnight *Gardnerella vaginalis* LMG 7832 culture was diluted to a final concentration that varied between 3 and 4 MacFarland units. Each culture was inoculated at 0.1 % (vol/vol) into medium. We performed eight different experiments in which *Lb. gasseri* V105C, *Lb. jenseniii* V94G, *Lb. iners* LMG 14328 and *Gardnerella vaginalis* LMG 7832 were cultivated together with *Lb. crispatus* PRL2021 and LB57 and one experiment where all microorganisms were co-cultivated altogether. Batch cultures were incubated under

anaerobic conditions for 6h, 10h, 24h and 30h at 37°C. After different time points cultures were centrifuged at 3,000 rpm for 8 min and the bacterial pellets harvested. The pellets were subjected to DNA extraction using the GeneElute bacterial genomic DNA kit (Sigma, Germany) following the manufacturer's instructions. Each sample was subjected to a different cycle of quantitative PCR (qPCR) using species specific primers: Crisp2_Fw (5'-GGTAATGACGTTAGGAAAGCG-3') and Crisp33_Rv (5'-GCTGATCATGCGATCTGC-3') for *Lb. crispatus* PRL2021 and LB57, for *Lb. gasseri* V105C gassI (5'-GAGTGCGAGAGCACTAAAG-3') and gassII (5'-CTATTTCAAGTTGAGTTTCTCT-3'), for *Lb. jensenii* V94G LjensF (5′-AAGTCGAGCGAGCTTGCCTATAGA-3′) and LjensR (5′-CTTCTTTCATGCGAAAGTAGC-3′) (64), for *Lb. iners* LMG 14328 LinersF (5'-CTCTGCCTTGAAGATCGGAGTGC-3') and LinersR (5'-ACAGTTGATAGGCATCATCTG-3') (65) and for *G. vaginalis* Gard_vaginalis_154-454 FW (5'-CTCTTGGAAACGGGTGGTAA-3') and Gard_vaginalis_154-454 RV (5'-TTGCTCCCAATCAAAAGCGGT-3') (66).

qPCR was performed using qPCR Green Master Mix (SensiFAST™ SYBR® No-ROX kit, US) on a CFX96 system (BioRad, CA, USA) following previously described protocols (67) (66). qPCR was performed using qPCR Green Master Mix (SensiFAST™ SYBR® No-ROX kit, US) on a CFX96 system (BioRad, CA, USA) following previously described protocols (67). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: one cycle of 95 °C for 2-3 min, followed by 40 cycles of 95 °C for 15 s and 60-65°C for 30 s. The melting curve was 65 °C to 95 °C with increments of 0.5 °C/s. In each run, negative controls (no DNA) were included. A standard curve was built using the CFX96 software (BioRad).

Glycogen Measurement

Free glycogen in co-culture experiments was measured colorimetrically using the Glycogen Assay Kit (BioVision, Milpitas, CA). 10 µl of surnatants (diluted 1:10) was added to each well in a 96-well microplate with 2 µl of hydrolysis enzyme and volume was adjusted to 50 µl with hydrolysis buffer. Samples were incubated according to the manufacturer's instructions and absorbance (OD_{570nm}) was measured using a plate reader (BioTek, Winooski, VT, USA).

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Chapter 4

The core genome evolution of *Lactobacillus crispatus* as a driving force for niche competition in the human vaginal tract

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Abstract

The lower female reproductive tract is notoriously dominated by *Lactobacillus* species, among which *Lactobacillus crispatus* emerges for its protective and health promoting activities. Although previous comparative genome analyses highlighted genetic and phenotypic diversity within the *L. crispatus* species, most studies have focused on the presence/absence of accessory genes. Here, we investigated the variation at the single nucleotide level within protein-encoding genes shared across a human-derived *L. crispatus* strain selection, which includes 200 currently available human-derived *L. crispatus* genomes as well as 41 chromosome sequences of such taxon that have been decoded in the framework of this study. Such data clearly pointed out the presence of intra-species micro-diversities that could have evolutionary significance contributing to phenotypical diversification by affecting protein domains. Specifically, two single nucleotide variations in the type II pullulanase gene sequence led to specific amino acid substitutions, possibly explaining the substantial differences in the growth performances and competition abilities observed in a multi-strain bioreactor culture simulating the vaginal environment.

Accordingly, *L. crispatus* strains display different growth performances suggesting that the colonization and stable persistence in the female reproductive tract between the members of this taxon is highly variable.

INTRODUCTION

Bacteria evolved over millions of years to colonize different districts of the human body, e.g., skin, pulmonary, gastrointestinal, and vaginal tracts, giving rise to complex and dynamic populations of microorganisms engaged in close relationships with the human host, referred to as the microbiota (Blum, 2017). In particular, the gut microbiota, with its vastity of microbial genera and species, has attracted increasing interest in the last decades for its ability to impact several aspects of human health, development, and systemic physiology from infancy to adulthood (Turroni et al., 2021; Sommer & Bäckhed, 2013; Strati & Facciotti, 2022; Tarracchini, Fontana, et al., 2022; Turroni et al., 2022a; Warner, 2019). In contrast, the vaginal microbiome is typically manifested by a low degree of (bio)diversity and is commonly dominated by members of the *Lactobacillus* genus, such as *Lactobacillus iners*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactobacillus crispatus*. This latter is regarded as the primary determinant of vaginal health (Lepargneur, 2016; Tachedjian et al., 2017). Indeed, in healthy cervicovaginal microbiota, *L. crispatus* species prevails, producing D- and L-lactic acid, hydrogen peroxide, and bacteriocins, which prevent the overgrowth of possible pathogens, hence preventing upper genital tract infections in the host (Sanozky-Dawes & Barrangou, 2022; Stapleton et al., 2011). For such reason, probiotic supplements based on *L. crispatus* are widely used as vehicles of health-promoting strains in the vaginal environment (Bohbot et al., 2018; Cohen et al., 2020; Mändar et al., 2023).

Recently, the evolution of the genome sequences of *L. crispatus* species has been studied in relation to its adaptation to the human vaginal niche, underlining strain-dependent efficiency to grow on glycogen as well as to inhibit pathogens (Abdelmaksoud et al., 2016; Argentini et al., 2022; Mendes-Soares et al., 2014; Ojala et al., 2014; Puebla-Barragan et al., 2021; Van Der Veer et al., 2019).

Moreover, besides the human vaginal tract, *L. crispatus* have also been identified and isolated from various (sub)niches, ranging from healthy poultry gut to various districts of the human body, including the oral cavity, rectum, and urinary tract, highlighting within-species genetic diversity, and variegated metabolic capabilities (France et al., 2016; Mancabelli, Mancino, Lugli, Milani, et al., 2021; Pan et al., 2020; Q. Zhang et al., 2020). Taken together, this evidence suggests the presence of distinct evolutive trajectories underlying the observed phenotypic diversification within this species. However, comparative genomic analyses involving chromosome sequences of *L. crispatus* species have often focused on the relationship between the presence/absence of accessory genes and a particular phenotype (Pan et al., 2020; Q. Zhang et al., 2020), overshadowing the importance of mutations to within-species evolution (Juhas et al., 2011; Martínez-Carranza et al., 2018; Rousset et al., 2021).

In this framework, the aim of this study is to evaluate genome sequence variations at the single nucleotide level within protein-encoding genes shared across non-identical *L. crispatus* chromosomes, providing a close-up view of genetic (micro)diversity, which can contribute significantly to strain diversification within this species. In addition, to investigate the possible implications of the identified genetic differences in the *L. crispatus* intraspecies competition within the vaginal microbiota, we performed *in vitro* experiments consisting of carbohydrate-growth assays involving *L. crispatus* multi-strain co-cultivation in a bioreactor simulating the vaginal tract.

Our findings revealed inter-strain genotypic variation and phenotypic differences between *L. crispatus* strains, highlighting distinct evolutionary developments that may provide this species with differential abilities to long persist and predominate in the human vaginal tract.

RESULTS AND DISCUSSION

Identification of representative *Lactobacillus crispatus* genomes. To investigate the genomic differences between human *L. crispatus* strains, an extensive comparative genome analysis was performed on *L. crispatus* genomes recovered from human specimens of healthy donors, including fecal, vaginal, saliva, and urine samples (Supplementary Table S1). Specifically, seven *L. crispatus* strains were obtained from international bacteria culture collection (Table 1), and their genomes were sequenced along with those of 34 strains isolated from the human vaginal tract in the context of a previous study (Table 1). Additionally, with the aim of expanding the overview of the genetic variability of this taxon, 200 genome sequences (complete and draft) of *L. crispatus* strains isolated from human biological samples were selected from public repositories (Table S1). Following dereplication aimed at removing the genomic redundancy by grouping essentially identical genomes (using dRep tool, version 2.2.0, with average nucleotide identity > 99 %, [https://drep.readthedocs.io/en/latest/choosing_parameters.html]), 22 *L. crispatus* chromosomes with average completeness of 98.97 % ± 0.14 % were retained as representatives of the sequence variation observed in our genome repertoire and therefore used for comparative analysis (Supplementary Table S2).

The general features of the 22 representative *L. crispatus* genomes are reported in Table 1 and include an average of 2,105 ± 179 predicted Coding Sequences (CDSs) per chromosome (ranging from 2,632 to 1,865), with an average genome length of 2.20 ± 0.18 Mbp.

Table 1. Genome features of the 22 representative *L. crispatus* genomes.

Assembly ID	**Strain** name	**Genome size** Mbp	**CDS** number	**Genome Completeness** (%)	**Isolation** source
GCF_000162255.1	125-2-CHN	2.30525	2,032	99.03	human vagina
GCF_000162315.1	MV-3A-US	2.43708	2,252	98.38	human vagina
GCF_002861805.1	UMB0824	2.17405	2,061	99.03	human urine
GCF_002861815.1	UMB0085	2.17506	2,081	99.03	human urine
GCF_009857395.1	Indica2	2.20949	2,028	99.03	human vagina
GCF_007713895.1	NCK1350	2.04734	1,932	99.03	human stool
GCF_013456995.1	B4	2.03959	1,902	99.03	human stool
GCF_000160515.1	JV-V01	2.2172	1,992	98.03	human vagina
GCF_014654865.1	BC5	2.06419	1,901	99.03	human vagina
GCF_015669875.1	D31t1	2.2782	2,120	99.03	human stool
GCF_018987235.1	ATCC 33820	2.23909	2,020	99.03	human saliva
GCF_020042005.1	Lc1700	2.81896	2,632	98.86	human vagina
GCF_021278925.1	lc83	2.30843	2,112	98.9	human vagina
GCF_025194085.1	CIRM-BIA 2111	2.00737	1,865	99.03	human stool
GCF_025194045.1	CIRM-BIA 2233	2.24513	2,127	99.03	human vagina
This study	LMG11440	2.032412	2,087	98.86	human vagina
This study	LMG12005	2.019682	2,014	98.94	human vagina
This study	LMG18189	2.094399	2,079	99.03	human saliva
This study	LMG11415	2.030901	2,004	99.03	human saliva
This study	LMG18200	2.208098	2,182	99.03	human stool
This study	LB93	2.202822	2,340	99.03	human vagina
This study	LB97	2.263389	2,422	99.03	human vagina

Intra-species genetic variability within the *Lactobacillus* genus. To investigate the level of genomic diversity among *L. crispatus* strains compared with other species of the *Lactobacillus* genus, we selected publicly accessible chromosomes belonging to seven different *Lactobacillus* species known to inhabit various human body sites. Notably, for a robust comparison with the dereplicated 22 representative *L. crispatus* genomes, we focused on *Lactobacillus* species for which at least 20 independent conspecific genomes with ANI values between 95% and 98% were retained after accounting for genome completeness > 95 % (Supplementary Table S3). Accordingly, 499 *Lactobacillus* chromosomes were collected and combined with the 22 representative genomes of *L. crispatus* for pangenome analysis, which led to the identification of 159 core genes, defined as the set of gene families (clusters of orthologous groups [COGs]) shared by each *Lactobacillus* chromosome tested (Figure 1a).

Exploiting this set of 159 core genes, the level of variability at the single nucleotide level was evaluated individually within each *Lactobacillus* species. In detail, sequences homologous to the 159 core genes (corresponding to an average of 46,760 ± 987 nucleotides) were recovered from each collected genome and compared between strains belonging to the same *Lactobacillus* species, eventually recording Single Nucleotide Polymorphisms (SNPs) at each nucleotide position.

Considering the nucleotide variations with an occurrence rate above 20% to exclude sequencing errors, the eight inspected *Lactobacillus* lineages exhibited a total number of intra-species SNPs per Mbp ranging from 20.6 to 442.3, with *L. johnsonii* and *L. acidophilus* showing the lowest and the highest number of SNPs, respectively (Figure 1a). Thus, these data highlighted how, on average, the various species of the *Lactobacillus* genus display different levels of intra-species genetic diversity, which has the potential to translate into intra-species phenotypic variability.

Remarkably, the *L. crispatus* core gene set returned an average of 364.7 SNPs per Mbp, emerging among the species with the higher genetic variation, even compared with other notorious *Lactobacillus* species inhabiting the human vaginal tract, such as *L. gasseri*, *L. iners*, and *L. jensenii* (Figure 1a). Specifically, among *L. crispatus* members, most SNPs were found in gene sequences coding for transmembrane transport mechanisms (26 %), followed by genes predicted to be involved in the biosynthesis of extracellular protein components (14.5 %) and carbohydrate metabolism (10.4 %). In contrast, within more niche-specialized *Lactobacillus* species, e.g., *L. gasseri*, the genes with higher number of SNPs were predicted to be involved DNA-related processes (21.4 %) and protein-protein interaction (12 %).

Pan- and Core-genome analysis of the *L. crispatus* species. Chromosome sequences of the 22 non-identical *L. crispatus* strains were submitted to gene re-annotation and subsequently analyzed from a pangenome perspective, providing information on the ubiquitous genetic backbone of conspecific chromosomes and the intra-species genetic diversity (Tettelin et al., 2005). In total, the pangenome of *L. crispatus* includes 6,512 COGs, whose accumulation curve, depicting the expansion of the pangenome as a function of the number of genomes included, is still far from being saturated. Thus, indicating that *L. crispatus* species is characterized by an open pangenome where the total gene pool obtainable for this species has not yet been fully disclosed (Figure 1b). Moreover, we determined the current *L. crispatus* core genome to be comprised of 959 COGs that were conserved across all the 22 analyzed strains (15 % of the pangenome), while an average of 157.3 ± 54.3 genes per genome were associated with only one strain.

Based on the core gene sequences obtained from the 22 non-redundant *L. crispatus* genomes, a phylogenetic tree was constructed to evaluate the evolution of the species (Figure 1c). According to the clustering relationship, the 22 strains assessed

were divided into two main clusters, one of which was intriguingly composed only of *L. crispatus* strains isolated from the female reproductive tract (Figure 1c, violet shadows). Moreover, this phylogenetic tree also displayed a second phylogenetic cluster of strains isolated from different human body districts, encompassing vagina, gut, saliva, and urine (Figure 1c, green shadow). Notably, this mixed group may include a few strains that can survive/colonize in closely related niches, like different human body districts.

To investigate the intra-species genomic variability of *L. crispatus* taxon, we measured the genetic diversity at the single-nucleotide level by comparing the whole genome sequences (wgSNPs) and the corresponding core genome (cgSNPs). Specifically, from the core genome-based phylogenomic tree (Figure 1), we selected the *L. crispatus* strain placed at the deepest split, i.e., the most divergent chromosome (RefSeq genome assembly GCF_015669875), which was used as a reference sequence to compute pairwise alignments and SNPs extraction. Overall, the 22 *L. crispatus* strains showed an average of 28,811 wgSNPs (representing about 2 % of the genome sequence), most of which (about 90 %) resided within coding sequences.

For cgSNPs evaluation, each of the 22 homologous nucleotide sequences obtained by concatenating the 959 COGs shared among all the non-identical *L. crispatus* strains (corresponding to an average of 904,903 bases) were examined for sequence variations against the concatenated (core) gene set of the reference *L. crispatus* assembly. This procedure resulted in the identification of an average of 15,007 cgSNPs (representing a variation rate of 1 for every 56 nucleotides) that was lowered when compared with previous analyses of polymorphic sites within clinically relevant microorganisms such as *Pseudomonas aeruginosa* and *Escherichia coli* (showing 159,609 SNPs within the concatenated core genes of

3,629,979 bp and 266,969 SNPs within a core genome of 2,159,296 bp, respectively) (Muthukumarasamy et al., 2020; Shakya et al., 2020). Albeit the core genome resulted rather conserved within these 22 *L. crispatus* strains, it might be worth mentioning that the observed micro-diversity lies within DNA sequences that code for proteins. Therefore, it could have evolutionary importance contributing to intra-species diversity by affecting protein domains. Indeed, the nucleotide sequence variation among the *L. crispatus* core genome was not randomly distributed, but phylogenetically co-clustered strains showed common patterns of SNP profiles (Figure 1c), thus indicating that the observed SNPs are representing evolutionary trajectories and not mere random mutations or sequencing errors.

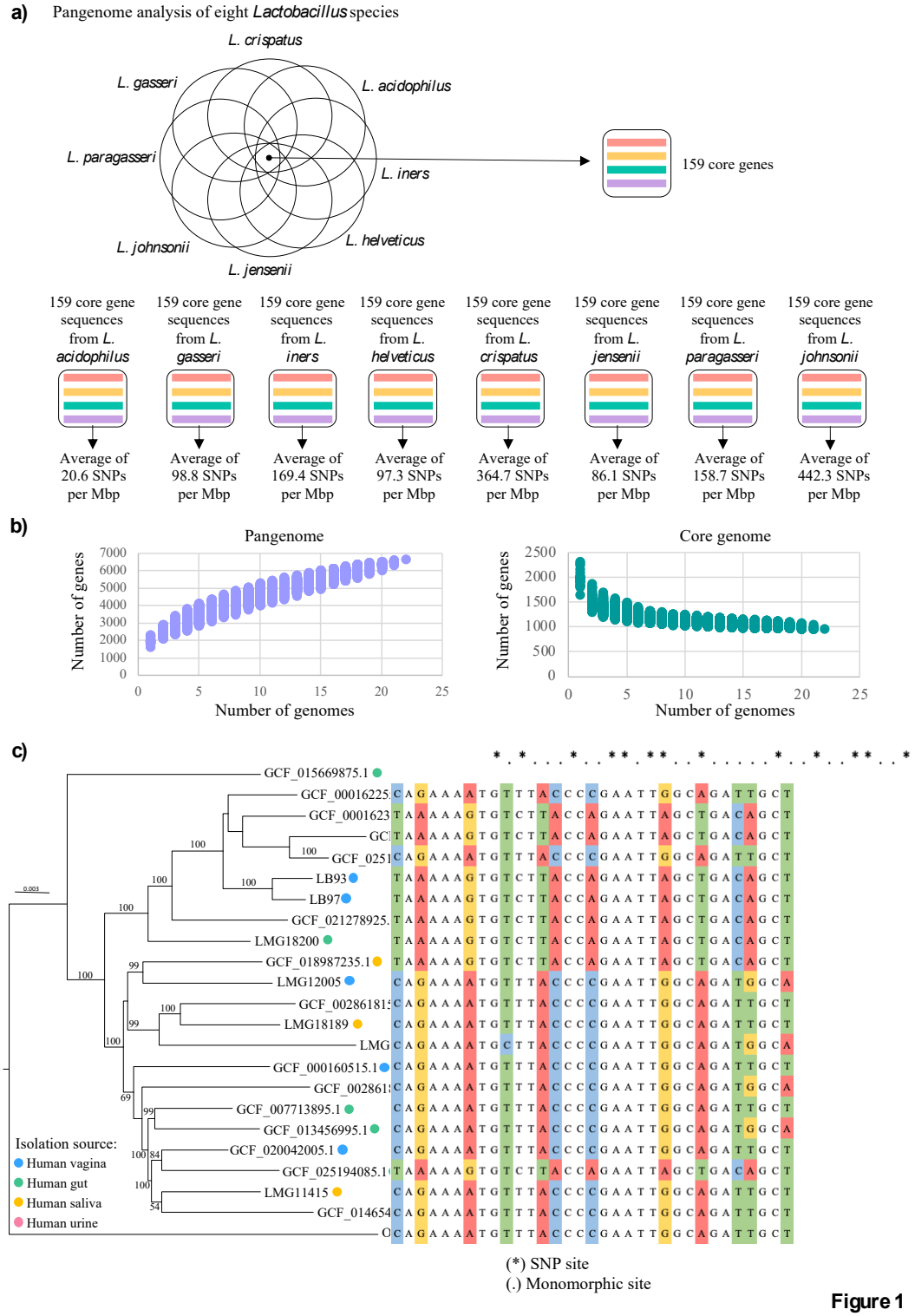
a)
Pangenome analysis of eight Lactobacillus species
L. crispatus
L. gasseri
L. acidophilus
L. paragasseri
L. iners
L. johnsonii
L. helveticus
L. jensenii
159 core genes
159 core gene sequences from L. acidophilus
159 core gene sequences from L. gasseri
159 core gene sequences from L. iners
159 core gene sequences from L. helveticus
159 core gene sequences from L. crispatus
159 core gene sequences from L. jensenii
159 core gene sequences from L. paragasseri
159 core gene sequences from L. johnsonii
Average of 20.6 SNPs per Mbp
Average of 98.8 SNPs per Mbp
Average of 169.4 SNPs per Mbp
Average of 97.3 SNPs per Mbp
Average of 364.7 SNPs per Mbp
Average of 86.1 SNPs per Mbp
Average of 158.7 SNPs per Mbp
Average of 442.3 SNPs per Mbp
b)
Pangenome
Number of genes
Number of genomes
Core genome
Number of genes
Number of genomes
c)
GCF_015669875.1
LB93
LB97
LMG18200
LMG12005
LMG18189
LMG11415
0.003
Isolation source:
Human vagina
Human gut
Human saliva
Human urine
(*) SNP site
(.) Monomorphic site

Figure 1

Figure 1. Comparative analysis between different *Lactobacillus* species and between 22 non-identical *L. crispatus* strains. In panel a, Venn diagram shows the eight *Lactobacillus* species sharing the 159 genes used to measure the magnitude of intra-species genetic diversity. Below, the species-specific number of SNPs identified within the common protein-encoding genes is reported for each of the considered *Lactobacillus* species. Panel b depicts the *L. crispatus* pan- and core-genome size. The number of discovered genes (vertical axe) is plotted as a function of the number of sequentially added genomes (horizontal axe). In panel c, the phylogenomic tree based on the concatenated 959 core genes shared among the 22 non-identical *L. crispatus* genomes showed the relationships between evolutive trajectories and SNPs patterns. The tree was constructed by the neighbor-joining method. Bootstrap percentages based on 1,000 replicates above 50 are shown at node points. For each strain, the isolation source is highlighted with a colored circle.

Exploration of the micro-diversity in the ubiquitous features of *L. crispatus* genome and identification of fast evolving genes.

With the aim of defining whether and which categories of genes are more concerned by a rapid sequence evolution, we calculated the level of polymorphisms for each protein-coding gene constituting the *L. crispatus* core genome. Like what has been performed above, the homologous gene sequences from the *L. crispatus* GCF_015669875 were used as reference in pairwise comparisons of each individual core gene. Accordingly, the number of SNPs resulting from the average of all alignment pairs ranged from zero to 347.45 ± 150.30 per gene (Supplementary Table S4).

Among the genes with the lower average number of SNP sites (lower than 5.8, corresponding to the data below the 25th percentile, Figure 2a), we identified protein-coding sequences involved in putative housekeeping functions, including ribosome assembly and function, central glycolysis regulation, as well as DNA replication and cell division (Supplementary Table S4).

In contrast, by considering the data above the third quartile (Q3+1.5·Inter-Quartile Range, Figure 2a), we identified 52 genes with the highest average number of SNP sites (ranging from 347.45 ± 150.30 to 90.25 ± 28.76), i.e., the most highly variable genes (HVGs), which therefore represented the set of genes that have been likely under the strongest selection pressure (Figure 2b, Supplementary Table S4). Interestingly, among the HVGs, we identified mainly genes involved in the biosynthesis and rearrangement of cell wall components, such as lipoteichoic acids and peptidoglycan, as well as transmembrane transport of a variety of substrates, including carbohydrates and micronutrients (Figure 2b, Supplementary Table S4).The presence of such micro-diversity in proteins directly mediating interactions with the environment likely reflects adaptive mechanisms to the changing biotic and abiotic components, thereby leading to possible different competitive abilities

and (sub-)niche specialization. Indeed, the intra-species heterogeneity observed in the *L. crispatus* core genes emerged less marked when the genomes of 22 *L. crispatus* strains were compared based on their ecological niche, thus showing greater gene sequence homogeneity among genomes sharing the same environment (Figure 2a). However, vaginal-derived *L. crispatus* strains showed a significantly higher number of HVGs than those isolated from the human gut (Mann-Whitney test, *p*-value = 0.007), indicating that the vaginal environment exerts crucial ecological forces driving the *L. crispatus* genome evolution.

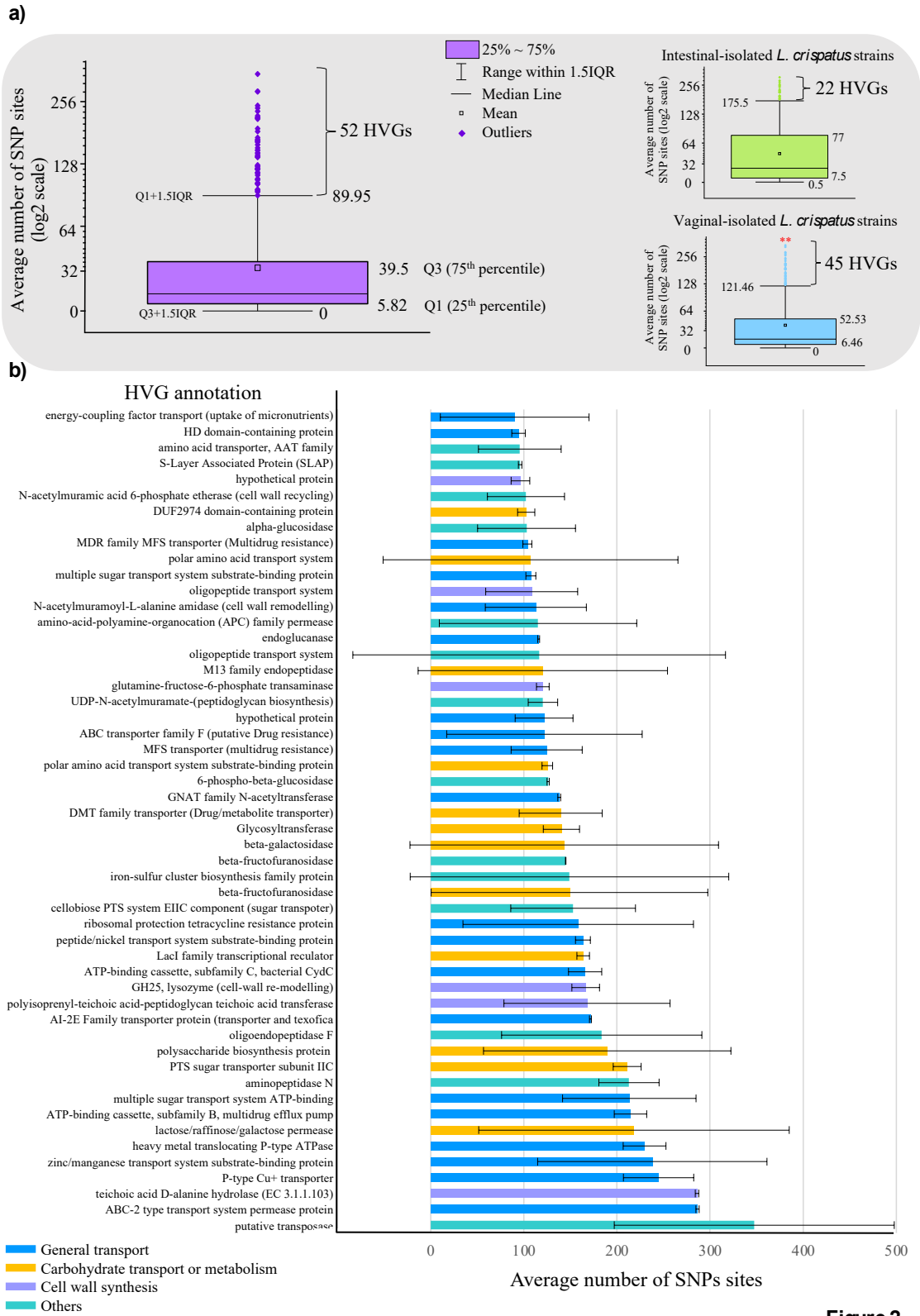

Figure 2

pg. 100

Figure 2. Identification of the 52 HVGs. In panel a, Box-Whisker plot was used to represent the gene distribution based on the number of SNP sites obtained by comparing the nucleotide sequence of every 959 protein-coding genes shared among all the 22 *L. crispatus* chromosomes. For each gene, the number of SNP sites was expressed as the average of all the pairwise comparisons against the reference sequence (homologous gene sequence of *L. crispatus* GCF_015669875). The Q3+1.5IQR was used as a cut-off to select the 52 HVGs. Panel b reports the functional annotation and the number of SNP sites of each HVG.

A new phylogenomic tree, representing the evolutionary outcomes determined by mutational hotspots within the *L. crispatus* species, was generated employing the nucleotide sequences of the 52 HVGs (Figure 3). Specifically, this tree was composed of three main clusters, where not all the strains maintained the same distribution compared to the original phylogenomic tree based on the whole core genome (Figure 3). Indeed, the HVG-based tree better distinguished among strains from closely related niches, highlighting that the evolution of the HVGs does not strictly follow the overall strain speciation, probably reflecting a relatively recent adaptation to specific environmental stimuli, such as multiple human body site colonization or inter-strain niche competition. Accordingly, based on the picture emerging from the HVG-derived phylogenetic distribution, we selected four highly divergent *L. crispatus* strains, i.e., LB97, LMG11440, LMG18200, and LMG11415, that were used for *in vitro* phenotypical assays aimed at investigating the link between evolutionary trajectories, grow performances, and competitive abilities.

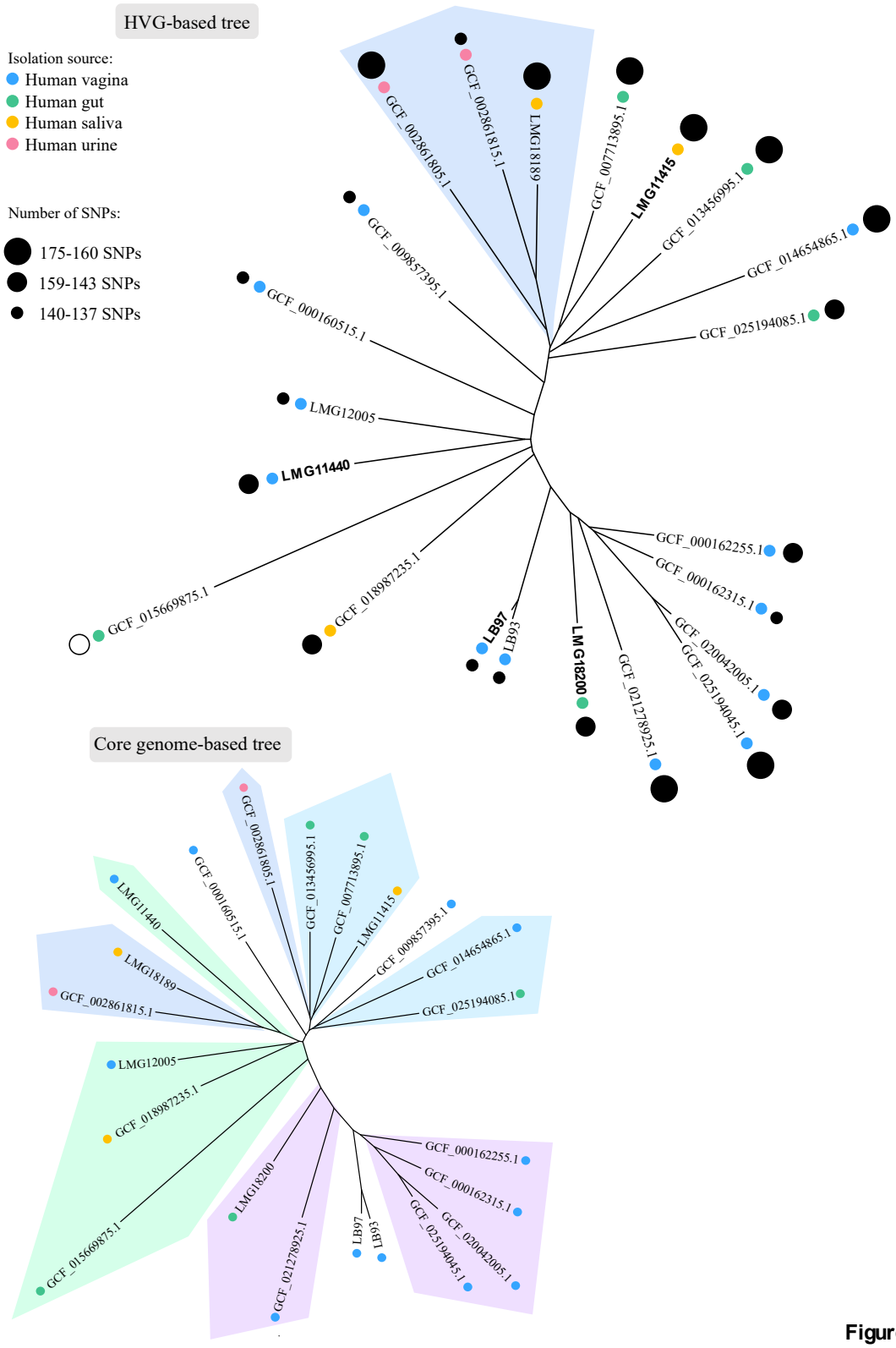
HVG-based tree
Isolation source:
Human vagina
Human gut
Human saliva
Human urine
Number of SNPs:
175-160 SNPs
159-143 SNPs
140-137 SNPs
GCF_002861805.1
GCF_002861815.1
LMG18189
GCF_007713895.1
LMG11415
GCF_013456995.1
GCF_014654865.1
GCF_025194085.1
GCF_009857395.1
GCF_000160515.1
LMG12005
LMG11440
GCF_015669875.1
GCF_018987235.1
LB97
LB93
LMG18200
GCF_021278925.1
GCF_000162255.1
GCF_000162315.1
GCF_020042005.1
GCF_025194045.1
Core genome-based tree
GCF_002861805.1
GCF_000160515.1
LMG11440
LMG18189
GCF_002861815.1
GCF_013456995.1
GCF_007713895.1
LMG11415
GCF_009857395.1
GCF_014654865.1
GCF_025194085.1
LMG12005
GCF_018987235.1
GCF_015669875.1
LMG18200
GCF_021278925.1
LB97
LB93
GCF_000162255.1
GCF_000162315.1
GCF_020042005.1
GCF_025194045.1

Figure 3

Figure 3. Phylogenetic analysis based on the 52 HVG sequences. Proteomic tree based on concatenating the 52 protein encoding core genes identified as highly variable across the 22 non-identical *L. crispatus* genomes. Phylogenetic groups are highlighted in different colors. For comparison, the phylogenomic tree resulting from the whole core genome (presented in Figure 1) is visualized using the redial layout. For each strain, the colored circle represents the isolation source, while the diameter of the black circle is proportional to the number of SNPs identified within the core genome using the GCF_015669875.1 genome sequence as reference.

***In vitro* evaluation of *L. crispatus* growth performances on selected carbohydrate sources in an *in vitro* human vaginal model.** To investigate how the high level of genetic heterogeneity identified within *L. crispatus* could influence the respective growth abilities, the four selected *L. crispatus* strains (LB97, LMG11440, LMG18200, and LMG11415, whose genome diversity corresponded to ANI pairwise values < 98 %, Supplementary Table S2) were cultivated on six different carbohydrate sources including glycogen, which is the primary bacterial nutritional source in the vaginal lumen (Tester & Al-Ghazzewi, 2018; J. Zhang et al., 2022), along with other glycogen-like α-glucans which may also represent a substrate for the bacterial enzymatic arsenal involved in carbohydrates breakdown of the vaginal environment (Figure 4a, Supplementary Table S5). The optical density (OD) was registered after 48h of anaerobic cultivation, and the growth on MRS was used as control condition (Supplementary Table S5). Upon one-way ANOVA test with Bonferroni correction (cut-off p-value < 0.05), the comparative growth assay showed widespread statistically significant differences across the four *L. crispatus* strains. In particular, *L. crispatus* LB97, isolated from the human vagina, showed greater growth performances on most of the carbohydrates tested, including glycogen (final OD > 1.2; all Bonferroni-corrected p-values < 0.05), thus demonstrating a metabolic specialization consistent with its isolation niche. Conversely, the gut-derived *L. crispatus* LMG18200 exhibited the lowest growth when glycogen, starch, and pullulan were used as the unique carbon source (all final OD measures ~ 0.3; all Bonferroni-corrected p-values < 0.05), suggesting the incapability of this strain to metabolize long-chain α-glucans (Figure 4a, Supplementary Tables S5, S6). Moreover, all strains appeared nearly equally limited in mucin utilization (all final OD < 0.3; Bonferroni-corrected p-values > 0.05) (Figure 4a, Supplementary Tables S5, S6).

Furthermore, to determine the reciprocal competitive ability of the different *L. crispatus* strains, the growth performances of the four selected strains were evaluated through a co-cultivation experiment involving a bioreactor model simulating the nutritional and chemical-physical conditions of the vaginal environment (Pan et al., 2020) (Figure 4b). The proliferation trend of each strain was followed for 48h by mapping the sequenced metagenomic reads at multiple time points against a set of strain-specific marker genes (Supplementary Table S7, Supplementary Table S8). In accordance with what was observed in the carbohydrate grow assay, the vaginal isolate LB97 showed a notable proliferation ability, over dominating the four-strain *L. crispatus* community at every co-cultivation time-point (Supplementary Table S8, all Bonferroni-corrected *p*-values < 0.05) (Figure 4c).

In contrast, the strains LMG11415 (isolated from the human saliva) and LMG18200 (isolated from the human intestine) were clearly overwhelmed (Figure 4c). Consistently, close examination of the genomes of these four *L. crispatus* strains revealed that these latter were lacking in any glycogen-degrading encoding gene, while the proliferating vaginal-derived LB97 and LMG11440 strains carried a type II pullulanase acting on both α-1,6- and α-1,4- glycosidic bonds, which therefore achieves complete glycogen degradation, as reported in recent studies (Van Der Veer et al., 2019; J. Zhang et al., 2022). Nevertheless, LB97 and LMG11440 substantially differ in their proliferation capabilities (Supplementary Table S8, Bonferroni-corrected *p*-values < 0.05) (Figure 4c).

Accordingly, the metabolic potential of the gene sequences identified in the comparative genome analysis as associated uniquely with the *L. crispatus* LB97 was investigated using the MetaCyc database (Caspi et al., 2018). The results revealed that the predicted proteome of this strain is characterized by the presence of protein-encoding genes involved in the uptake and metabolism of galactitol and

polyamines, as well as a locus encoding proteins dedicated to the ascorbate degradation, which can contribute to the maintenance of the host's vaginal health (Linares et al., 2011) (Supplementary Table S9). Moreover, the unique gene repertoire of the LB97 strain also contained a gene encoding for a mucin-binding protein (MucBP domain), thus corroborating for this strain the hypothesis of a host mucin role as adhesion site rather than a carbon source, as previously reported in the literature (29–31) and confirmed by the growth assay described above (Figure 4a). However, our functional investigation did not detect genes possibly involved in the metabolism of the nutritional sources constituting the used glycogen-based culture medium (Supplementary Table S9).

Interestingly, when SNPs were calculated between these latter two *L. crispatus* genome sequences, a total of 27,906 nucleotide positions showed differences, of which 8,238 (29 % of the total SNPs) corresponded to amino acid replacements, thus resulting in strain-specific intragenic variants, which can contribute to generating phenotypic differences (Supplementary Table S10). Moreover, alignment of their type II pullulanase gene revealed four variations at single nucleotide level (Supplementary Table S11, Supplementary Figure S1), two of which resulted in amino acid substitutions (Figure 4d, Figure S1). In more detail, these non-synonymous SNPs lie within the protein Carbohydrate Binding Module (CBM) (Figure 4d), with possible repercussions on the efficiency of the protein binding to its substrate, as also evidenced by the 3D protein structure prediction (Supplementary Figure S2).

Overall, the finding of isolate-specific intragenic SNPs, and particularly those within the pullulanase-encoding gene, possibly explain the growth and competitiveness differences observed between the vaginal-isolated *L. crispatus* LB97 and LMG11440 strains cultivated on the simulated vaginal medium.

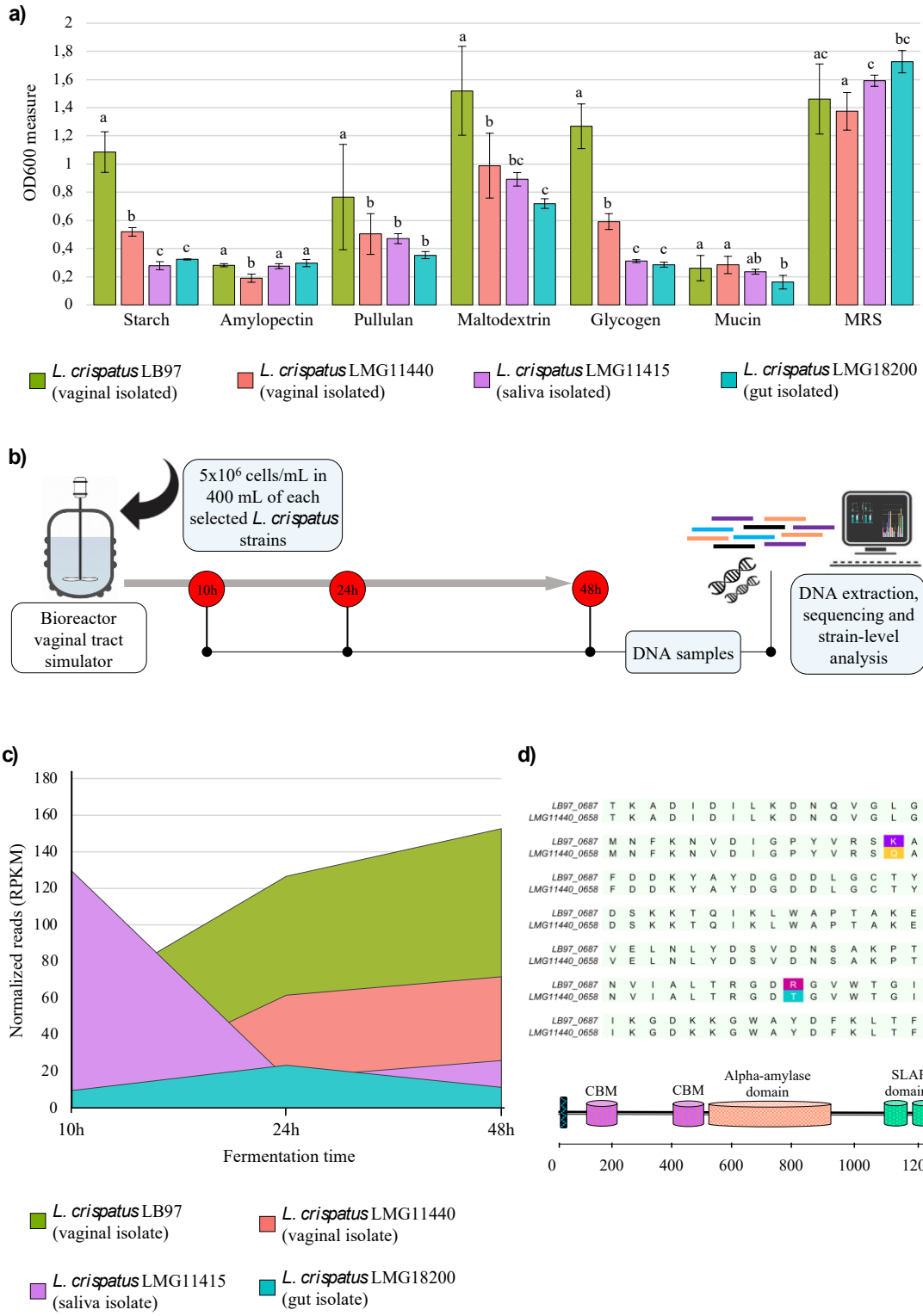
a)
OD600 measure
Starch
Amylopectin
Pullulan
Maltodextrin
Glycogen
Mucin
MRS
L. crispatus LB97 (vaginal isolated)
L. crispatus LMG11440 (vaginal isolated)
L. crispatus LMG11415 (saliva isolated)
L. crispatus LMG18200 (gut isolated)
b)
5x10⁶ cells/mL in 400 mL of each selected L. crispatus strains
Bioreactor vaginal tract simulator
10h
24h
48h
DNA samples
DNA extraction, sequencing and strain-level analysis
c)
Normalized reads (RPKM)
Fermentation time
L. crispatus LB97 (vaginal isolate)
L. crispatus LMG11440 (vaginal isolate)
L. crispatus LMG11415 (saliva isolate)
L. crispatus LMG18200 (gut isolate)
d)
CBM
CBM
Alpha-amylase domain
SLAP domains

Figure 4

Figure 4. Differential growth and competitive abilities between *L. crispatus* strains.

Panel a shows the optical density (OD) registered after 48h of anaerobic growth in different nutritive substrates. Panel b illustrates the design of the bioreactor-based experiment simulating the vaginal environment. In panel c, the bar chart reports the quantification of the metagenomic reads (using average RPKM measures) mapping marker genes unique to each *L. crispatus* strain throughout the 48 hours of growth in the bioreactor.

The standard deviations are plotted as error bars. Different lowercase letters indicate significant differences at p-value < 0.05 according to the Bonferroni test.

In panel d, alignment of partial amino acid sequences corresponding to the type II pullulanase genes of *L. crispatus* LB97 and LMG11440 strains genes highlights two amino acid substitutions Gln (Q) to Lys (K) and Arg (R) to Thr (T).

CONCLUSION

In this study, a comparative genome analysis involving 41 newly decoded human *L. crispatus* genomes coupled with 200 publicly available genome sequences from this species allowed us to deeply investigate the *L. crispatus* core gene evolution by connecting data from single nucleotide variations, phylogenomic reconstructions, and *in vitro* experiments.

Compared with other *Lactobacillus* species, including those inhabiting the human vaginal tract, i.e., *L. iners, L. gasseri*, and *L. jensenii*, a higher level of sequence variation at the single nucleotide level was observed within the gene pool shared among the inspected *L. crispatus* strains, thus highlighting a within-species diversity driven by conserved genes evolution.

Interestingly, the genetic heterogeneity observed within the *L. crispatus* species appears to be reflected at the phenotypic level. In fact, when different *L. crispatus* strains were co-cultivated in a bioreactor-based model simulating the vaginal environment, substantial differences were noted in the colonization and competition efficiency. Although members of the *L. crispatus* species was previously thought to utilize the glycogen hydrolysis products generated in the vaginal environment by the human α-amylase (Mirmonsef et al., 2014; Tester & Al-Ghazzewi, 2018), recent evidences showed that members of this taxon produce the enzyme to independently degrade glycogen, annotated as a type II pullulanase (Van Der Veer et al., 2019; J. Zhang et al., 2022). In this context, while the absence of this gene was noted for those *L. crispatus* strains unable to stably proliferate on glycogen under *in vitro* conditions, we identified two amino acid substitutions within the type II pullulanase carbohydrate-binding module arising from non-synonymous single nucleotide polymorphisms (SNPs) which could explain the different proliferation and dominance abilities observed *in vitro* for the *L. crispatus* strains investigated in this study.

Remarkably, while the strain-specific accessory genetic content has been historically pointed out as one of the main sources of variability resulting from intra-species evolution, data collected in the framework of this study revealed that the evolution of the core genome could contribute to generate marked strain-specific phenotypic traits. Thus, understanding this evolutionary driving force could be relevant for unraveling strain-specific capabilities to successfully dominate the female reproductive tract and, ultimately, selecting suitable *L. crispatus* strains that could be applied for novel bacterial therapy strategies.

MATERIAL AND METHODS

Isolation of *L. crispatus* strains and retrieval of publicly available genome sequences. Candidate *Lactobacillus* strains were obtained from an isolation effort performed in a framework a previous study.

Identification of newly isolated *L. crispatus* strains was achieved through the Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) Biotyper Sirius (Bruker, Germany) using the manufacturer's software FlexControl and the MALDI-Biotyper software (MBT). In detail, a single bacterial colony grown on MRS agar was transferred onto a spot of the MSP 96 target polished steel BC MALDI target plate (Bruker, Germany). Subsequently, the bacterial sample was overlaid with 1 μL of matrix solution containing 10 mg/mL HCCA (a-cyano-4-hydroxycinnamic acid, Sigma-Aldrich, Poland) resolved in 50% acetonitrile (Carlo Erba, Italy) and 2.5% TFA (trifluoro-acetic acid, Carlo Erba, Italy) and air-dried (Schulthess et al., 2014; Werner et al., 2012). The MALDI target plate was then introduced into the spectrometer for automated measurement and data interpretation. The mass spectra were processed with the MALDI Biotyper 3.0 software package (Bruker, Germany) containing reference spectra, including different lactobacilli species. According to the criteria recommended by the manufacturer, a score of ≥ 2.000 indicates a significant similarity between the obtained spectrum and the database entry. Each sample was analyzed in duplicate (2 spots for each sample).

The default parameter settings are as follows: positive linear mode, laser frequency 200Hz, ion source 1 = 19.84 kV, ion source 2 = 18.07 kV, Bruker's MBT_FC and MBT_AutoX methods, mass range: 2000–20000Da. Moreover, before analysis, calibration was performed with a bacterial test standard (Bruker, Germany) containing an extract of *Escherichia coli* DH5 alpha.

A total of 34 *L. crispatus* strains were identified and taken forward for whole genome sequencing. In addition, seven *L. crispatus* strains isolated from human biological samples were purchased from international bacterial collections. To perform chromosomal DNA extraction, the 41 *L. crispatus* strains were cultivated in MRS broth supplemented with 0.05% (wt/vol) L-cysteine hydrochloride in an anaerobic atmosphere at 37°C (2.99% [vol/vol] H_2, 17.01% [vol/vol] CO_2, and 80% [vol/vol] N_2) for 12 h. Subsequently, cells from 10 ml of the culture were harvested by centrifugation at 6,000 rpm for 8 min, and the obtained cell pellet was used for DNA extraction using the GenElute bacterial genomic DNA kit (Sigma-Aldrich) following the manufacturer's guide.

Furthermore, 200 *L. crispatus* genome assemblies with completeness > 95% derived from strains isolated from biological material of human subjects were retrieved from the NCBI database.

Genome Sequencing, Assembly, and Annotation. The DNA extracted from the 41 *L. crispatus* strains was subjected to whole-genome sequencing using MiSeq (Illumina, UK) at GenProbio srl, Parma, Italy (www.genprobio.com) according to the supplier's protocol (Illumina, UK). Individual genome libraries were generated using the Nextera XT preparation kit and loaded into a 600-cycle (250-bp paired-ends) flow cell version 3 (Illumina). Raw DNA sequence reads (fastq files) obtained from genome sequencing were assembled using the MEGAnnotator pipeline (Lugli, Milani, Mancabelli, van Sinderen, et al., 2016). Briefly, SPAdes software was used for *de novo* assembly of the genome sequences with the pipeline option "--carefull" and a list of k-mer sizes of 21; 33; 55; 77; 99; 127 (Bankevich et al., 2012) and protein-encoding genes were predicted for contig greater than 1,000 bp using Prodigal (Hyatt et al., 2010a). Functionally annotation of the predicted genes was achieved through RAPSearch2 (cut-off E value, 1×10^{-5}; minimum alignment

length, 20 amino acids) (Zhao, Tang, et al., 2012) and hidden Markov model profile (HMM) searches (cut-off E value, $1 \times 10{-}10$) (http://hmmer.org/) performed against the NCBI nr database and the manually curated Pfam-A database, respectively. Moreover, tRNA genes were determined using tRNAscan-SE version 1.4 (Lowe & Eddy, 1997), and rRNA loci were identified with RNAmmer version 1.2 (Lagesen et al., 2007).

Furthermore, to obtain comparable quality standards for the analyzed genomes, all the 200 *L. crispatus* genomes retrieved from the NCBI database were re-annotated employing the same approach based on MEGAnnotator pipeline used for the 41 *L. crispatus* genomes decoded in the current study.

Pangenome analyses and phylogenomic tree reconstruction. All pangenome calculations were performed using PGAP [PanGenomes Analysis Pipeline, (Zhao, Wu, et al., 2012a)] as described previously (Lugli et al., 2019a; Tarracchini et al., 2020). In detail, orthologous protein sequences were identified in genome sequences using BLAST analysis (cut-off E-value = 1×10^{-5}; 50% identity over at least 80% of sequence coverage) and then organized into functional Clusters of Orthologous Groups (COGs) through the MCL algorithm (graph-based Markov clustering algorithm) using the gene family (GF) method. Pangenome profiles were produced through an optimized procedure integrated into the PGAP software, based on a presence/absence matrix including all COGs identified in the given genomes. The concatenated protein sequences of core genes were aligned using Mafft v7.453 (Katoh et al., 2002) and then employed to build correspondent phylogenomic trees through the neighbor-joining method in ClustalW version 2.1. Visual core genome-based phylogenomic trees were developed using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

Single-Nucleotide Polymorphism identification. The species-specific level of polymorphisms within the *Lactobacillus* genus was assessed exploiting the identified core gene set shared between *L. crispatus,* and seven different *Lactobacillus* species. In detail, 159 core-shaping genes were concatenated and aligned using the multiple genome aligner Mafft v7.453 (Katoh et al., 2002). Nucleotide variants at each sequence position were then extracted through the SNP-sites program (version 2.5.1) (Page et al., 2016). Assuming that, unlike sequencing errors, real genetic variants should be observed in a quite number of independent genomes assembly, we considered only sequence positions in which two or more alternatives were observed in at least 20% of genome collection. The number of intra-species SNPs obtained for each *Lactobacillus* species was converted into SNPs per Mbp to account for variation in genome length.

In a similar fashion, both concatenated and individual gene nucleotide sequences comprised within the *L. crispatus* core genome were aligned with Mafft v7.453 and parsed with the SNP-site software. For these analyses, the genome sequence of the most divergent *L. crispatus* strain (assembly number GCF_015669875.1) was used as reference sequence. Synonymous and non-synonymous nucleotide variations were discriminated using the ParaAT 2.0 software (Z. Zhang et al., 2012) combined with KaKs Calculator 3.0 toolkit (Z. Zhang, 2022). Whole-genome SNPs were extracted by combining the short-reads aligner BWA and the VarScan tool (version 2.3.6)

Carbohydrate growth assay. *In vitro* growth assays with different carbon sources, such as starch, amylopectin, pullulan, maltodextrin, glycogen, and mucin, were performed on selected *L. crispatus* strains, i.e., LB97, LMG11440, LMG18200, and LMG11415. In detail, the four *L. crispatus* strains were cultivated overnight on a semisynthetic MRS medium supplemented with 0.05% (w/vol) L-cysteine

hydrochloride at 37 °C under anaerobic conditions. Subsequently, cells were diluted in MRS without glucose to obtain an $OD_{600\,nm}$=1, and 15 µl of the diluted cells were inoculated in 135 µl of MRS without glucose supplemented with 1% (wt/vol) of a particular sugar in a 96-well microtiter plate and incubated in an anaerobic cabinet. Specifically, each carbohydrate was dissolved in MRS without glucose previously sterilized by autoclaving at 121°C for 15 min. Subsequently, the obtained solutions were sterilized using a 0.2 µm filter size before use. Cell growth was evaluated by monitoring the optical density at 600 nm using a plate reader (Biotek, VT, USA). Each plate was read in discontinuous mode, with absorbance readings performed thrice at 3-min intervals after 48h of growth, and each reading was ahead of 30s of shaking at medium speed. Cultures were performed in triplicates for each strain, and the resulting growth data were expressed as the average OD_{600nm} of these independent biological replicates. Carbohydrates tested in this study were purchased from Merck (Germany) and Fisher Scientific, ACROS Organics (USA) and include soluble starch from potato, amylopectin from maize, pullulan, maltodextrin, glycogen from beef liver, mucin from porcine stomach. The semisynthetic MRS medium was used as the control medium.

Co-culture using a bioreactor system. The four selected *L. crispatus* strains (reported above) were grown anaerobically at 37°C for 24h in simulated vaginal fluid (SVF) (Pan et al., 2020) to adapt the microorganisms to the medium. Next, revitalized cells were inoculated in a bioreactor system (Solaris Biotech Solutions, Italy) to obtain a final concentration per bacterial strain of $5x10^6$ cells/mL in 400 mL of SVF. The co-culture of the four *L. crispatus* strains was performed with a non-continuous supply of the growth medium for the first 12h to stabilize the microbial community. Subsequently, the cultivation was shifted to a continuous mode to provide fresh SVF medium and continued for 48h under anaerobic

conditions at 37°C with a mechanical agitation set at 180 rpm. In addition, the pH was maintained at 4.5 by adding 2.5M NaOH to mimic the pH of the human vaginal environment (Pan et al., 2020). During bacterial growth, culture aliquots were collected at 10h, 24h, and 48h.

DNA extraction and shotgun metagenomic sequencing. Each aliquot collected from bioreactor cultivation was subjected to DNA extraction using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, D4300) following the manufacturer's instructions. Then, after assessing DNA concentration and purity using a BioPhotometer D30 (Eppendorf, Germany), each DNA sample was sequenced by GenProbio srl, Parma, Italy (www.genprobio.com) employing next-generation sequencing technique (shotgun metagenomic sequencing). The preparation of DNA libraries was performed using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions, using 1 ng of DNA from each metagenomic sample. The isolated DNA underwent fragmentation, adapter ligation, and purification. The ready-to-go libraries were pooled equimolarly and diluted to a sequencing concentration of 650 pM. On-board DNA denaturation and sequencing were performed on a NextSeq 2000 instrument (Illumina, San Diego, CA), according to the manufacturer's instructions, using the 2x150 bp NextSeq 1000/2000 P2 Reagents (300 Cycles) v3 and spike-in of 1 % PhiX control library. Whole-metagenome shotgun (WMGS) sequencing of the three bioreactor culture aliquots produced an average of 21,861,838 ± 7,995,330 paired-end 150 bp reads per sample. Raw metagenomic sequencing reads were trimmed and quality filtered with fastq-mcf software supplied by Illumina Inc (minimum mean quality score, 20; window size, 5 bp; and minimum length, 100 bp). Following quality filtering, an average of

18,662,746 ± 5,671,267 quality-filtered microbial reads per sample were retained (Supplementary Table S8).

***L. crispatus* strain-level profiling of the bioreactor-derived cultures.** To disentangle the different *L. crispatus* strains in the co-culture aliquots collected at different time points (10h, 24h, and 48h) during bioreactor growth, the filtered metagenomic reads obtained from each shotgun sequencing effort were mapped against specific distinctive regions of every *L. crispatus* genome using the software BBMap (https://sourceforge.net/projects/bbmap/) with 100% homology (perfectmode=t flag). Notably, to avoid mis-mapping of the metagenomic short reads, it was used *L. crispatus* genomes that returned pairwise ANI values < 98%, as advised in a previous qualified study (https://drep.readthedocs.io/en/latest/choosing_parameters.html). In detail, to identify suitable discriminative genes, the whole set of genes unique to each *L. crispatus* strain detected in the PGAP analysis were mapped against the combined genomes of all strains using the Bowtie2 --very-sensitive mode (Langmead & Salzberg, 2012). Genes that did not return any hits other than those corresponding to the genome to which they belong were retained as candidate strain-specific marker genes. This selection was then manually inspected to exclude genes corresponding to transposases, phage genes, and genes located alongside the contig ends. This procedure identified a set of roughly ten unique marker genes for each *L. crispatus* strain that were used in downstream analyses on the bioreactor-derived metagenomic reads (Supplementary Table S7). A proxy measure of each strain abundance was calculated by normalizing the mapped read count on the corresponding marker gene length and library size using the RPKM mathematical formula [(10^9 * Number of mapped reads to a gene)/(Total mapped reads * gene length in base-pairs]). Moreover, the set of genes associated uniquely with each of

the four co-cultivated strains was functionally investigated to discover potential accessory protein-encoding sequences conferring peculiar growth abilities in the cultivation medium. To this scope, we employed the MetaCyc database (https://metacyc.org/), which allowed us to assign a detailed functional annotation to each scrutinized gene. In addition, the Transporter Classification Database (TCDB) was exploited to characterize transport systems and identify their possible substrates (https://tcdb.org/).

Statistical analysis. The software SPSS version 25 and OriginPro version 2023 (www.ibm.com/software/it/analytics/spss/) (https://www.originlab.com/) were used for statistical data analyses and graphing. One-way ANOVA with Bonferroni correction was used to determine the statistical significance of differences in the OD_{600} measures (growth assay) and normalized read counts (bioreactor-based co-cultivation experiment). AlphaFold (Jumper et al., 2021) and PyMOL software (https://pymol.org/2/) were used to observe SNPs within the predicted 3D protein structure of the pullulanase type II gene derived from the *L. crispatus* LB97.

Data availability. Genome sequences of the 41 newly sequenced *L. crispatus* were deposited in NCBI-SRA (Short Read Archive) repository with accession number PRJNA947599.

Conflicts of interest.

The authors declare no conflict of interest.

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Chapter 5

Exploring the ecological effects of naturally antibiotic-insensitive bifidobacteria in the recovery of the resilience of the gut microbiota during and after antibiotic treatment

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Abstract

Amoxicillin-clavulanic acid (AMC) is the most widely used antibiotic, being frequently prescribed to infants. Particular members of the genus *Bifidobacterium* are among the first microbial colonizers of the infant gut, and it has been demonstrated that they elicit various beneficial activities upon their human host, including promotion/maintenance of the human gut microbiota homeostasis. It has been shown that natural resistance of bifidobacteria toward AMC is limited to a small number of strains. In the current study, we investigated the mitigation effects of AMC-resistant bifidobacteria in diversity preservation of the gut microbiota during AMC-treatment. In detail, an *in vitro* co-culture experiment based on infant fecal samples and an *in vivo* study employing a rodent model were perfomed. The obtained results confirmed the ability of AMC-resistant bifidobacterial strains to bolster gut microbiota resilience, while specific covariance analysis revealed strain-specific and variable impacts on the microbiota composition by individual bifidobacterial taxa.

Importance

The first microbial colonizers of the infant's gut are members of the genus *Bifidobacterium*, which elicit different beneficial activities to their host. Amoxicillin-clavulanic acid (AMC) is the most frequently prescribed antibiotic during infancy and few strains of bifidobacteria are known to show a natural resistance to this antibiotic. In the present work, we evaluated the possible positive effects of AMC-resistant bifidobacterial strains in maintaining gut microbiota diversity during AMC exposure, performing an *in vitro* and *in vivo* experiment based on an infant gut model and a rodent model, respectively. Our results suggested the ability of AMC-resistant bifidobacterial strains to support gut microbiota restoration.

Introduction

The term 'microbiota' refers to the complex population of microorganisms that colonize a specific ecological niche (1). In recent decades, it has become clear that the gut microbiota composition affects the health status of the (human) host (2). In fact, various studies have reported the crucial role played by the gut microbiota in maintaining physiological homeostasis of the host as well as in modulating nutritional and immunological functionalities (1, 3). Upon birth microorganisms very rapidly colonize the neonatal gut and the composition of this early life intestinal microbiota is reported to be influenced by several factors, such as mode of delivery, diet, and gestational age, in addition to antibiotic treatment (4). An abberant gut microbiota composition, sometimes referred to as dysbiosis, may influence human health directly, but may also impose long-term host health consequences. Several factors can lead to a dysbiotic state, such as diet, environment (5) and the use of antimicrobial agents, like antibiotics (6). In this context, oral antibiotics have been widely reported to exert a marked effect on the human gut microbiota composition, frequently causing dysbiosis (7, 8) and contributing to the onset of several metabolic and intestinal disorders (9-12). Amoxicillin is a beta-lactam antibiotic which is typically administered together with clavulanic acid, a beta-lactamase inhibitor (AMC), and this dual drug combination represents the most widely used antibiotic, commonly administered during infancy (13, 14). Interestingly, microbiota analysis of healthy human adult volunteers treated with AMC revealed a significant decrease in abundance of bifidobacterial species (15), which are dominant members of the human gut microbiota in the early stages of life until weaning (16). In this regard, it has recently been discovered that AMC resistance is rare among bifidobacteria and appears to be a species-independent feature (17, 18). In order to evaluate possible ecological roles played by three previously identified AMC-insensitive bifidobacterial strains, i.e.,

Bifidobacterium breve 1891B, *Bifidobacterium breve* M1D and *Bifidobacterium longum* subsp. *longum* 1898B, in maintaining and/or re-establishing homeostasis of the gut microbiota during or following AMC therapy, we assayed the mitigation effects of these strains on the gut microbiota in the presence of AMC using an *in vitro* infant gut model as well as an *in vivo* rodent study. For this purpose, qPCR and shallow metagenomics approaches were used to evaluate the impact over time of these AMC-resistant strains on the intestinal microbiota

Materials and methods

Ethics Statement.

All experimental procedures and protocols involving animals were approved by the Veterinarian Animal Care and Use Committee of Parma University (approved protocol 370/2018-PR) and conducted in observance with the European Community Council Directives dated 22 September 2010 (2010/63/UE). The study protocol for fecal samples was approved by the Ethics Committee number 2016/0028558. Signed informed consent was obtained from the legally authorized representative(s) of each infant enrolled in this study.

Strains and cultivation conditions.

Bifidobacterium strains used in this study were *Bifidobacterium breve* 1891B, *Bifidobacterium breve* M1D, *Bifidobacterium longum* subsp. *longum* 1898B (18). Strains were grown anaerobically in De Man, Rogosa, Sharpe (MRS) medium (Scharlau) supplemented with 0.05% L-cysteine-HCl and incubated at 37°C for 24 h. Anaerobic conditions were achieved using an anaerobic cabinet (Ruskin), in which the atmosphere consisted of 17% CO_2, 80% N_2, and 2.99% H_2.

In vitro gut microbiota cultivation.

For the purpose of this study, fecal samples from three infants (aged from 0 to 6 months) were collected, which consisted of approximately 10 g of fresh fecal material, were kept on ice, shipped under subzero conditions to the laboratory, and stored at −20°C until further processing. These collected fecal samples were used for *in vitro* cultivation of the gut microbiota employing a medium essentially as described by Macfarlane *et al.* (19). A 96-deep well plate was prepared as previously described (20). Briefly, 1.5 g of a freshly thawed infant fecal sample was dropped into a 50 mL tube containing 7 mL of sterile phosphate-buffered saline

(PBS) solution. Subsequently, the sample was homogenized with a vortex mixer. Sample homogenates were pipeted into each well in the medium for a batch cultivation at a 10^7 CFU/mL final inoculum concentration (cell density was determined by means of flow cytometry (21). The final volume of the medium in each well was 1.5 mL. We tested four different growth conditions: the first condition involved inoculation of only the fecal sample in the medium, the second condition consisted of the fecal sample together with the antibiotic AMC at a final concentration of 20μM (7, 18), the third condition consisted of the fecal sample inoculum with each single AMC-insensitive bifidobacterial strain, i.e., *B. breve* M1D, *B. breve* 1891B and *B. longum* subsp. *longum* 1898B, at a 10^5 CFU/mL final inoculum concentration. The fourth condition consisted of the fecal sample with each of the three AMC-insensitive bifidobacterial strains in the medium supplemented with 20μM AMC. For each growth condition, six replicates were collected at three different time points, i.e., T0, 12h and 24h (Fig. S1a). Each aliquot was subjected to DNA extraction using the QIAamp DNA Stool Mini kit following the manufacturer's instructions (Qiagen, UK) for sequence library preparation.

Evaluation of *Bifidobacterium* cell numbers by qPCR and PCR analysis.

In order to evaluate *Bifidobacterium* cell numbers in rodent (rat) fecal samples and the presence of *Bifidobacterium* strains in co-culture experiments we used strain-specific primers: 1891B _FW (5'-GGTTGAGCTTACCGAAGACC-3') and 1891B _RV (5'-TAAGGCTCCTTCTGGTGTGG-3') for *B. breve* 1891B. For *B. breve* M1D we used primers M1D_FW (5'-CGCTATCGACACCGACTACA-3') and M1D_RV (5'-GATATCGGCCTTGGAACAGA-3'). For *B. longum* subsp. *longum* 1898B we employed primers B1898_0685_Fw (5'-GACGCGCAAGGTTCAATAAC-3) and B1898_0685_Rev (5'-ACTATACAATGCGCCGTTGG-3'). qPCR was performed using qPCR Green

Master Mix (Biotech Rabbit, Germany) on a CFX96 system (BioRad, CA, USA) following previously described protocols (22). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: one cycle of 95 °C for 2-3 min, followed by 40 cycles of 95 °C for 15 s and 60-65°C for 30 s. The melting curve was 65 °C to 95 °C with increments of 0.5 °C/s. In each run, negative controls (no DNA) were included. A standard curve was generated using the CFX96 software (BioRad).

Each 12.5 μL PCR reaction contained 30–40 ng of genomic DNA, 2X PCRBIO HS Taq Mix (PCR Biosystems, USA) and 100 μM of each oligo. Each PCR reaction consisted of an initial denaturation step of 5 min at 94°C, followed by 30 amplification cycles as follows: denaturation at 94°C for 20 s, annealing at a temperature of 58°C for *B. longum* subsp. *longum* 1898B and *B. breve* M1D, and 60°C for *B. breve* 1891B for 30 s, 72°C for 30 s and finalized by an elongation step at 72°C for 5 min. PCRs were performed on a Verity Thermocycler (Applied Biosystems, USA). PCR products were analyzed by electrophoresis on a 1.5% agarose gel and visualized by SYBR Safe DNA gel stain (Invitrogen).

Animal housing and design of the *in vivo* experiment.

The *in vivo* rodent experiments involved 16 male, 5-week-old Wistar rats. After weaning, rats were housed in same-sex sibling groups in rooms under humidity (50±10%) and temperature (22±2°C) controlled conditions, at 12-h light-dark cycle (lights on at 7 a.m.) and with food and water available *ad libitum*. Experiments were conducted on 5-week-old male Wistar rats (n=16). In rats, this age corresponds to the beginning of the human perioadolescent phase (23) After weaning on post-natal day 28, rats were housed in sibling groups in rooms under humidity (50±10%) and temperature (22±2°C) controlled conditions, under a 12-h light-dark cycle (lights

on at 7 a.m.) and with food and water available *ad libitum,* and did not undergo any type of treatment (Fig. 1a).

On day 35, experimental rats were housed individually in polymethyl methacrylate (Plexiglas®) cages (39 cm × 23 cm × 15 cm), continued to consume a standard chow and were orally given a 500µl sucrose solution (2%) with a syringe to adapt them to this form of administration. This time point represents the reference control of the experiment, also considered the baseline for microbiota analyses (24) (Fig. 1a). For the next 12 days, the rats were randomly assigned to four different treatment groups, i.e., G1, G2, G3, and G4. All four groups were orally administred with Augmentin® (amoxicillin-clavulanic acid) at a dosage of 35 mg kg^{-1} dissolved in water, two times per day using a syringe (25) (Fig. 1a). Furthermore, groups G2, G3 and G4 were also orally administred with *B. breve* 1891B, *B. breve* M1D or *B. longum* subsp. *longum* 1898B, respectively, while group G1 received only the antibiotic and 500µl sucrose solution (2%) without any *Bifidobacterium* strain. *Bifidobacterium* strains were cultivated as previously described (26). The resulting cell cultures were subsequently centrifugated, washed and resuspended in 500µl of sucrose solution (2%). Body Weight (BW), Food Intake (FI) and fresh fecal samples were collected at five different time points. The first fecal sample collection was performed before the oral administration of AMC and bifidobacteria (T0), then fecal samples were collected on days 2, 5, 8, 10, and 12 (T1, T2, T3, T4, and T5, respectively) (Fig. 1a). The sawdust was changed 1h before each fecal collection. Afterward, fresh fecal samples were collected in the morning and stored at −20°C until analysis. BW was measured as previously described (27) (Fig. 1b-c). Food Intake (FI) was calculated as the amount (g) of food consumed over 24 hours. BW and FI data were collected every two days at five different time points (T0, T1, T2, T3, T4, and T5).

Fecal bacterial DNA extraction and shallow shotgun metagenomics.

Rodent fecal samples were subjected to DNA extraction using the QIAamp DNA Stool Mini Kit following the manufacturer's instructions (Qiagen). Extracted DNA was then processed according to the Illumina Nextera XT protocol. DNA samples were enzymatically fragmented, barcoded and purified involving magnetic beads. Then, samples were quantified using fluorometric Qubit quantification system (Life Technologies, USA), loaded on a 2200 Tape Station Instrument (Agilent Technologies, USA) and normalized to 4nM. Paired-end sequencing was performed using an Illumina MiSeq sequencer with flow cell v3 600 cycles (Illumina Inc., San Diego, USA). The obtained fastq files were filtered for quality (> 20) and length (> 80 bp) of the reads. Filtered data were then used to reconstruct the taxonomic profile of the analyzed samples (28) using the bioinformatic software platform METAnnotatorX2 (29). In detail, the taxonomic classification of each read was obtained by megablast analysis (30) using as a reference the database of non-redundant genome sequences retrieved from the database at the National Center for Biotechnology Information (NCBI). In addition, the beta-diversity among the analyzed samples was calculated by means of the Bray-Curtis dissimilarity and based on species abundance. The results of this analysis were represented by 3D-PCoA (Principal Coordinates Analysis), using the QIIME2 software (31, 32).

Evaluation of cell density by flow cytometry assay.

For bacterial cell counting, 0.1 g of a rat fecal sample was diluted in a physiological solution (phosphate-buffered saline, PBS). Subsequently, bacterial cells were stained with 1 µl SYBR green I and incubated in the dark for at least 15 min before measurement. All count experiments were performed using an Attune NxT flow cytometer (Invitrogen, Thermo Fisher Scientific) equipped with a blue laser set at 50 mW and tuned to an excitation wavelength of 488 nm. Multiparametric analyses

were performed on both scattering signals (forward scatter, FSC, and side scatter, SSC) and SYBR green I fluorescence was detected on the FL1 channel. Cell debris and eukaryotic cells were excluded from acquisition analysis by a sample-specific FL1 threshold. All data sets were statistically analyzed with Attune NxT flow cytometer software. Utilizing these cell counts to normalize the sequencing data into absolute abundance of each profiled taxon, we were able to perform quantitative microbiome profiling using a previously described method (21).

Statistical analysis.

Two-way ANOVA for repeated measures with "group" as the between-subject factor (4 groups) was performed for both BW and FI data, with "time" as the within-subject factor (six levels: T0, T1, T2, T3, T4 and T5). All statistical analyses were performed with SPSS v. 25 software (www.ibm.com/software/it/analytics/spss/). In particular, ANOVA analysis was performed to evaluate the relative abundance differences of bacterial species.

Data Deposition.

Raw sequences of the Shallow-shotgun metagenomics profiling experiments are accessible through SRA study accession numbers PRJNA803045.

Results and Discussion

Impact of AMC-resistant bifidobacterial strains on the infant gut microbiota in the presence of AMC.

In order to evaluate the possible effects of AMC treatment on a gut microbiota encompassing AMC-resistant *Bifidobacterium* strains, co-culture experiments involving infant fecal samples inoculated with *B. breve* 1891B, *B. breve* M1D or *B. longum* subsp. *longum* 1898B (18) were carried out using the Mipro model (20). Specifically, each fecal sample was used to test four different co-culture conditions for each AMC-insensitive strain, i.e., i) fecal sample, ii) fecal sample with AMC-insensitive strain, iii) fecal sample with AMC, iv) fecal sample with AMC and each AMC-insensitive strain (see Materials & Methods) (Fig. S1a). The co-cultures were monitored at two different time points, i.e., 12h and 24h after the inoculum (T0). For each time point and for each cultivation condition tested, the changes in the microbiota composition were assessed by shallow-shotgun metagenomics analysis, while the absolute abundance of bacterial cells was evaluated by flow cytometry. The PCoA (Principal Coordinates Analysis) based on absolute bacterial abundance at species level revealed a clear difference between each T0 and corresponding 12h and 24h, revealing a general selection of certain bacteria during cultivation (Fig. S1b). Notably, cultures of fecal samples from Infant-1 in all cases seemed to select bacteria belonging to *Clostridium paraputrificum*, *Enterococcus faecalis*, *E. faecium*, *Escherichia coli* and *Klebsiella michiganensis* (prevalence > 80 %) (Table S1 in the supplemental material). Similarly, cultures of fecal samples from Infant-2 and Infant-3 revealed a selection of species belonging to the *Escherichia* genus (prevalence > 80 %) and *Bacteroides fragilis* (prevalence > 80 % in Infant-3) (Table S1). Furthermore, analysis of cell number variations between 12h and 24h indicated that 83% of AMC supplemented co-culture showed a decrease in cell number over time, while 75% of co-culture without AMC treatment revealed an opposite trend.

These results confirm the previously reported impact of antibiotic treatment on the microbiota by decreasing microbial abundance and complexity (7, 8). In order to confirm the ability of the three AMC-insensitive *Bifidobacterium* strains to resist antibiotic treatment, we applied strain-specific PCR to all fecal samples, which revealed as expected the absence of the AMC-resistant bacterial strains in the original fecal samples used for the experiments, yet highlighted the presence of these strains in the cultures of fecal samples at 12h and 24h (Fig. S1c).

Assessment of bifidobacterial colonization following antibiotic treatment.

In order to validate the capability of AMC-insensitive *Bifidobacterium* strains in preserving the gut microbiota as indicated by the *in vitro* analyses, an *in vivo* study based on a rodent model was performed. In detail, to assess the level of intestinal microbial colonization by AMC-resistant strains of bifidobacteria, we applied a qPCR approach to quantify bacterial DNA extracted from fecal samples of each rat included in our study. In detail, qPCR analysis revealed the presence of these bifidobacterial strains in each fecal sample from time point T1. The two applied *B. breve* strains, i.e., M1D and 1891B, showed a relatively linear trend of microbial load across the different experimental times (Fig. 1d), thus such persistence data confirmed their insensitivity to AMC (18). Furthermore, *B. breve* 1891B exhibited a higher microbial load than *B. breve* M1D, in accordance with the previously published *in vitro* experiments that reported greater MIC values for *B. breve* 1891B when compared with other *B. breve* strains (18) (Fig. 1d). Conversely, evaluation of the microbial load of *B. longum* subsp. *longum* 1898B revealed a non-linear variation (Fig. 1d). In particular, the qPCR analysis demonstrated that the microbial load for this strain increases at time T3, decreases at time T4 followed by a second considerable increase at time T5 (ANOVA p-value < 0.05 at T3 and T5) (Fig.1d). Notably, previously reported MIC analyses involving the used AMC-insensitive

strains revealed that *B. longum* subsp. *longum* 1898B is associated with a lower MIC value when compared to strains belonging to the *B. breve* species (18), while under the conditions used here this strain showed the highest microbial load when compared to *B. breve* 1891B and M1D strains (Fig. 1d), suggesting superior *in vivo* colonization ability of this strain despite antibiotic treatment.

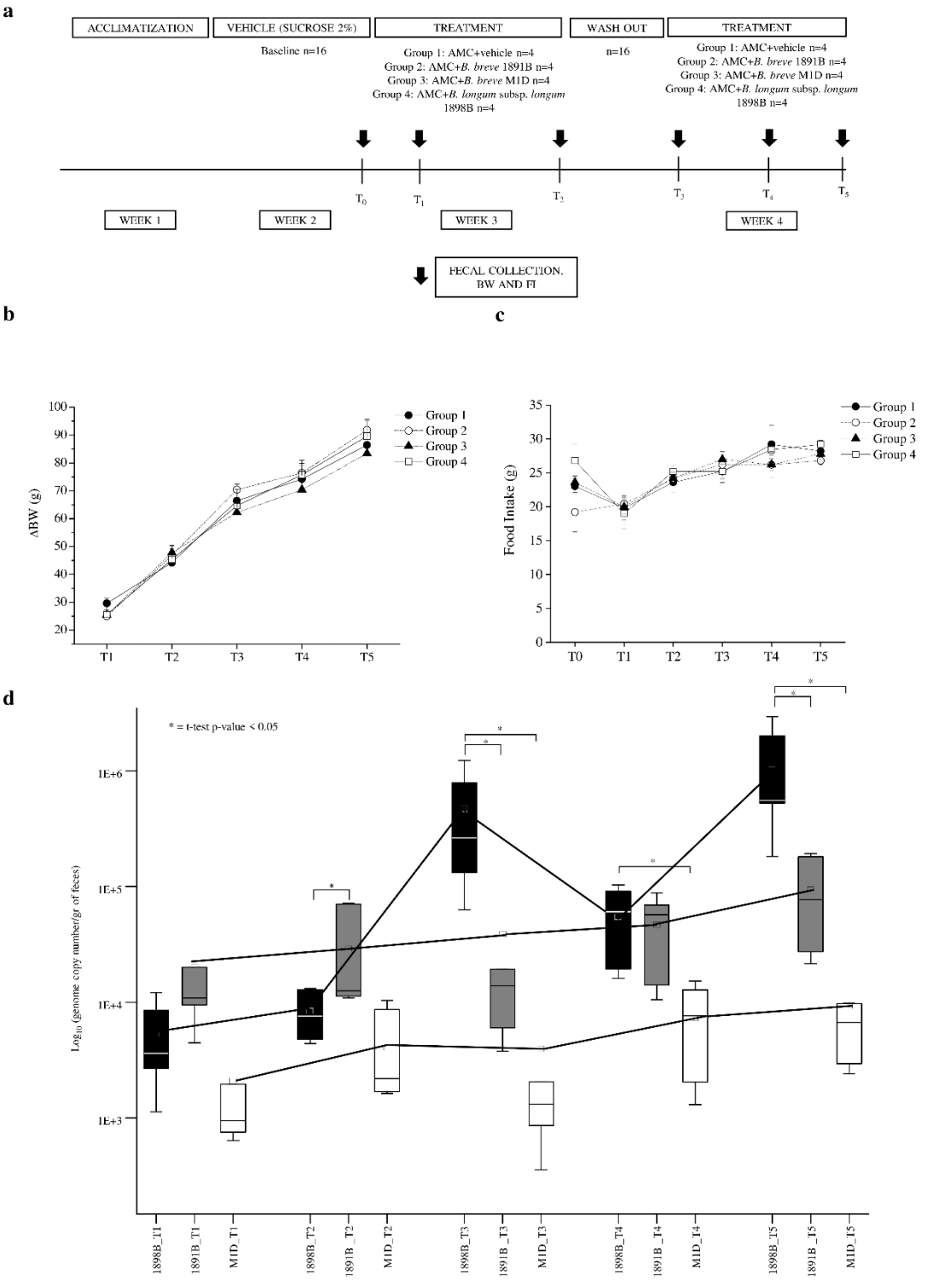
a
ACCLIMATIZATION
VEHICLE (SUCROSE 2%)
TREATMENT
WASH OUT
TREATMENT
Baseline n=16
Group 1: AMC+vehicle n=4
Group 2: AMC+B. breve 1891B n=4
Group 3: AMC+B. breve M1D n=4
Group 4: AMC+B. longum subsp. longum 1898B n=4
n=16
Group 1: AMC+vehicle n=4
Group 2: AMC+B. breve 1891B n=4
Group 3: AMC+B. breve M1D n=4
Group 4: AMC+B. longum subsp. longum 1898B n=4
T0
T1
T2
T3
T4
T5
WEEK 1
WEEK 2
WEEK 3
WEEK 4
FECAL COLLECTION, BW AND FI
b
ABW (g)
Group 1
Group 2
Group 3
Group 4
T1
T2
T3
T4
T5
c
Food Intake (g)
Group 1
Group 2
Group 3
Group 4
T0
T1
T2
T3
T4
T5
d
* = t-test p-value < 0.05
Log10 (genome copy number/gr of feces)
1E+6
1E+5
1E+4
1E+3
1898B_T1
1891B_T1
M1D_T1
1898B_T2
1891B_T2
M1D_T2
1898B_T3
1891B_T3
M1D_T3
1898B_T4
1891B_T4
M1D_T4
1898B_T5
1891B_T5
M1D_T5

Figure 1

Figure 1. Timeline and experimental results of the *in vivo* study.

Panel a shows the timeline of the experimental procedures in rats.

Panels b and c depict body weight and food intake measurements at different time points (from T0 to T5). Values are expressed as means ± SEM. Statistical results are reported in the "Supplementary Data" section.

Panel d displays qPCR evaluation of the numerical load of bifidobacterial strains in stool samples of rats. The graph reports the average abundance of *B. breve* 1891B, *B. longum* subsp. *longum* 1898B and *B. breve* M1D as calculated by qPCR at T0, T1, T2, T3, T4 and T5.

Impact of AMC-based antibiotic therapy on the gut microbiota of rats.

The gut microbiota is reported to be strongly influenced by antibiotic therapy (33, 34). In order to evaluate the impact of AMC-based antibiotic therapy in association with AMC-resistant bifidobacterial strains on rodent physiology, body weight (BW) (Fig. 1b) and food intake (FI) (Fig. 1c) parameters were measured at different time-points during the experiment for each rat. However, no statistically significant differences were found between the investigated groups (Supplementary Data). In order to evaluate the impact of AMC-based antibiotic therapy on the gut microbiota of rats when associated with administration of AMC-insensitive bifidobacterial strains, shallow shotgun metagenomics analysis on the fecal samples of each rat included in this study was performed. In detail, a total of 64 fecal samples were analyzed, resulting in a total of 5,626,619 reads, with an average of 87,916 ± 52,213 reads per sample (Table S2). Analysis of bacterial species richness indicated a significantly higher number of species in untreated control samples at T0 compared to samples from treated rats at subsequent time points (ANOVA p-value < 0.01) (Fig. 2a). In contrast, only rats treated with AMC in association with *B. breve* 1891B strain (G2 group) showed at T1 a non-significant difference from the control (Tukey HSD post-hoc p-value > 0.05). These results indicate that the presence of this strain impacts on the complexity of the microbiota. Furthermore, analysis of beta-diversity based on the Bray-Curtis dissimilarity, represented through a principal coordinates analysis (PCoA) representation, revealed three different clusters associated with each time point (PERMANOVA p-value < 0.05) (Fig. 2b). In detail, the three clusters appear to be independent of the bifidobacterial strain administered along with AMC treatment and strongly correlated with time. In particular, while T0, T3 and T5 samples grouped as three condensed independent clusters, samples at T1 comprise an heterogeneous group, indicating a severe initial impact of antibiotic treatment on the gut microbiota composition of the rats (35). In order to

evaluate the metagenomic-based microbial composition divergence between samples and the T0 group, a specific analysis based on a modified dysbiosis score (36) was performed (Fig. 2c). This analysis assessed the difference of the median Bray-Curtis dissimilarity of the T0 reference group and each treated sub-groups, i.e., G1, G2, G3 and G4, in order to detect any divergence in microbial composition. The obtained findings revealed that rats treated with AMC in association with *B. breve* 1891B strain (G2) at T1 show a less divergent microbiota compared to the reference T0 group (Fig. 2c), indicating that this strain mitigates the disruptive effect of AMC treatment on the stability of the microbiota. Intriguingly, for subsequent time points, i.e., T3 and T5, analysis indicated a lower divergence of the samples belonging to groups 3 (G3) and 4 (G4) compared to T0 reference (Fig. 2c), apparently reflecting the stabilizing influence of *B. breve* M1D and *B. longum* subsp. *longum* 1898B on the microbial composition over the course of the experiment.

In order to obtain a comprehensive biological interpretation of the analyzed fecal microbiome complexity, we performed a quantitative microbiome profiling experiment based on flow cytometric analyses for the enumeration of microbial cells present in each sample at T0, T1, T3 and T5. Interestingly, comparison of all fecal samples absolute abundance at different time points revealed a 3.1-fold decrease of microbial cells at T1 compared to T0 (ANOVA p-value < 0.01), highlighting a depletion of the bacterial community that is most likely due to AMC treatment (Fig. 2d). Conversely, samples at T3 and T5 revealed an increase of 0.4- (ANOVA p-value < 0.01) and 0.7-fold (ANOVA p-value < 0.05), respectively, of microbial cells compared to T0 samples (Fig. 2d), probably due to microbial resilience and proliferation of AMC-insensitive strains. Focusing our interest on the most abundant species representative of each fecal sample, the metagenomic analysis revealed that the fecal microbiota composition of rats at T0 was relatively

stable and was mainly characterized by species belonging to the genus *Duncaniella*, such as *Duncaniella muris* (absolute average of 6.93E+07 ± 5.59E+07, prevalence of 56 %), *Duncaniella dubosii* (absolute average of 5.72E+07 ± 3.67E+07, prevalence of 69 %) and an as yet unclassified species of *Duncaniella* (absolute average of 2.47E+08 ± 1.72E+08, prevalence of 100 %) (Table S3). Remarkably, T1 samples showed a substantial change in microbiota composition compared to T0 (Table S3), indicating an inter-specific diversity between samples that is presumed to be due to the effect of antibiotic treatment on the microbiota composition (37, 38). Conversely, analysis of fecal samples at T3 and T5 highlighted a partial restoration of bacterial taxa characteristic of those present in T0 samples, mainly represented by uncharacterized species of *Duncaniella* (Table S3). Moreover, T3 and T5 samples highlighted a homogeneous microbiota composition characterized by species belonging to *Bacteroides* genus, such as *Bacteroides congonensis* (absolute average of 5.11E+08 ± 6.85E+08, prevalence of 72 %) and *Bacteroides uniformis* (absolute average of 3.30E+08 ± 2.16E+08, prevalence of 97 %) (Table S3), suggesting a possible bacterial adaptation to AMC treatment.

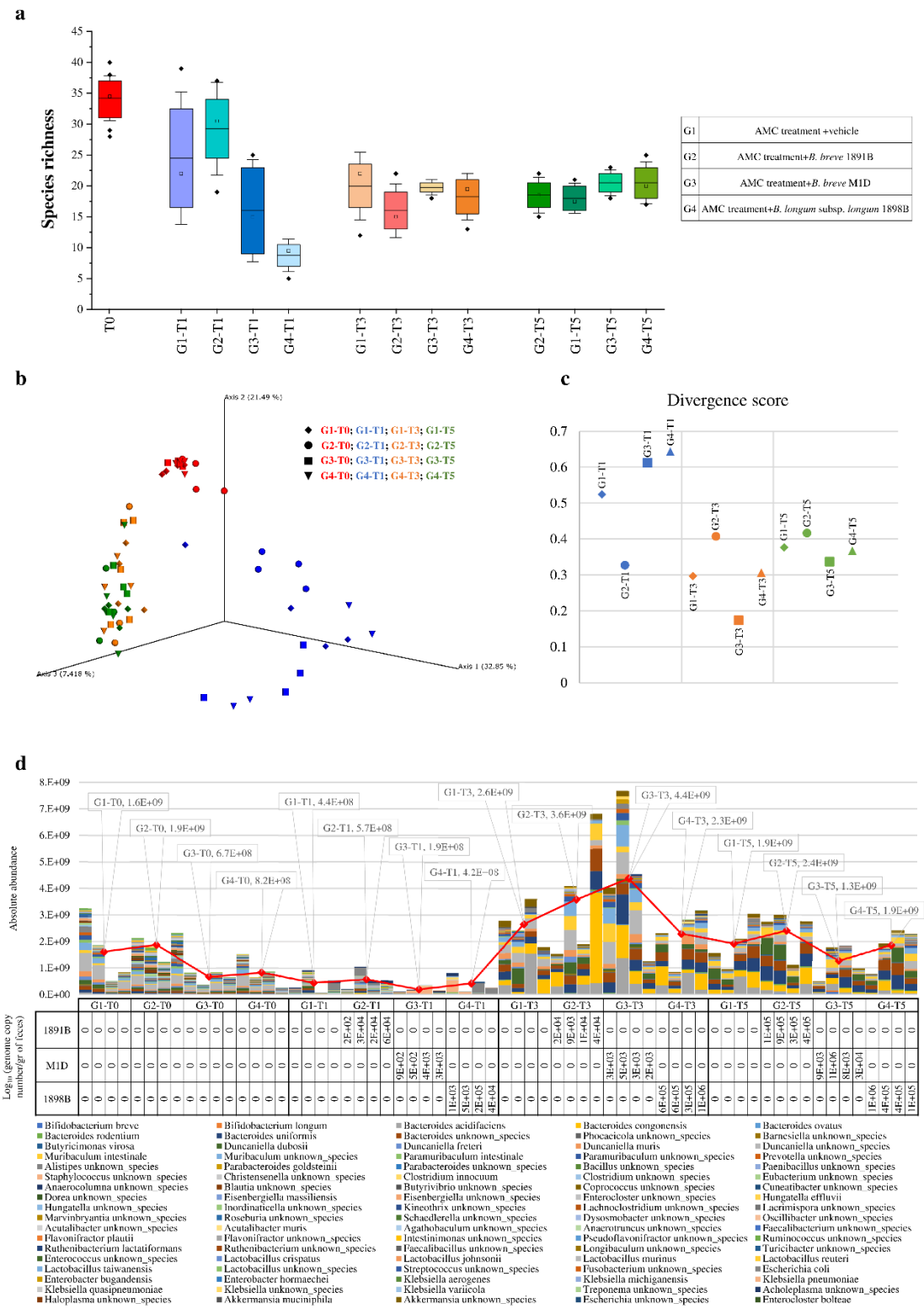
a
Species richness
G1 AMC treatment +vehicle
G2 AMC treatment+B. breve 1891B
G3 AMC treatment+B. breve M1D
G4 AMC treatment+B. longum subsp. longum 1898B
T0
G1-T1
G2-T1
G3-T1
G4-T1
G1-T3
G2-T3
G3-T3
G4-T3
G2-T5
G1-T5
G3-T5
G4-T5
b
Axis 2 (21.49 %)
Axis 1 (32.85 %)
Axis 3 (7.418 %)
G1-T0; G1-T1; G1-T3; G1-T5
G2-T0; G2-T1; G2-T3; G2-T5
G3-T0; G3-T1; G3-T3; G3-T5
G4-T0; G4-T1; G4-T3; G4-T5
c
Divergence score
d
Absolute abundance
G1-T0, 1.6E+09
G2-T0, 1.9E+09
G3-T0, 6.7E+08
G4-T0, 8.2E+08
G1-T1, 4.4E+08
G2-T1, 5.7E+08
G3-T1, 1.9E+08
G4-T1, 4.2E−08
G1-T3, 2.6E+09
G2-T3, 3.6E+09
G3-T3, 4.4E+09
G4-T3, 2.3E+09
G1-T5, 1.9E+09
G2-T5, 2.4E+09
G3-T5, 1.3E+09
G4-T5, 1.9E+09
Log10 (genome copy number/gr of feces)
1891B
M1D
1898B
Bifidobacterium breve
Bifidobacterium longum
Bacteroides acidifaciens
Bacteroides congonensis
Bacteroides ovatus
Bacteroides rodentium
Bacteroides uniformis
Bacteroides unknown_species
Phocaicola unknown_species
Barnesiella unknown_species
Butyricimonas virosa
Duncaniella dubosii
Duncaniella freteri
Duncaniella muris
Duncaniella unknown_species
Muribaculum intestinale
Muribaculum unknown_species
Paramuribaculum intestinale
Paramuribaculum unknown_species
Prevotella unknown_species
Alistipes unknown_species
Parabacteroides goldsteinii
Parabacteroides unknown_species
Bacillus unknown_species
Paenibacillus unknown_species
Staphylococcus unknown_species
Christensenella unknown_species
Clostridium innocuum
Clostridium unknown_species
Enterocloster unknown_species
Anaerocolumna unknown_species
Blautia unknown_species
Butyrivibrio unknown_species
Coprococcus unknown_species
Cuneatibacter unknown_species
Dorea unknown_species
Eisenbergiella massiliensis
Eisenbergiella unknown_species
Enterocloster unknown_species
Hungatella effluvii
Hungatella unknown_species
Inordinaticella unknown_species
Kineothrix unknown_species
Lachnoclostridium unknown_species
Lacrimispora unknown_species
Marvinbryantia unknown_species
Roseburia unknown_species
Schaedlerella unknown_species
Dysosmobacter unknown_species
Oscillibacter unknown_species
Acutalibacter unknown_species
Acutalibacter muris
Agathobaculum unknown_species
Anaerotruncus unknown_species
Faecalibacterium unknown_species
Flavonifractor plautii
Flavonifractor unknown_species
Intestinimonas unknown_species
Pseudoflavonifractor unknown_species
Ruminococcus unknown_species
Ruthenibacterium lactatiformans
Ruthenibacterium unknown_species
Faecalibacillus unknown_species
Longibaculum unknown_species
Turicibacter unknown_species
Enterococcus unknown_species
Lactobacillus crispatus
Lactobacillus johnsonii
Lactobacillus murinus
Lactobacillus reuteri
Lactobacillus taiwanensis
Lactobacillus unknown_species
Streptococcus unknown_species
Fusobacterium unknown_species
Escherichia coli
Enterobacter bugandensis
Enterobacter hormaechei
Klebsiella aerogenes
Klebsiella michiganensis
Klebsiella pneumoniae
Klebsiella quasipneumoniae
Klebsiella unknown_species
Klebsiella variicola
Treponema unknown_species
Acholeplasma unknown_species
Haloplasma unknown_species
Akkermansia muciniphila
Akkermansia unknown_species
Escherichia unknown_species
Enterocloster bolteae

Figure 2

Figure 2. Evaluation of fecal microbiota of rats at different time points.

Panel a displays the Whiskers plot reporting the species richness value for each experimental group at different time points. The x-axis represents the different time points of each group, while the y-axis indicates the number of species. The boxes are determined by the 25th and 75th percentiles. The whiskers are determined by SD (Standard Deviation). The line in the boxes represents the average, while the square represented the median. The rhombus indicates the outlier.

Panel b displays the principal coordinates analysis (PCoA) of the rat samples, subdivided by treatment group and time point.

Panel c shows the divergence in microbial composition (divergence score) calculated through the difference of the median Bray-Curtis dissimilarity between the T0 group reference and each treated sub-group.

Panel d reports the bar plots of the absolute abundance of each rat fecal sample based on the treatment group and the results of the qPCR analysis based on the bifidobacterial strains.

Covariances between AMC-insensitive bifidobacterial strains and the gut microbiota of AMC-treated rats.

In order to determine if administration of AMC-insensitive bifidobacterial strains is involved in delineating the overall taxonomic composition of the rat fecal microbiota, we performed a covariance analysis through Spearman's rho coefficient. In detail, we evaluated the presence of AMC-insensitive bifidobacterial strains in the rodent fecal samples through a qPCR approach to overcome our failure to detect these strains by shallow shotgun metagenomic analysis (presumed to be due to their abundance being below the detection limit of this metagenomic technique). Thus, we correlated the absolute abundance observed for all taxa and qPCR results, revealing a variable impact of AMC-insensitive bifidobacterial strains on the gut microbiota composition of the experimental rats. In detail, *B. breve* 1891B revealed five positive and one negative correlation with other bacteria, suggesting a rather modest interaction of this strain with other gut microbiota members. Conversely, correlation analysis showed that *B. breve* M1D and *B. longum* subsp. *longum* 1898B elicit the highest ability to negatively impact the presence of other bacteria, as indicated by negative correlations (p-value <0.05) with 16 % of the taxa identified by the analysis (Table 1). Moreover, *B. breve* M1D positively correlates with 13 % of the species included in the analysis and seems to promote the presence of species belonging to *Enterobacter*, *Klebsiella,* and *Clostridium* genera (Table 1), which may contribute to intestinal dysbiosis (14, 39).

Table 1. Covariance analysis between AMC-insensitive bifidobacterial strains and fecal samples microbiota calculated through Spearman's rho coefficient. Only significant correlations are reported. In detail, the dark-grey color highlighted significant negative correlations, while the light-grey color highlighted significant positive correlations.

Phylum	Species	1898B	M1D	1891B
Actinobacteria	*Bifidobacterium longum*	-0.086	0.436	-0.086
Actinobacteria	*Bifidobacterium breve*	-0.123	0.078	0.371
Bacteroidetes	*Bacteroides ovatus*	0.251	-0.078	0.145
Bacteroidetes	*Bacteroides rodentium*	0.142	0.060	0.272
Bacteroidetes	*Duncaniella freteri*	-0.316	-0.089	-0.120
Bacteroidetes	*Paramuribaculum intestinale*	-0.356	-0.001	-0.312
Bacteroidetes	*Prevotella unknown*_species	-0.248	0.052	-0.137
Firmicutes	*Clostridium innocuum*	0.382	0.289	-0.204
Firmicutes	*Eubacterium unknown*_species	-0.286	-0.281	-0.173
Firmicutes	*Blautia unknown*_species	-0.217	-0.297	-0.210
Firmicutes	*Eisenbergiella unknown*_species	-0.287	-0.356	-0.113
Firmicutes	*Enterocloster bolteae*	0.315	-0.060	-0.060
Firmicutes	*Enterocloster unknown*_species	-0.269	-0.226	-0.158
Firmicutes	*Hungatella unknown*_species	-0.293	-0.293	-0.076
Firmicutes	*Inordinaticella unknown*_species	-0.060	0.255	-0.060
Firmicutes	*Kineothrix unknown*_species	-0.293	-0.293	-0.157
Firmicutes	*Marvinbryantia unknown*_species	-0.25	-0.25	-0.083
Firmicutes	*Roseburia unknown*_species	-0.346	-0.292	-0.161
Firmicutes	*Schaedlerella unknown*_species	-0.265	-0.346	-0.170
Firmicutes	*Dysosmobacter unknown*_species	-0.226	-0.314	-0.099
Firmicutes	*Acutalibacter unknown*_species	-0.282	-0.282	-0.059
Firmicutes	*Flavonifractor unknown*_species	-0.253	-0.346	-0.070
Firmicutes	*Intestinimonas unknown*_species	-0.253	-0.346	-0.069
Firmicutes	*Pseudoflavonifractor unknown*_species	-0.193	-0.282	-0.116
Firmicutes	*Ruminococcus unknown*_species	-0.215	-0.318	-0.206
Firmicutes	*Ruthenibacterium unknown*_species	-0.060	-0.060	0.275

Firmicutes	*Longibaculum unknown*_species	-0.060	0.255	-0.060
Firmicutes	*Lactobacillus crispatus*	-0.060	-0.060	0.275
Firmicutes	*Lactobacillus taiwanensis*	-0.106	-0.106	0.253
Proteobacteria	*Escherichia unknown*_species	0.336	-0.086	-0.086
Proteobacteria	*Enterobacter bugandensis*	-0.060	0.265	-0.060
Proteobacteria	*Enterobacter hormaechei*	-0.153	0.373	0.000
Proteobacteria	*Klebsiella aerogenes*	-0.060	0.265	-0.060
Proteobacteria	*Klebsiella pneumoniae*	0.072	0.269	-0.148
Proteobacteria	*Klebsiella quasipneumoniae*	0.115	0.258	-0.139
Proteobacteria	*Klebsiella variicola*	0.115	0.258	-0.139
Tenericutes	*Haloplasma unknown*_species	-0.060	0.255	-0.060

Conclusions

Antimicrobial agents, including antibiotics, are known to influence the composition and the complexity of the human gut microbiota. One of the most frequently recommended antibiotics during infancy and adolescence is the combination of amoxicillin and clavulanic acid (AMC-antibiotics), with consequent major effects in reducing the complexity of the gut microbiota composition and thus promoting the development of gut dysbiosis. Our hypothesis is that the reinforcement of the gut microbiota during AMC treatment with naturally AMC-insensitive strains prevents or reverses dysbiosis and/or re-establish the natural resilience of the gut microbiota. This notion was verified here using three AMC-insensitive bifidobacterial strains, i.e., *Bifidobacterium breve* 1891B, *B. breve* M1D and *B. longum* subsp. *longum* 1898B, by assessing these strains employing *in vitro* and *in vivo* models. In this context, interesting effects pertaining to the recovery of the original microbial diversity were observed for all these strains, even if with different and strain-specific consequences. The biological explanation of these important ecological effects may be linked to the abilities of these bifidobacterial strains in supporting growth of other members of the gut microbiota by establishing mutualistic trophic interactions such as those previously observed for various bifidobacterial strains (40-45). Several metagenomic-based studies have reported the establishment of positive correlations between bifidobacteria and the other members of the gut microbiota, which are ultimately important to promote homeostasis of the microbial communities (3, 46-48). Recent studies have reported the positive effect of the occurrence of members of some bifidobacterial species, such as *B. breve* and *B. longum*, in modulating the transcriptome of other members of the gut microbiota (24). Therefore, correlations between different bacterial species may explain the positive ecological effects of bifidobacteria in driving the

establishment of the early life and in recovering the natural resilience of the gut microbiota during and following antibiotic treatment.

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Chapter 6

Contribution of the capsular polysaccharide layer to antibiotic resistance in bifidobacteria

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Abstract

Bifidobacteria have been shown to produce exopolysaccharides (EPS), which are polymeric structures composed of various carbohydrates, commonly containing glucose, galactose and rhamnose. EPS are produced by different bifidobacterial taxa commonly identified in the human gut, such as *Bifidobacterium breve* and *Bifidobacterium longum* subsp. *longum*, and have been suggested to modulate the interaction of bifidobacterial cells with other members of the human gut microbiota as well as with their host. In this study, we evaluated if bifidobacterial EPS production of four selected EPS-producing strains is associated with enhanced resistance to antibiotic treatments through MIC analysis when compared to bacterial cultures that do not produce exopolysaccharides. Our results showed that an increase in EPS production by modifying the growth medium with different carbon sources, i.e., glucose, galactose or lactose and/or by applying stressful conditions, such as bile salts and acidity, is associated with a tolerance enhancement of bifidobacterial cells toward various beta-lactam antibiotics. In addition, after analyzing the production of EPS at the phenotypic level, we explored the genes involved in the production of these structures and evaluated their expression, in presence of various carbon sources, using RNAseq. Overall, this study provides preliminary experimental evidence showing how bifidobacterial EPS modifies the level of susceptibility of these bacteria towards antibiotics.

Introduction

The ability to produce extracellular polysaccharides is wide-spread among bacteria, these polymers include capsular polysaccharides known as exopolysaccharides (EPS), which are loosely attached to the cell surface and may also be secreted into the environment (Ruas-Madiedo & de los Reyes-Gavilan, 2005). EPS can be composed of a single type of saccharide subunit (homopolysaccharides) or of different carbohydrates (heteropolysaccharides), and bifidobacterial EPS typically belong to the latter category, consisting of different types of monosaccharides, e.g., D-glucose, D-galactose, and L-rhamnose (Hidalgo-Cantabrana*, et al.*, 2014). In the gastrointestinal environment human gut commensals like bifidobacteria are exposed to different stressful conditions such as hypertonic and low pH environments to which these microorganisms need to adapt. Intestinal microorganisms have developed various strategies to counteract these harsh conditions, one of which could be the production of EPS in order to create a physical barrier that protects the bacterial cell against these stresses (Ruas-Madiedo*, et al.*, 2009, Salazar*, et al.*, 2009, Castro-Bravo*, et al.*, 2018, Kelly*, et al.*, 2020). In addition, these molecules are involved in host-microbiota interactions (Alp & Aslim, 2010, Fanning*, et al.*, 2012) and have been reported to elicit beneficial effects on human health, such as cholesterol lowering ability (Pigeon*, et al.*, 2002), immunomodulatory and anticancer activities (Kitazawa*, et al.*, 1998), and modulation of the human gut microbiota composition through their prebiotic activity (Fernandez-Julia, 2022) (Bello*, et al.*, 2001, Korakli*, et al.*, 2002). A bifidobacterial *eps* locus encompasses genes encoding the biosynthetic machinery responsible for EPS subunit production, transport, and polymerization. Previously, the existence of nine distinct types of bifidobacterial *eps* loci was described, exhibiting considerable variability in number and predicted functions of the *eps* genes (Ferrario*, et al.*, 2016). It has been shown that the capsule (Strain*, et al.*, 2022)

of *Acinetobacter baumanii,* which represents a virulence factor of this microorganism, is involved in increased resistance to antimicrobial agents, including antibiotics (Geisinger & Isberg, 2015) and this prompted us to evaluate whether antibiotic tolerance of bifidobacteria is somehow influenced by EPS production (Geisinger & Isberg, 2015). Here, we evaluated how different growth conditions may affect the susceptibility of bifidobacterial strains toward different antibiotics (Linehan, 2022) and stresses through modulation of EPS production. We focused on human bacterial species that commonly occur in the infant gut (Turroni*, et al.*, 2021), in particular *Bifidobacterium bifidum*, *Bifidobacterium breve* and *Bifidobacterium longum*. These latter microbes have been known to be resistant to antibiotics such as amoxicillin and clavulanic acid (Mancabelli*, et al.*, 2021). EPS production could therefore play a defining role in the resilience of the infant gut microbiota following antibiotic treatment. For these reasons, trials involving both human-derived, EPS-producing bifidobacterial strains as well as strains that do not produce EPS, were executed, and the molecular effects of antibiotics on the production of exopolysaccharides were monitored by RNAseq experiments.

Materials and methods

Strains and cultivation conditions.

Bifidobacterium strains used in this study are *Bifidobacterium longum* subsp. *longum* 1898B, *Bifidobacterium breve* M1D, *B. longum* subsp. *longum* 39B (Mancabelli*, et al.*, 2021), *B. breve* UCC2003, *B. breve* UCC2003 EPS::Bbr_0430 (*B. breve* UCC2003 EPS::Bbr_0430 is a derivative of UCC2003 in which a non-replicating plasmid with a fragment of the target gene (Bbr_0430) and the *tet* gene is inserted in the Bbr_0439 gene of the chromosome causing its disruption and its inability to produce EPS; this strain therefore does not harbour a functional plasmid and is resistant to tetracycline) (Fanning*, et al.*, 2012), *B. breve* LMG 13208 and *Bifidobacterium bifidum* PRL2010 (Table 1). Strains were grown anaerobically in De Man, Rogosa, Sharpe (MRS) medium (Scharlau) supplemented with 0.05% L-cysteine-HCl and incubated at 37°C for 24 h. Media were supplemented with 10 µg/mL of tetracycline where relevant. Anaerobic conditions were achieved using an anaerobic cabinet (Ruskin), in which the atmosphere consisted of 17.01% CO_2, 80% N_2, and 2.99% H_2. Growth experiments using different carbon sources such as galactose and lactose were carried out in a modified MRS without glucose (w/o glu), with a final sugar concentration of 2% (wt/vol). Growth experiments employing different environmental conditions, i.e., pH and bile salts, were performed in MRS with the addition of HCl to obtain pH 5 and 0.1% (wt/vol) or 0.05% (wt/vol) of bovine bile (bovine bile; Sigma B3883), respectively. For phenotypic characterization of EPS-producing strains, ruthenium red-milk (RRM) plates were prepared as previously described (Mora*, et al.*, 2002). Another qualitative evaluation for EPS-producing strains was performed by growing bifidobacteria 24h in MRS, tubes were vortexed and left overnight without stirring. Subsequently, OD_{600nm} of the upper part of the cultivation tube, i.e., the supernatant,

was measured using a spectrophotometer (Eppendorf, Germany). The experiment was conducted in triplicate.

Antibiotic susceptibility assays.

The MIC breakpoints (micrograms per milliliter) of antibiotic were determined using the broth microdilution method (MDIL) according to the established EFSA rules. Antibiotics tested were ampicillin, vancomycin, gentamicin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol, amoxicillin and amoxicillin-clavulanic acid (AMC) (Sigma-Aldrich, US). Microplates were incubated under anaerobic conditions for 48 h at 37°C. Cell density was monitored by optical density measurements at 600nm (OD_{600nm}) using a plate reader (BioTek, VT, USA). The MIC breakpoint represents the highest concentration of a given antibiotic to which a particular bacterial strain was shown to be resistant. The MIC breakpoint determinations were performed in triplicate.

***In silico* analysis of *eps* loci in *B. longum* strains.**

Screening for *eps* loci in *B. longum* 1898B, and *B. longum* 39B was performed through homology searches using BLASTP analyses (cutoff E-value of $<1 \times 10^{-5}$ and 50% identity over at least 80% of sequence coverage). The predicted *eps* loci were manually inspected through Artemis Informatics Software (Welcome Trust Sanger Institute). Functional annotation of *eps*-encoding genes was performed using the HHpred server, which allows to search multiple databases, such as the PDB, SCOP, Pfam, SMART, COGs and CDD (https://toolkit.tuebingen.mpg.de/tools/hhpred) (Soding*, et al.*, 2005). TM finder (http://tmfinder.research.sickkids.ca/cgi-bin/TMFinderForm.cgi) was used to predict transmembrane regions present in queried proteins.

Growth assays performed at different pH values and bile salt concentrations.

B. longum 1898B, *B. breve* M1D, *B. longum* 39B and *B. breve* UCC2003 were cultivated on semisynthetic MRS medium supplemented with different concentrations (wt/vol) of bile salts, i.e., 2%, 1%, 0.5%, 0.45%, 0.4%, 0.35%, 0.3%, 0.25%, 0.2%, 0.15%, 0.1%, 0.05%, 0.04%, 0.03% and 0.02% to simulate bile stress (being also representative of osmotic and oxidative stress). Moreover, the same strains were cultivated on MRS medium (whose pH correspond to 6.2±0.2) at different pH values, i.e., pH of 6.8, 6, 5.5, 5, 4.5, 4 and 3.5 to impose acid stress. The optical densities (OD_{600nm}) were recorded using a plate reader (BioTek, Winooski, VT, USA). Optical densities were read in intermittent mode, with absorbance readings performed at 3-min intervals for three times after 48h of growth, where each reading was preceded by 30 s of shaking at medium speed. Cultures were grown as biologically independent triplicates, and the resulting growth data were expressed as the means of these replicates.

Growth of *B. longum* 1898B on galactose and sub-MIC AMC and RNA-Seq analysis.

Cells were inoculated with an initial OD_{600nm} of 0.1 (bacterial cell counts standardized to 2×10^7 CFU/mL). Following inoculation, growth was monitored and at an OD_{600nm} between 0.6 and 0.8 (bacterial cell counts of each strain correspond to $3.00\pm0.5 \times 10^8$ CFU/mL) (exponential phase) cells were harvested by centrifugation at 6000 rpm for 5 min. Growth assays were carried out in triplicate. Total RNA (Berkhout, 2022) was isolated from cultures grown in the presence of sublethal concentration (sub-MIC), i.e., 4 μg/mL of antibiotic (AMC) in MRS w/o glucose at 2% (wt/vol) of galactose or MRS w/o glucose at 2% (wt/vol) of galactose (reference condition) and in MRS (reference condition for glucose) using previously described methods (Turroni*, et al.*, 2010). Briefly, cell pellets were

resuspended in 1 ml of QUIAZOL (Qiagen, United Kingdom) and placed in a tube containing 0.8 g of glass beads (diameter, 106 μm; Sigma). The cells were lysed by shaking the mix on a BioSpec homogenizer at 4°C for 2 min (maximum setting). The mixture was then centrifuged at 12,000 rpm for 15 min, and the RNA-containing sample was recovered from the upper phase. The RNA sample was further purified by using the RNeasy mini kit (Qiagen) as reported in the manufacturer's instructions, including DNase treatment in order to remove all genomic DNA. The quality of the RNA was verified employing a Tape station 2200 (Agilent Technologies, USA). RNA amount was evaluated using a spectrophotometer (Eppendorf, Germany). For RNA sequencing (RNA-Seq), 200 ng of extracted RNA of each sample was treated to remove rRNA by using the QIAseq FastSelect – 5S/16S/23S following the manufacturer's instructions (Qiagen, Germany). The yield of rRNA depletion was checked by employing a Tape station 2200 (Agilent Technologies, USA). Subsequently, a transcriptome library was constructed using the TruSeq Standard mRNA Sample preparation kit (Illumina, San Diego, USA). Samples were processed by a NextSeq high-output v2.5 kit (150 cycles, single end) (Illumina) according to the technical support guide. The experiment was conducted employing biologically independent triplicates. Demultiplexed reads were quality filtered (with overall quality and length filters) (Table S8) and aligned to the genome sequence of *B. longum* 1898B (with predicted ORFs) using the Bowtie2 software (with option: --very-sensitive --score-min C, -13,0) (Langmead & Salzberg, 2012), while quantification of reads overlapping *B. longum* genes was achieved using the htseq-count script of HTSeq software (Anders*, et al.*, 2015). For differential expression analyses, the TMM (Trimmed Mean of M-Values) method implemented in the EdgeR package (https://bioconductor.org/packages/release/bioc/html/edgeR.html) was applied at each sample to account for differences in library sizes and RNA composition. Read

counts normalized by the effective (adjusted) library size were obtained using Counts Per million (CPM) and differences in the *eps*-encoding gene expression level between different experimental conditions was evaluated as $\log_2$ fold change (logFC). Statistical significance of differences in read counts between the tested conditions was assessed through the exact test of EdgeR package and FDR correction (Benjamini-Hochberg, cut-off p-value = 0.05).

Statistical analysis.

All statistical analyses were performed with the software SPSS v. 25 (www.ibm.com/software/it/analytics/spss/). In differential gene expression analysis, EdgeR package was used to normalize read counts (TMM method) and to calculate fold changes (log2FC). Adjust significance threshold according to the FDR (Benjamini & Hochberg) method were obtained through the p.adjust function in R.

Data availability.

All RNAseq raw data from this study are accessible through SRA under study accession number PRJNA864568.

Results.

Potential involvement of exopolysaccharides in antibiotic resistance in bifidobacteria.

B. longum subsp. *longum* CRC002 has previously been reported to modulate the amount of EPS produced depending on the available carbon source in the growth medium (Audy*, et al.*, 2010). Therefore, in order to evaluate a possible impact of EPS production on antibiotic susceptibility, we cultivated different bifidobacterial strains (Table 1) in the presence of various antibiotics (see below) on different

carbon sources, i.e., glucose, galactose and lactose (Audy*, et al.*, 2010), then the tolerance toward antibiotics was assayed through MIC analyses (Hidalgo-Cantabrana*, et al.*, 2014). We decided to use these sugars because they represent those carbohydrates constituting the EPS and therefore probably used by EPS-producing microorganisms for EPS biosynthesis. Susceptibility to different antibiotics (i.e., ampicillin, vancomycin, gentamicin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol, amoxicillin and amoxicillin-clavulanic acid) was assessed for *B. longum* 1898B, *B. breve* M1D, *B. longum* 39B and *B. breve* UCC2003 (Table 1) when cultivated in the presence of various carbon sources. Evaluation of the antibiotic susceptibility of UCC2003 grown in the presence of galactose at 2% (wt/vol) revealed a beta-lactam MIC increase ranging from two to 4-fold when compared to the MIC value when the strain had been cultivated in the presence of glucose as the sole carbon source (Fig. 1 and Table S1). When such tests were performed using the EPS-negative mutant of UCC2003 strain, i.e., UCC2003::Bbr_0430 (Fanning*, et al.*, 2012), no increase for AMC and amoxicillin MIC values respect to the reference condition (glucose) were observed (Fig. 1 and Table S1), thus suggesting that EPS production is involved in this variable antibiotic sensitivity phenotype. Some of the assessed bifidobacterial strains such as *B. breve* M1D, *B. bifidum* PRL2010 and *B. breve* LMG 13208 did not show any changes in the MIC values irrespective of the carbohydrate present in the growth medium, in fact these strains have previously been described not to produce EPS (Ferrario*, et al.*, 2016). It is known that the amount of EPS produced by bifidobacterial strains differs between sugar substrates present in the media (Audy*, et al.*, 2010). These results therefore highlight how some strains, unable to produce exopolysaccharides, do not use the carbon sources present in the media to build their EPS. In contrast, though in agreement with the findings for *B. breve* UCC2003, in the case of strain 1898B challenged with amoxicillin-clavulanic acid

it was observed that the MIC (Fig. 1 and Table S1) values were doubled compared to the reference condition (i.e., cultivated on glucose) when this strain was grown in galactose as the sole carbon source. Similarly, the MIC values for ampicillin were shown to increase two-fold when 1898B cells were cultivated on lactose. Interestingly, both AMC and ampicillin are part of the class of beta-lactams that target the bacterial wall of Gram-positive bacteria (Yocum*, et al.*, 1980, Huttner*, et al.*, 2020). Similar results were found for *B. longum* 39B (Fig. 1 and Table S1), suggesting that this representative of the *B. longum* species varies its tolerance toward AMC and ampicillin by the presence of specific carbon sources, perhaps due to the presence of varying levels of EPS as a protective layer around the bifidobacterial cell envelope. Clindamycin, tetracycline and chloramphenicol are involved in inhibition of protein synthesis, their MIC values were higher compared to those observed for the control when 1898B cells were grown in the presence of galactose as the only carbon source (Fig. S1 and Table S1). It will be relevant to keep in mind that the cut-off MIC values as defined by EFSA do not take into account on what carbohydrate strains are cultivated.

Table 1. Strains used in this study.

Species	Strain	Reference	*eps* locus/loci[a]	Phenotypic EPS evidence: Cell growth[b]	Phenotypic EPS evidence: Growth on RRM[c]
Bifidobacterium longum subsp. *longum*	1898B	(Mancabelli, *et al.*, 2021)	+	+	W
Bifidobacterium longum subsp. *longum*	39B	(Mancabelli, *et al.*, 2021)	+	+	W
Bifidobacterium breve	M1D	(Mancabelli, *et al.*, 2021)	-	-	T
Bifidobacterium breve	UCC2003	(Fanning, *et al.*, 2012)	+	+	W
Bifidobacterium breve	UCC2003::Bbr_0430	(Fanning, *et al.*, 2012)	-	-	T
Bifidobacterium breve	LMG 13208	(Ferrario, *et al.*, 2016)	+	-	P
Bifidobacterium bifidum	PRL2010	(Ferrario, *et al.*, 2016)	-	-	T

[a] + presence of *eps* locus/loci, - absence of *eps* locus/loci; [b] values were assigned as follows: + planktonic growth, - cells sediment during growth; [c] colony aspect: W: white, P: pink, T: transparent.


Amoxicillin-clavulanic acid
UCC2003 MRS
UCC2003 GAL
UCC2003 LAC
UCC2003::Bbr_030 MRS
UCC2003::Bbr_030 GAL
UCC2003::Bbr_030 LAC
0.25
1
1
0.5
0.5
0.5
0
0.5
1
1.5
2
Amoxicillin
UCC2003 MRS
UCC2003 GAL
UCC2003 LAC
UCC2003::Bbr_030 MRS
UCC2003::Bbr_030 GAL
UCC2003::Bbr_030 LAC
0.5
1
0.5
0.5
0.5
0.5
0
0.5
1
1.5
2
Ampicillin
UCC2003 MRS
UCC2003 GAL
UCC2003 LAC
UCC2003::Bbr_030 MRS
UCC2003::Bbr_030 GAL
UCC2003::Bbr_030 LAC
0.5
1
1
0.25
0.5
0.25
0
0.5
1
1.5
2
Amoxicillin-clavulanic acid
1898B MRS
1898B GAL
1898B LAC
39B MRS
39B GAL
39B LAC
M1D MRS
M1D GAL
M1D LAC
8
16
8
0.5
1
1
16
16
16
0
2
4
6
8
10
12
14
16
Amoxicillin
1898B MRS
1898B GAL
1898B LAC
39B MRS
39B GAL
39B LAC
M1D MRS
M1D GAL
M1D LAC
8
8
8
1
1
1
16
16
16
0
2
4
6
8
10
12
14
16
Ampicillin
1898B MRS
1898B GAL
1898B LAC
39B MRS
39B GAL
39B LAC
M1D MRS
M1D GAL
M1D LAC
4
8
8
0.5
1
0.5
8
8
8
0
1
2
3
4
5
6
7
8

Figure 1

Figure 1. Evaluation of antibiotic resistance. Bar chart indicating Minimum Inhibitory Concentration (MIC) values for beta-lactams antibiotics of *B. breve* UCC2003, *B. breve* UCC2003::Bbr_0430 *B. longum* 39B, *B. longum* 1898B and *B. breve* M1D cultivated with different carbon sources. The red dashed line indicates the cut-off value defined by EFSA. On the x axis is reported the MIC value (μg/mL).

Evaluation of bifidobacterial EPS production.

A phenotypic analysis for EPS-producing strains was achieved using a previously described approach commonly applied for the detection of EPS producing bifidobacteria, involving the ruthenium-red milk (RMM) agar plates (Ruas-Madiedo & de los Reyes-Gavilan, 2005) (Table 1). Both *B. longum* strains, i.e., 39B and 1898B and *B. breve* UCC2003 display a white mucoid phenotype, indicative of EPS production. This phenotype is in contrast to the non-EPS producing strains and the mutant UCC2003 EPS::Bbr_0430 that produce non-mucoid transparent colonies. In detail, for EPS-producing strains, the presence of the polymer prevents the uptake of the ruthenium stain and colonies appear white and with a mucoid aspect. On the other hand, ruthenium stains the bacterial cell wall, producing pink colonies for non-EPS producing strains (Geisinger & Isberg, 2015). We used both negative and positive control, included *B. bifidum* PRL2010 that is not predicted to encompass *eps* genes in its genome and *B. reuteri* DSM 23975 that showed a mucoid white phenotype in previous observations (Geisinger & Isberg, 2015). Another phenotypic evaluation for EPS-producing strains was performed by the evaluation of the cell sedimentation rate of the growth-supernatant of culture, the supernatant turbidity was checked reading OD_{600nm} values using a spectrophotometer, which is an approach that was previously applied for the discrimination between bifidobacterial EPS-producer strains vs. non-producer strains (Ferrario*, et al.*, 2016). EPS-producing strains sediment less fast and this phenotype is consequently associated with a higher OD_{600nm} value of the growth medium when compared to non-producing strains, which sediment faster and is correlated with low OD_{600nm} value of the growth medium. When we assayed bifidobacterial strains (Table 1) with this method, *B. longum* 1898B, *B. longum* 39B and *B. breve* UCC2003 display a high OD_{600nm} value, i.e., average of 3.04 (Table S2), clearly indicative of EPS-production. On the other hand, *B. breve* M1D, *B.*

breve UCC2003::Bbr_0430, *B. bifidum* PRL2010 and *B. breve* LMG 13208 display low OD_{600nm} values, i.e., average of 1.02 (Table S2), suggesting that these strains are unable to produce EPS (Fanning*, et al.*, 2012). Notably, such phenotypic profiling approaches for the production of EPS provided consistent results, which were further validated by analyzing the genetic determinants of EPS production in the genomes of the same set of bifidobacterial strains.

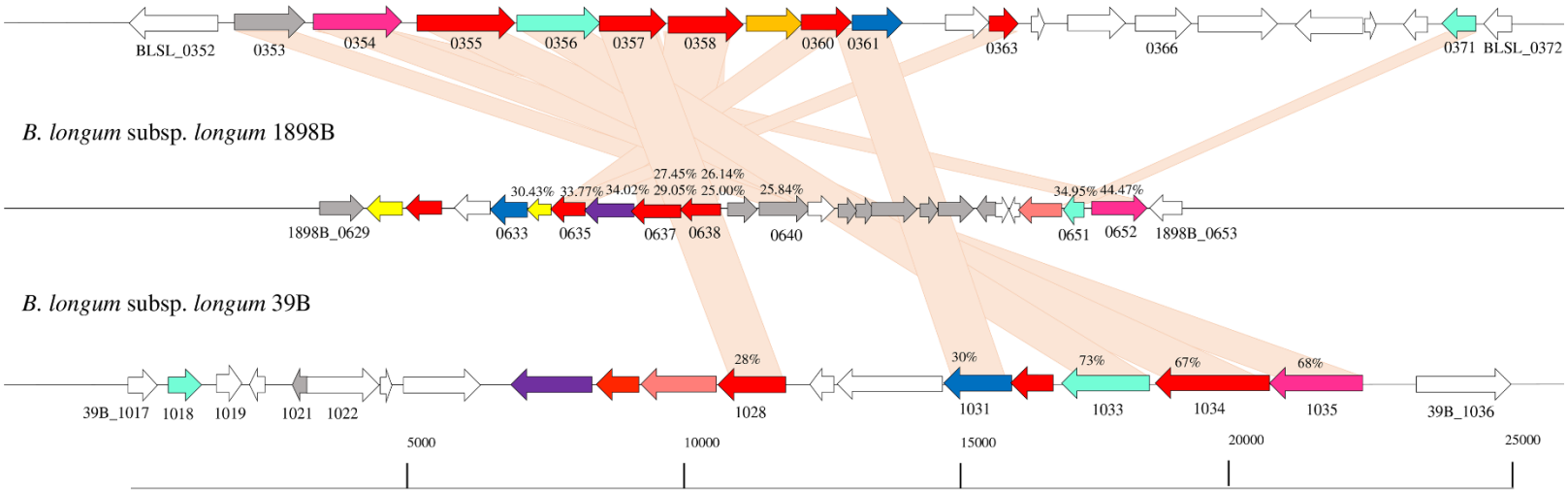
Identification of *eps* loci in bifidobacterial genomes.

The genome sequences of all bifidobacterial strains employed in our study (Table 1) were subjected to *in silico* analyses in order to identify possible *eps* encoding loci in their genome sequences. Such *in silico* analyses, which consisted of a BLASTp approach based on the annotated and conserved *eps*7 locus of *B. longum* subsp. *longum* LMG 13197 (BLSL 0353 to BLSL 0371) (Ferrario*, et al.*, 2016), revealed the occurrence of an homologous *eps* locus in strains *B. longum* 1898B and *B. longum* 39B, whereas no identifiable *eps* locus was detected in *B. bifidum* PRL2010 and *B. breve* M1D strains (Ferrario*, et al.*, 2016) (Fig. 2). Comparative genomics attempts highlighted that the various *eps* loci identified in the analyzed bifidobacterial chromosomes displayed genetic similarity restricted to a small number of genes such as those predicted to encode the priming glycosyltransferase (pGTase), epimerases and glycosyltransferases (Fig. 2a and Table S3). Indeed, locus organization varies considerably between strains, reinforcing the notion that genetic determinants for EPS production in bifidobacteria are strain-specific, while it also indicates that these clusters have been acquired by horizontal gene transfer (Ferrario*, et al.*, 2016). Notably, when the genetic data concerning EPS production were correlated with the phenotypic findings involving the antibiotic susceptibility of bifidobacterial strains, we find that those that are genetically equipped to produce EPS display the typical phenotype of an EPS-producer microorganism, i.e., the

mucoid phenotype and a low sedimentation rate of the culture, suggesting that these bifidobacterial strains do indeed produce EPS. The *in silico* analyses revealed that the genome of *B. longum* 1898B harbors a truly unique EPS encoding locus, encompassing a 25.3 kb region that contains 31 genes whose functional prediction was achieved through HHpred software (Soding*, et al.*, 2005) (Table S4), including genes predicted to encode various glycosyltransferases (1898B_1182, 1898B_1190, 1898B_1196, 1898B_1199, 1898B_1200, 1898B_1202, 1898B_1203, 1898B_1204, 1898B_1205), the pGtase (1898B_1185), acyltransferase (1898B_1181, 1898B_1192, 1898B_1201) and flippase or ABC transporters (1898B_1179, 1898B_1187) (Ferrario*, et al.*, 2016) (Fig. 2b and Table S4). Moreover, the deviation in GC content compared to the genome average (Table S4) suggests that the genes encompassing the *eps* locus were acquired by horizontal gene transfer, as has also been proposed for other EPS-encoding loci (Bourgoin*, et al.*, 1999).

a)

B. longum subsp. *longum* LMG 13197

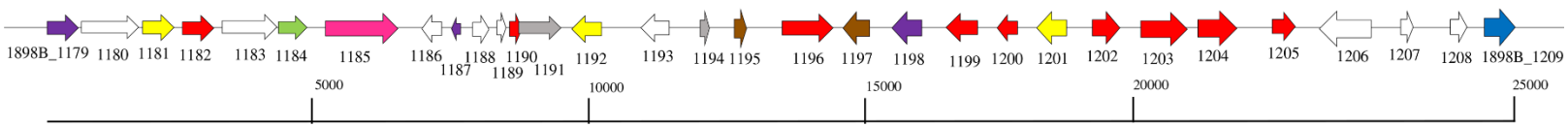


B. longum subsp. *longum* 1898B

B. longum subsp. *longum* 39B

b)

B. longum subsp. *longum* 1898B



	Epimerase		Glycosylhydrolase
	pGtase		Flippase or ABC transporter
	Glycosyltransferase		Cell wall binding protein
	Transposase		Other functions
	Acyltransferase		Tyrosine phosphatase or kinase
	Polymerase		Polysaccharide biosynthesis/chain length determination

Figure 2

Figure 2. The genetic of EPS production of bifidobacteria. Panel a) displays a schematic comparative representation of *eps*-encoding loci of various bifidobacterial strains. Each arrow indicates an ORF, the size of which is proportional to the length of the arrow. Coloring of the arrows represents the different function of the gene as indicated in Table S3 in the supplemental material. Related proteins are linked by pink shading (<80%) according to amino acid similarities; percent amino acid identities are shown. Panel b) represents the unique *eps*-locus of *B. longum* 1898B. The legend below shows the color code of the arrows associated with genes function.

Modification of EPS production of bifidobacteria upon application of stressful conditions and associated change in antibiotic susceptibility.

Considering that EPS production could contribute to cell protection and survival, we investigated the potential relationships between bifidobacterial EPS formation and environmental stresses, i.e., bile salts and acidity. We did this by evaluating the growth performance of *B. longum* strains i.e., 1898B and 39B and *B. breve* strains, i.e., *B. breve* M1D and *B. breve* UCC2003 (Table 1) on different amounts of bile salts and exposure to different pH values. When *B. longum* 1898B and *B. breve* UCC2003 were grown in the presence of 0.1% (wt/vol) bovine bile salts, they exhibited a higher OD_{600nm}, i.e., average value of 0.786, compared to *B. longum* 39B and *B. breve* M1D, i.e., average value of 0.269 (Table S5). The latter two strains were inhibited in their growth in the presence of amounts of bile salts greater than 0.05% (wt/vol) (Student t-test *p*-value <0.05). All bifidobacterial strains elicited statistically significant growth at pH 5 (Student t-test *p*-value <0.05) when compared to the corresponding reference conditions (MRS) (Table 2 and Table S5). These results suggest differential EPS production, as demonstrated by slow sedimentation and mucoid colony formation on RRM agar (described above) and conferring a protective role of EPS to the bifidobacterial cell at low pH conditions and/or in the presence of bile salts (Fanning*, et al.*, 2012) (Table 2). In order to evaluate if different stressful conditions commonly encountered by intestinal bifidobacteria in their ecological niche, i.e., low pH conditions and presence of bile salts, might impact on the tolerance toward different antibiotics, MIC assays were performed involving the same set of antibiotics mentioned above. Notably for vancomycin, an antibiotic targeting the cell wall, most of the EPS-producing strains, i.e., *B. longum* 39B, *B. longum* 1898B and *B. breve* UCC2003 doubled their MIC values upon exposure to different environmental stresses (Fig. S2 and Table S6). The results show a certain level of diversity, therefore it seems that the imposed

environmental stress conditions influence bifidobacterial EPS production, though not to the same extent and for the same antibiotics for all assayed strains.

Table 2. Growth performances of different strains under different pH value and bile salts amounts.

	1898B		UCC2003		M1D		39B	
pH values	24h	48h	24h	48h	24h	48h	24h	48h
pH 6.8	+++	+++	+++	+++	+++	+++	+++	+++
pH 6	+++	+++	+++	+++	+++	+++	+++	+++
pH 5.5	+++	+++	++	+++	++	+++	+++	+++
pH 5	++	+++	++	++	++	++	+++	+++
pH 4.5	-	-	-	-	-	-	-	-
pH 4	-	-	-	-	-	-	-	-
pH 3.5	-	-	-	-	-	-	-	-
Bile salts								
Bile salts 0.25%	-	-	-	-	-	-	-	-
Bile salts 0.2%	-	-	-	-	-	-	-	-
Bile salts 0.15%	-	-	-	-	-	-	-	-
Bile salts 0.1%	++	++	++	+++	-	-	-	-
Bile salts 0.05%	+++	+++	+++	+++	++	++	+++	+++
Bile salts 0.04%	+++	+++	+++	+++	++	+++	+++	+++
Bile salts 0.03%	+++	+++	+++	+++	+++	+++	+++	+++
Bile salts 0.02%	+++	+++	+++	+++	+++	+++	+++	+++

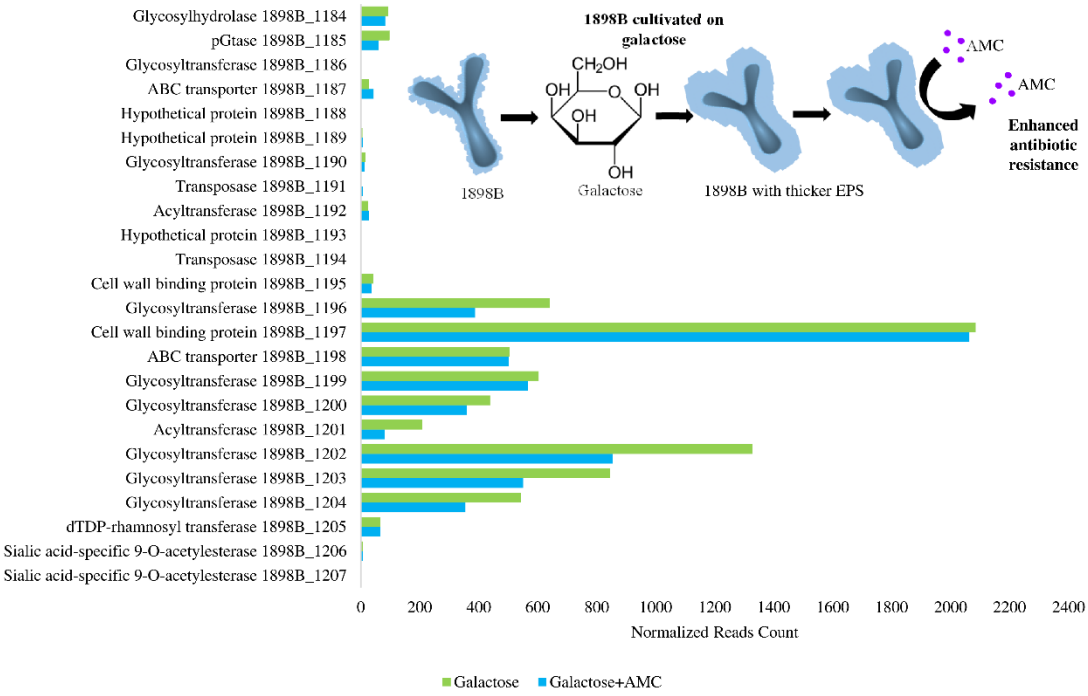
-, no growth; +, poor growth ($0.5 < OD_{600nm}$); ++, growth ($0.51 < OD_{600nm} < 0.8$); +++, good growth ($0.81 < OD_{600nm} < 1.9$); +++, very good growth ($OD_{600nm} > 1.91$).

Transcriptomics analyses of *eps* loci and antibiotic.

In order to evaluate if exposure to antibiotics influences the transcription of *eps* genes in bifidobacteria, we performed a RNAseq analysis involving *B. longum* 1898B, cultivated up to the exponential phase (OD600nm ~ 0.6-0.8) in the presence of galactose with and without AMC. Comparison of the transcriptome profiles of the *eps* locus of 1898B when the strain was cultivated on galactose with AMC vs. without AMC, no statistically significant transcriptional increase (adjusted Benjamini–Hochberg p-value > 0.05) of the genes that are considered essential for EPS production was observed (Fig. 3a and Table S7). This finding indicates that exposure to sub-MIC AMC does not modify *eps* gene transcription when the strain was cultivated on galactose. Furthermore, RNAseq experiments were performed on the strain cultivated on glucose, with the aim to evaluate if galactose is a transcriptional inducer of the *eps* locus. Notably, comparison of the transcriptome profiles of the *eps* locus of 1898B when the strain was cultivated on galactose vs. when it was grown on glucose, highlighted a statistically significant transcriptional increase (adjusted Benjamini–Hochberg p-value < 0.05) of the main genes involved in the EPS production, such as gene predicted to encode a cell wall binding protein (1898B_1195) and various glycosyltransferases (1898B_1186, 1898B_1190, 1898B_1196, 1898B_1200 and 1898B_1204). Particularly, transcription of 1898B_1186 and 1898B_1187, and 1898B_ 1190, when strain 1898B was cultivated on galactose, were shown to be enhanced from 2- to 3-fold (log_2FC between 1 and 1.6, Table S7) as compared to growth on glucose. These findings therefore reinforce the notion that cultivation of *B. longum* 1898B in the presence of galactose increases EPS production. However, as mentioned above, the presence of the antibiotic does not in itself represent a factor triggering *eps* gene expression (Fig. 3b and Table S7). Beyond *eps*-encoding genes, a total of 181 genes were uniquely overexpressed when the transcriptome of *B. longum* 1898B grown on

galactose was compared to the grown condition on glucose (Table S8 and S9). Among these we could find genes commonly involved in galactose metabolism, in particular beta-galactosidase (1898B_0355), arabinogalactan endo-1,4-beta-galactosidase (1898B_0660) and galactose-1-phosphate uridylyltransferase (1898B_1372), which displayed a change of expression ranging from 1.8 to 5-fold (log_2FC between 0.9 to 2.7, Table S7), corroborating how the strain utilizes this carbon source. On the other hand, 249 genes were identified as uniquely over expressed when *B. longum* 1898B was cultivated on galactose supplemented with AMC (Table S9). Interestingly, among these genes, we identified genes putatively involved in antibiotic resistance, such as ABC transporter (1898B_1159) UDP-N-acetylglucosamine 1-carboxyvinyltransferase (1898B_1232) and tetracycline resistance protein (1898B_1131) (Table S9), however we were unable to identify any gene specifically related to resistance to amoxicillin and clavulanic acid.

a)


Glycosylhydrolase 1898B_1184
pGtase 1898B_1185
Glycosyltransferase 1898B_1186
ABC transporter 1898B_1187
Hypothetical protein 1898B_1188
Hypothetical protein 1898B_1189
Glycosyltransferase 1898B_1190
Transposase 1898B_1191
Acyltransferase 1898B_1192
Hypothetical protein 1898B_1193
Transposase 1898B_1194
Cell wall binding protein 1898B_1195
Glycosyltransferase 1898B_1196
Cell wall binding protein 1898B_1197
ABC transporter 1898B_1198
Glycosyltransferase 1898B_1199
Glycosyltransferase 1898B_1200
Acyltransferase 1898B_1201
Glycosyltransferase 1898B_1202
Glycosyltransferase 1898B_1203
Glycosyltransferase 1898B_1204
dTDP-rhamnosyl transferase 1898B_1205
Sialic acid-specific 9-O-acetylesterase 1898B_1206
Sialic acid-specific 9-O-acetylesterase 1898B_1207
1898B cultivated on galactose
1898B
Galactose
1898B with thicker EPS
AMC
AMC
Enhanced antibiotic resistance
0 200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400
Normalized Reads Count
Galactose
Galactose+AMC

b)

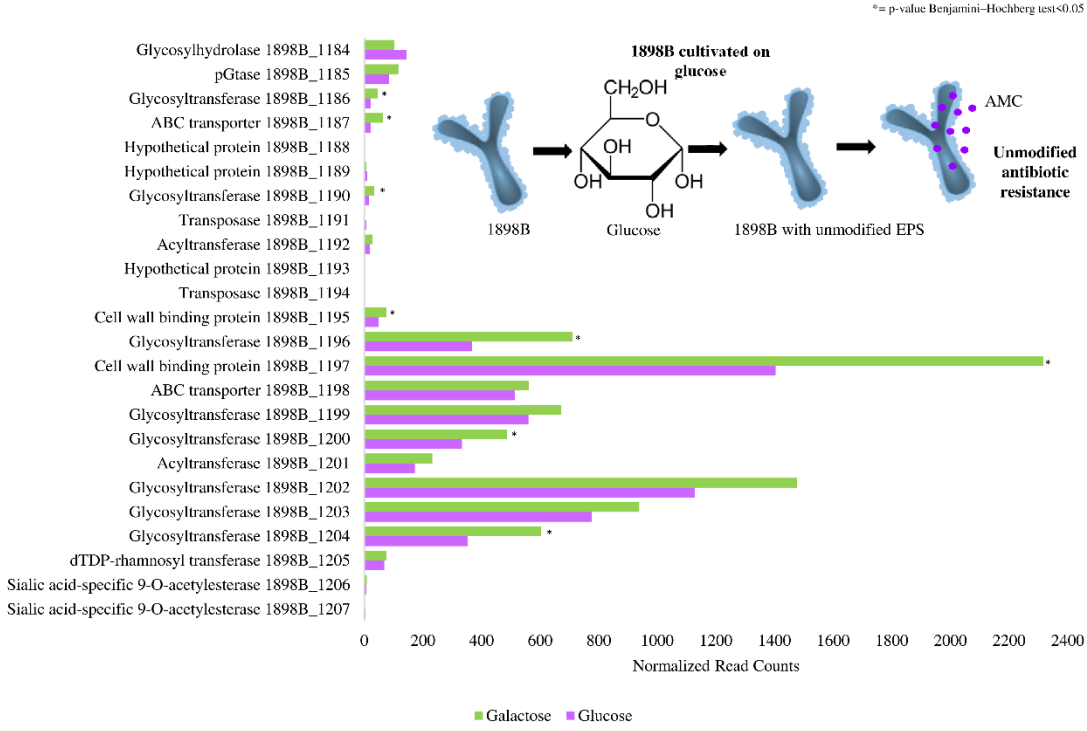

*= p-value Benjamini–Hochberg test<0.05
Glycosylhydrolase 1898B_1184
pGtase 1898B_1185
Glycosyltransferase 1898B_1186
ABC transporter 1898B_1187
Hypothetical protein 1898B_1188
Hypothetical protein 1898B_1189
Glycosyltransferase 1898B_1190
Transposase 1898B_1191
Acyltransferase 1898B_1192
Hypothetical protein 1898B_1193
Transposase 1898B_1194
Cell wall binding protein 1898B_1195
Glycosyltransferase 1898B_1196
Cell wall binding protein 1898B_1197
ABC transporter 1898B_1198
Glycosyltransferase 1898B_1199
Glycosyltransferase 1898B_1200
Acyltransferase 1898B_1201
Glycosyltransferase 1898B_1202
Glycosyltransferase 1898B_1203
Glycosyltransferase 1898B_1204
dTDP-rhamnosyl transferase 1898B_1205
Sialic acid-specific 9-O-acetylesterase 1898B_1206
Sialic acid-specific 9-O-acetylesterase 1898B_1207
1898B cultivated on glucose
1898B
Glucose
1898B with unmodified EPS
AMC
Unmodified antibiotic resistance
0 200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400
Normalized Read Counts
Galactose
Glucose

Figure 3

Figure 3. Evaluation of the mRNA levels of the *eps* genes of *B. longum* 1898B cultivated in the presence of different carbon sources and upon treatment with AMC. Panel a) shows the transcriptomics profiles of the *eps* encoding genes of 1898B strain cultivated in the presence of 2% (wt/vol) of galactose with or without AMC. Panel b) displays the transcriptomics profiles of the *eps* encoding genes of 1898B strain grown on galactose at 2% (wt/vol) respect when cultivated on glucose.

Discussion.

Exopolysaccharides (EPS) have been shown to be synthetized by different bifidobacterial taxa commonly identified in the gut microbiota such as *B. breve* and *B. longum* subsp. *longum*, which have been documented to act as a key extracellular structure in the modulation of the interactions of bifidobacteria with the host as well as with other members (Henderickx JGE*, et al.*, 2022) of the human gut microbiota (Fanning*, et al.*, 2012, Fanning*, et al.*, 2012). However, nothing is known about the potential effects of EPS produced by bifidobacteria in terms of enhancing their tolerance toward various antibiotics commonly used in antibiotic therapies. For some pathogens, i.e., *Acinetobacter baumanii*, *Pseudomonas* spp. and *Klebsiella pneumoniae*, it has been shown that EPS can play an important role in allowing bacterial cells to become more resistant to antibiotics and the expression of EPS in these bacteria is increased by the presence of antibiotics (Held*, et al.*, 1995, Geisinger & Isberg, 2015). Based on this prior knowledge, we evaluated if the EPS production by bifidobacteria is influenced by the carbon source present in the growth medium and if a higher EPS production had an influence on the susceptibility to antibiotics.

Our results showed that EPS producing strains appear to be involved in improving their tolerance towards specific classes of antibiotics i.e., beta-lactam antibiotics. In detail, we observed that *B. breve* UCC2003 grown in the presence of galactose at 2% (wt/vol) showed a beta-lactam MIC value increase ranging from two to 4-fold when compared to the MIC value when the strain was cultivated in the presence of glucose as the sole carbon source. Interestingly, its EPS-negative mutant, i.e., *B. breve* UCC2003::Bbr_0430 (Fanning*, et al.*, 2012), revealed no change of MIC values for AMC and amoxicillin when compared to the reference condition (glucose), suggesting that EPS production is involved in this variable antibiotic sensitivity phenotype. This phenotypic behavior was also observed for *B. longum*

1898B and 39B, strains whose MIC values doubled when they were grown in the presence of galactose, when compared to the reference condition (i.e., grown on glucose), while non-producing strains of EPS show unchanged MIC values. These results were observed for three beta-lactam antibiotics, such as AMC (combination between the beta-lactam antibiotic amoxicillin and the beta-lactamase inhibitor, clavulanic acid), amoxicillin alone and ampicillin, that are all part of the class of beta-lactams that target the bacterial wall of Gram-positive bacteria (Yocum*, et al.*, 1980, Huttner*, et al.*, 2020). These findings can be explained by the increase of the physical barrier of the EPS on the cell surfaces, which ultimately may protect cells from harsh conditions, including those generated by antibiotics and subsequently increase the tolerance against the antibiotic molecules.

Furthermore, lactose and consequently galactose are components of breast milk (Turroni, 2022) and these sugars may contribute to the increase in EPS production by infantile intestinal bifidobacteria and consequently help them to better resist beta-lactam antibiotics in the early stages of life. This hypothesis should be investigated in future studies involving EPS-producing strains and their impact on the infant intestinal microbiota (Ventura, 2022). Furthermore, it will be interesting to evaluate how the expected decrease in the source of galactose (which occurs after the weaning period) may cause an increased sensitivity of bifidobacteria to beta-lactam antibiotics.

In order to assess if antibiotics are involved in regulating transcription of *eps* genes, we performed transcriptomic experiments where we cultivated *B. longum* 1898B in glucose or in galactose with and without AMC. Beyond the *eps*-encoding genes, a total of 249 genes were identified as uniquely over expressed when *B. longum* 1898B was grown on galactose supplemented with AMC. Interestingly, among these we found genes putatively involved in antibiotic resistance, but none of these was shown to be correlated with amoxicillin and clavulanic acid resistance.

Therefore, it is not evident that exposure to antibiotics in itself induces expression of bifidobacterial EPS, as previously observed for pathogenic bacteria (Held*, et al.*, 1995, Geisinger & Isberg, 2015). This indicates that AMC is not recognized by bifidobacterial cells as an environmental stimulus triggering EPS production. However, other stimuli, e.g., exposure to stressful conditions or availability of particular carbon sources such as galactose and lactose are effective in the transcriptional upregulation of the *eps* locus.

Altogether these data support the notion that bifidobacterial tolerance toward antibiotics, and consequently determined MIC values, can be modified according to the growth condition and the environmental conditions applied to bifidobacterial cultures. Thus, interpretation of the results concerning varying levels of antibiotic susceptibility of bifidobacteria needs to be carefully scrutinized and their precise determination requires a very accurate setting of protocols by checking various variables including the physiological status of the cells and the thickness of EPS layer surrounding the bacterial cells assayed. In this context, the scientific validity of the EFSA-based rules on antibiotic resistance of bifidobacteria, which are currently not taking into consideration the above-mentioned variables, may need revision.

In conclusion, this study shows preliminary experimental evidence on the possible role played by bifidobacterial EPS in modifying the susceptibility level of these bacteria toward antibiotics and future studies will be aimed at evaluating how other extracellular structures in both bifidobacteria and other intestinal bacteria may modify the behavior of bacterial cells when exposed to antibiotics.

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Declaration of interests

The authors declare no competing interest.

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Chapter 7

Ecology- and genome-based identification of the *Bifidobacterium adolescentis* prototype of the healthy human gut microbiota

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Abstract

Bifidobacteria are among the first microbial colonizers of the human gut, being frequently associated with human health-promoting activities. In the current study, an *in silico* methodology based on an ecological and phylogenomic-driven approach allowed the selection of a *Bifidobacterium adolescentis* prototype strain, *i.e.*, *B. adolescentis* PRL2023, which best represents the overall genetic content and functional features of the *B. adolescentis* taxon. Such features were confirmed by *in vitro* experiments aimed at evaluating the ability of this strain to survive in the gastrointestinal tract of the host and its performance to interact with human intestinal cells and other microbial gut commensals. In this context, co-cultivation of *B. adolescentis* PRL2023 and several gut commensals revealed various microbe-microbe interactions and indicated co-metabolism of particular plant-derived glycans, such as xylan.

Importance

The usage of appropriate bacterial strains in experimental research becomes imperative in order to investigate bacterial behavior while mimicking the natural environment. In the current study, through *in silico* and *in vitro* methodologies, we were able to identify the most representative strain of the *B. adolescentis* species. The ability of this strain, *B. adolescentis* PRL2023, to cope with the environmental challenges imposed by the gastrointestinal tract, together with its ability to switch its carbohydrate metabolism to compete with other gut microorganisms, make it an ideal choice as a *B. adolescentis* prototype and a member of the healthy microbiota of adults. This strain possesses a genetic blueprint appropriate for its exploitation as a candidate for next-generation probiotics.

Introduction

The human gastrointestinal tract (GIT) encompasses an intricate community of different microorganisms representing the human gut microbiota (1). Millions of years of co-evolution between the intestinal bacterial community and its host are believed to have contributed to the establishment of multiple trophic interactions, including mutualistic relationships in which the host provides nutrients and a suitable environment for growth of its intestinal microbes while, in exchange, the latter perform multiple beneficial physiological and metabolic functions (2, 3). Among the different bacterial species residing in the human GIT are members of the genus *Bifidobacterium*, which represent an extensively studied microbial component due to their purported ability to exert health-promoting or probiotic effects upon their host (4–10).

Bifidobacterium species are considered to be among the first microbial colonizers of the infant gut (11–15), and, despite a decline in their relative abundance from infancy to adulthood, they reach stably maintained numbers until old age, thus in principle capable of eliciting beneficial activities during the entire lifespan of the host (1). Among the reported healthy features exerted by bifidobacteria are maturation of the host-immune system (1, 16, 17) and development of the intestinal barrier integrity, which assists in the protection against pathogen invasion and subsequent proliferation, while also maintaining gut homeostasis (18, 19). Furthermore, members belonging to the *Bifidobacterium* genus have been shown to generate various metabolites, such as the short chain fatty acid (SCFA) acetate, polyphenols, vitamins, and conjugated linoleic acids (17, 20). In addition, bifidobacteria are able to degrade complex host- and diet-derived glycans (21), which facilitates their gut colonization, while it also generates nutrients to both the host and other intestinal commensals through cross-feeding mechanisms (22–24).

Recently, it has been shown that “omics” technologies are crucial when investigating the composition and activities of commensal bacteria (20). In particular, genome analysis of bifidobacterial species instigated a new discipline called probiogenomics (25) which, together with functional genomic data, allows improved understanding of diversity, evolution, and beneficial effects of commensal bacteria (26–28). Nonetheless, to date, most bacterial strains included in supplements and added to certain fermented foods like yogurt have been selected based on their superior growth yields and/or survival levels in bioreactor scale production without providing any information on the interplay between bacterial strains and host or with other commensals of the human GIT. Recently, an ecological and phylogenomic-driven approach facilitated the identification of an optimal representative strain or prototype of the *Bifidobacterium longum* subsp. *longum* species, *i.e.*, *B. longum* subsp. *longum* PRL2022 (20, 29).

Here, we describe a comprehensive screening aimed at identifying a *Bifidobacterium adolescentis* prototype, *i.e.*, *B. adolescentis* PRL2023, of the human gut as based on the microbiota of 4,019 healthy subjects. The selected prototypical strain was assessed by means of metatranscriptomic experiments during co-cultivation with bacterial species that co-occur in the human microbiome (30), highlighting how PRL2023 is able to interact with other commensals inhabiting the intestinal environment (31).

Results and Discussion

Identification of key bifidobacterial species associated with the human gut of healthy adults. *In silico* analyses involving 4,019 publicly available human gut microbiome datasets (produced through shotgun metagenomic sequencing) belonging to healthy adults (ranging from 18 to 80 years of age) were investigated to identify the composition of their bifidobacterial communities (Table S1). Microbial profiling based on short-read taxonomic classification down to species level revealed the occurrence of *B. adolescentis* and *B. longum*, with a relative abundance of 1.5% ± 0.1% and 1.1% ± 0.1%, respectively, followed by *Bifidobacterium pseudocatenulatum* (relative abundance of 0.51% ± 0.03%) (Table 1). Among the analyzed human gut metagenomes, the prevalence of the latter species was 42%, 48%, and 25%, respectively, indicating that *B. adolescentis* and *B. longum* represent the most common bifidobacterial species across the adult human gut (Table 1). Notably, these same two species were also identified as the most abundant bifidobacterial taxa of the human gut microbiota of a smaller metagenomic dataset composed of 76 elderly (healthy humans of 80 years and over) (Table 1). Thus, these findings highlight that *B. adolescentis* and *B. longum* are important contributors of the human gut microbiota due to their prevalence and abundance in hosts ranging from adolescence through old age.

In silico analyses of the human gut microbiota representing 82 independent metagenomic studies confirmed that *B. adolescentis* is a typical bifidobacterial colonizer of the adult human gut, as previously shown (32). Furthermore, dissection of microbial profiles suggests that *B. adolescentis* and *B. longum* are the most prevalent and abundant bifidobacterial species inhabiting the gut microbiota of humans across their lifespans. Thus, we focused our interest on identifying a *B. adolescentis* prototype following a recently published approach (20, 29).

Table 1. Abundance of bifidobacteria in healthy subjects.

Species	Adults (> 18 years)		Elderly (> 80 years)	
	Average abundance	Prevalence	Average abundance	Prevalence
Bifidobacterium adolescentis	1.52% ± 0.06%	42.0%	1.64% ± 0.23%	29.5%
Bifidobacterium angulatum	0.06% ± 0.01%	3.5%	0.00% ± 0.00%	0.0%
Bifidobacterium animalis	0.03% ± 0.00%	2.9%	0.02% ± 0.01%	2.7%
Bifidobacterium bifidum	0.26% ± 0.02%	15.7%	0.36% ± 0.07%	15.5%
Bifidobacterium breve	0.02% ± 0.01%	2.4%	0.08% ± 0.03%	6.2%
Bifidobacterium catenulatum	0.13% ± 0.01%	10.0%	0.15% ± 0.05%	6.9%
Bifidobacterium dentium	0.02% ± 0.00%	2.7%	0.09% ± 0.02%	11.8%
Bifidobacterium gallinarum	0.00% ± 0.00%	0.2%	0.01% ± 0.00%	1.1%
Bifidobacterium longum	1.08% ± 0.06%	47.9%	1.73% ± 0.23%	42.2%
Bifidobacterium merycicum	0.00% ± 0.00%	0.1%	0.00% ± 0.00%	0.0%
Bifidobacterium pseudocatenulatum	0.51% ± 0.03%	25.1%	0.51% ± 0.13%	15.2%
Bifidobacterium pseudolongum	0.00% ± 0.00%	0.2%	0.00% ± 0.00%	0.0%
Bifidobacterium pullorum	0.00% ± 0.00%	0.0%	0.01% ± 0.01%	0.4%
Bifidobacterium ruminantium	0.00% ± 0.00%	0.6%	0.00% ± 0.00%	0.2%
Bifidobacterium saeculare	0.00% ± 0.00%	0.1%	0.00% ± 0.00%	0.4%
Bifidobacterium scardovii	0.00% ± 0.00%	0.2%	0.00% ± 0.00%	0.2%

Ecological and phylogenomic-driven identification of *B. adolescentis* prototypes. To evaluate the distribution of *B. adolescentis* species among the human gut microbiota, an InStrain-based profiling of 113 strains was performed, representing all currently available genome sequences for this bifidobacterial species. First, a de-replication procedure was applied using the dRep software among collected genome sequences, resulting in the identification of 79 distinct genetic lineages of *B. adolescentis* (Table 2). The occurrence of such a high number of genetically unique strains suggests a high level of genetic heterogeneity within the species, a finding which validates a similar report (33). Thus, their distribution across the gut microbiome of healthy individuals was investigated by means of a k-

mer based analysis, employing the same above-described datasets used for bifidobacterial profiling to explore. This analysis unveiled the ecological dissemination of each lineage among the metagenomic datasets of healthy adults. When detected, the *B. adolescentis* strain distribution among microbiomes ranged from 27.9% to 2.9% for PRL2023 and DSM 20087, respectively (Table 2). The wide distribution range of these 79 *B. adolescentis* lineages highlighted those that commonly inhabit the human gut, and others being rarely identified among the considered population (Table 2).

Then, an Average x Prevalence index (AxP index) was generated, integrating genetic data produced as ANI values between de-replicated genetically unique strains and ecological data based on lineage prevalence among metagenomes (20). This procedure allowed the selection of a reference strain from lineage 12, *i.e.*, *B. adolescentis* PRL2023, with the highest AxP score (98.77), corresponding to the most representative *B. adolescentis* strain inhabiting the GIT of healthy humans (Table 2). In contrast, the *B. adolescentis* type strain, *i.e.*, ATCC 15703 showed a much lower AxP value of 54.61. Thus, *in silico* analyses revealed that from a genomic perspective PRL2023 can be considered as the best representative *B. adolescentis* strain of the human gut, while this strain is also ecologically significant due to its global distribution among metagenomic samples. Based on these data, the proposed prototype of the *B. adolescentis* species, strain PRL2023, was further investigated through *in silico* genomic screenings and *in vitro* interaction with the intestinal microbiota community.

Table 2. *Bifidobacterium adolescentis* strain distribution among 4,019 publicly available datasets of human gut microbiome.

NCBI code	Strain	AVERAGE ANI (between dereplicated genomes)	Prevalence (when detected)	AxP (Average x Prevalence index)
GCA_002108035.1	PRL2023	97.88	27.9%	98.77
GCA_003030905.1	1-11	97.94	24.3%	85.82
GCA_003436185.1	TM06-4	98.07	23.5%	83.33
GCA_019131675.1	MSK.11.28	98.25	21.3%	75.66
GCA_003437735.1	TF06-2AC	97.97	21.3%	75.44
Local	780B	97.75	20.6%	72.68
GCA_016069975.1	VKPM Ac-1245	97.96	19.9%	70.23
Local	77B	98.25	18.4%	65.22
GCA_003466335.1	TM06-51	98.12	18.4%	65.14
GCA_002107975.1	AL12-4	98.01	18.4%	65.06
GCA_019041975.1	MSK.7.22	97.88	18.4%	64.98
GCA_019127835.1	MSK.20.45	98.32	17.6%	62.66
GCA_003472245.1	AM12-59	98.24	17.6%	62.61
GCA_003468385.1	AM36-3AC	98.19	17.6%	62.57
GCA_000154085.1	L2-32	98.17	17.6%	62.56
GCA_024460485.1	SL.1.01	98.16	17.6%	62.56
GCA_015553925.1	BSD2780061687_150420_A4	98.23	16.2%	57.38
GCA_002108095.1	AL46-7	98.21	15.4%	54.76
GCA_019972965.1	4-2	98.19	15.4%	54.75
GCA_000010425.1	ATCC 15703	97.94	15.4%	54.61
GCA_015552825.1	D53t1_180928_D4	98.33	14.7%	52.22
GCA_001406215.1	2789STDY5834850	98.32	14.7%	52.21
GCA_002107995.1	LMG 10734	98.30	14.7%	52.20
GCA_017815835.1	PRL2019	98.26	14.7%	52.18
GCA_003457765.1	AF28-4AC	98.24	14.7%	52.17
GCA_019734235.1	K09	98.23	14.7%	52.17
GCA_018785705.1	MCC258	98.23	14.7%	52.17
GCA_003469145.1	AM34-11	98.13	14.7%	52.11
GCA_015558415.1	D52t1_170925_B8	98.10	14.7%	52.10
GCA_015559505.1	D52t1_170925_G1	98.32	14.0%	49.60
GCA_015558085.1	BSD2780120874b_170522_D7	98.30	14.0%	49.59
GCA_019129365.1	MSK.17.32	98.28	14.0%	49.58
GCA_023497865.1	NB2B-16-TSAB	98.27	14.0%	49.58
GCA_003429385.1	6	98.21	14.0%	49.55
GCA_003473105.1	AM13-11	98.11	14.0%	49.50
GCA_003467335.1	AM41-17	98.31	13.2%	46.99
GCA_003472095.1	AM14-37	98.30	13.2%	46.98
GCA_015555595.1	D59t2_181005_G7	98.29	13.2%	46.98
GCA_001406735.1	2789STDY5608862	98.23	13.2%	46.95
GCA_022135325.1	DFI.7.20	98.19	13.2%	46.93
Local	235B	98.18	13.2%	46.93

GCA_015558745.1	1001713B170214_170313_A6	98.18	13.2%	46.93
GCA_003464325.1	AF15-3	98.13	13.2%	46.90
GCA_015554265.1	1001099B_141217_F6	98.31	12.5%	44.38
GCA_000817995.1	BBMN23	98.29	12.5%	44.37
GCA_018785715.1	MCC257	98.29	12.5%	44.37
GCA_005845205.1	1001271st1_A4	98.28	12.5%	44.36
GCA_001010915.1	150	98.24	12.5%	44.35
GCA_002108015.1	487B	98.20	12.5%	44.33
GCA_001406455.1	2789STDY5608824	98.19	12.5%	44.32
GCA_015549865.1	D31t1_170403_E3	98.09	12.5%	44.28
GCA_014524935.1	HD17T2H	98.29	11.8%	41.76
GCA_015553845.1	BSD2780061687b_171204_D1	98.23	11.8%	41.73
Local	946B	98.34	11.0%	39.17
GCA_002108165.1	LMG 18897	98.34	11.0%	39.17
GCA_015557685.1	1001262B_160229_D10	98.34	11.0%	39.17
GCA_002108045.1	AD2-8	98.26	11.0%	39.14
Local	14B	98.24	11.0%	39.13
GCA_015558565.1	1001275B_160808_D1	98.23	11.0%	39.12
GCA_003856735.1	P2P3	98.19	11.0%	39.11
GCA_015548665.1	1001270B_150601_E2	98.14	11.0%	39.09
GCA_022136605.1	DFI.5.12	97.88	11.0%	38.99
Local	740B	98.33	10.3%	36.55
GCA_015547885.1	BSD2780061688_150302_F11	98.30	10.3%	36.54
GCA_001756865.1	Km 4	98.29	10.3%	36.54
GCA_015558575.1	D46t1_190503_C9	98.18	10.3%	36.50
GCA_002108075.1	AL46-2	98.18	10.3%	36.50
Local	713B	98.29	9.6%	33.93
GCA_019127685.1	MSK.21.29	98.23	9.6%	33.91
Local	2419-10B	98.31	8.8%	31.33
GCA_015553955.1	1001095IJ_161003_A7	98.23	8.8%	31.30
Local	2141B	97.94	8.8%	31.21
GCA_004167585.1	ca_0067	98.31	8.1%	28.71
Local	75B	98.29	8.1%	28.71
GCA_015560095.1	1001270J_160509_E8	98.28	8.1%	28.71
GCA_000737885.1	22L	98.32	7.4%	26.11
GCA_003458805.1	AF21-27	97.94	5.9%	20.80
GCA_003465205.1	AF14-56	98.08	5.1%	18.23
GCA_000702865.1	DSM 20087	97.74	2.9%	10.38

Genetic features of *B. adolescentis* PRL2023. To obtain a comprehensive overview of the genetic traits of PRL2023, its genome sequence was decoded employing a combination of short- and long-read sequencing technologies (see Materials and Methods), resulting in a complete genome sequence (*i.e.,* a single contig representing a circular chromosome). Then, a comparative genomic analysis of the *B. adolescentis* species was performed, including the predicted proteome of each representative strain of the above-identified 79 lineages (Table 2). Thus, pangenome and core-genome analyses of this taxon were undertaken following a previously described method based on Clusters of Orthologous Groups (COGs) (34–36). This analysis resulted in the identification of 11,024 COGs, representing the pangenome of the species. Among them, 1,068 COGs were shared between all 79 *B. adolescentis* assessed genomes, thus constituting their core genome (Fig. 1a). Furthermore, Truly Unique Genes (TUGs) of each strain were detected with an average of 63 TUGs per genome. Interestingly, *B. adolescentis* PRL2023 was revealed to be the second strain with the highest abundance of TUGs when compared to members of the other lineages (Fig. 1b). Among the identified TUGs of strain PRL2023 three putative genes encoding carbohydrate-active enzymes were identified, and we therefore decided to further investigate the metabolic capabilities of *B. adolescentis* PRL2023.

An *in silico* prediction of the glycobiome of *B. adolescentis* PRL2023, as based on the Carbohydrate-Active enZYmes (CAZy) database (37), revealed that 60 genes were predicted to encode glycosyl hydrolases (GHs) encompassing 24 different families (Fig. 1c). Among them, 12 genes were predicted to belong to the GH13 family, which encompasses enzymes with amylase and pullulanase activities. The high abundance of GH13 in the genome of PRL2023 is in line with a previous investigation of the *B. adolescentis* glycobiome based on 18 strains that highlighted a rich repertoire of GHs belonging to the GH13 and GH45 families (33). In

accordance, members of the latter GH family were represented by the predicted products of nine genes among the PRL2023 proteome. Thus, glycobiome profiling revealed the tendency of PRL2023 to process various polysaccharides such as plant-derived starch or animal-derived glycans like glycogen. Furthermore, members of additional GH families were identified that are predicted to degrade various glycans, *e.g.*, β-xylosidase (GH3 and GH120), α-L-arabinofuranosidase (GH51), β-L-arabinofuranosidase (GH127), α-galactosidase (GH36), β-galactosidase (GH35 and GH42), α-mannosidase (GH38), and β-mannosidase (GH26). Overall, the predicted glycobiome of PRL2023 indicates a preponderance of this strain to degrade dietary glycans, seemingly supporting the establishment of a symbiotic relationship with humans.

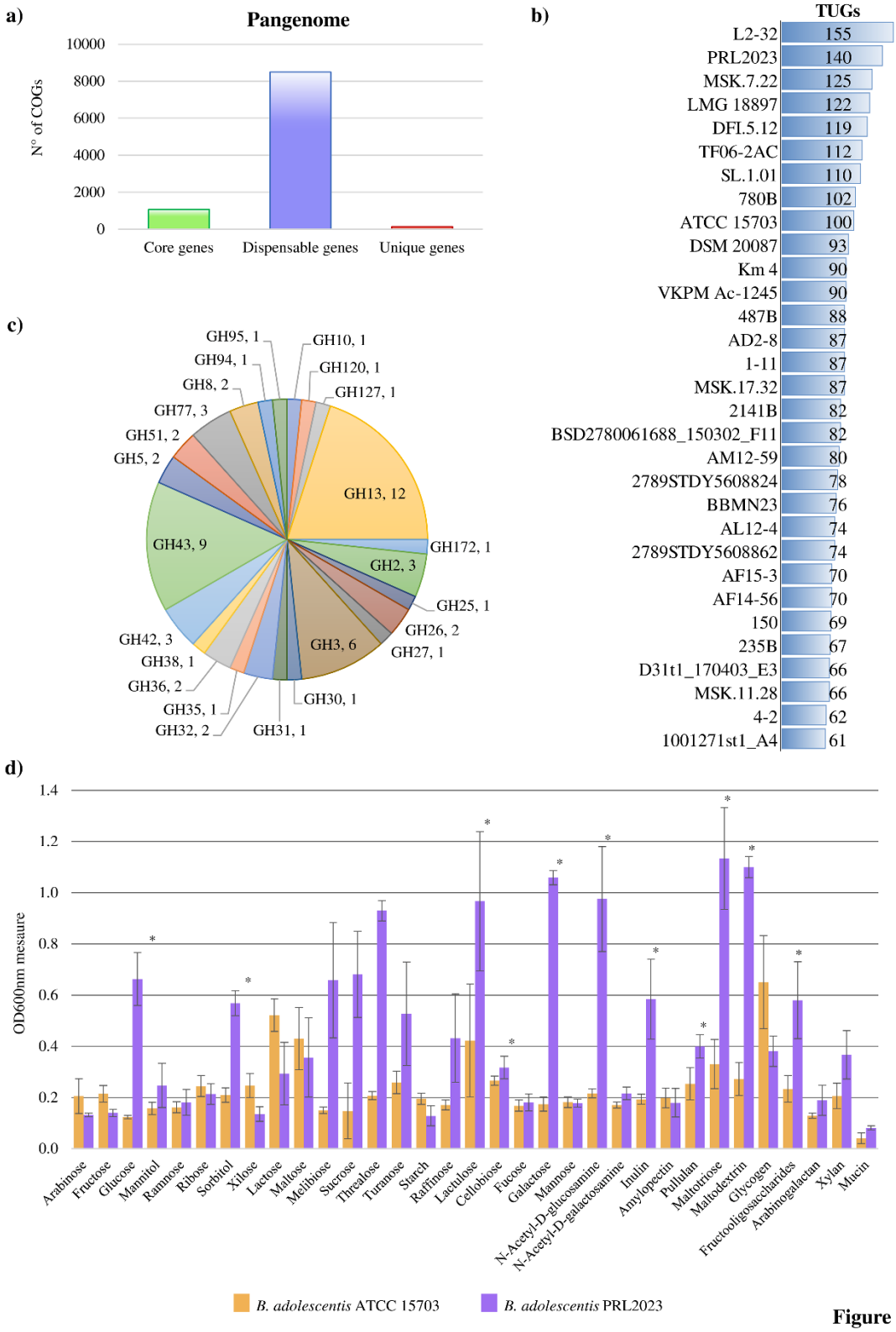
a)
Pangenome
N° of COGs
10000
8000
6000
4000
2000
0
Core genes
Dispensable genes
Unique genes
b)
TUGs
L2-32 155
PRL2023 140
MSK.7.22 125
LMG 18897 122
DFI.5.12 119
TF06-2AC 112
SL.1.01 110
780B 102
ATCC 15703 100
DSM 20087 93
Km 4 90
VKPM Ac-1245 90
487B 88
AD2-8 87
1-11 87
MSK.17.32 87
2141B 82
BSD2780061688_150302_F11 82
AM12-59 80
2789STDY5608824 78
BBMN23 76
AL12-4 74
2789STDY5608862 74
AF15-3 70
AF14-56 70
150 69
235B 67
D31t1_170403_E3 66
MSK.11.28 66
4-2 62
1001271st1_A4 61
c)
GH95, 1
GH10, 1
GH94, 1
GH120, 1
GH8, 2
GH127, 1
GH77, 3
GH51, 2
GH5, 2
GH13, 12
GH43, 9
GH172, 1
GH2, 3
GH25, 1
GH26, 2
GH27, 1
GH3, 6
GH42, 3
GH38, 1
GH36, 2
GH35, 1
GH32, 2
GH31, 1
GH30, 1
d)
OD600nm mesaure
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Arabinose
Fructose
Glucose
Mannitol
Rannose
Ribose
Sorbitol
Xilose
Lactose
Maltose
Melibiose
Sucrose
Threalose
Turanose
Starch
Raffinose
Lactulose
Cellobiose
Fucose
Galactose
Mannose
N-Acetyl-D-glucosamine
N-Acetyl-D-galactosamine
Inulin
Amylopectin
Pullulan
Maltotriose
Maltodextrin
Glycogen
Fructooligosaccharides
Arabinogalactan
Xylan
Mucin
B. adolescentis ATCC 15703
B. adolescentis PRL2023

Figure 1

Figure 1. Genomic analyses and carbohydrate profiling of members of the *B. adolescentis* taxon. Panel a) shows the number of core genes (green), unique genes (red), and dispensable genes (blue) identified in the pangenome of the *B. adolescentis* species. Panel b) displays the distribution of truly unique genes (TUGs) among putative *B. adolescentis* prototypes (only strains with more than 60 TUGs are reported). Panel c) depicts the glycosyl hydrolase (GH) distribution in PRL2023. Panel d) exhibits the growth performance of *B. adolescentis* PRL2023 and ATCC 15703 on different carbohydrates as measured by optical density at 600 nm (OD_{600nm}).

***B. adolescentis* PRL2023 growth profiles on different carbohydrates.** Aiming to validate the activity of the *in silico* identified carbohydrate-active enzymes, we performed growth experiments of *B. adolescentis* PRL2023 and the *B. adolescentis* type strain, *i.e.*, ATCC 15703, in a medium in which different carbohydrates including both plant- and host-derived glycans, were individually included as the sole carbon source (Fig. 1d). In detail, for growth-profiling experiments, we used a carbohydrate-free basic MRS medium, which was supplemented with one of a collection of 33 different sugars, as the sole carbon source (Table S2 and Fig. 1d). Upon Mann Whitney test with Benjamini–Hochberg correction (cut-off p-value < 0.05), the comparative growth assay showed widespread statistically significant differences in growth performances across the two *B. adolescentis* strains. In detail, *B. adolescentis* PRL2023 displays greater ability to grow on inulin, pullulan, maltotriose, maltodextrin, and fructooligosaccharides (FOS) (final OD value from 0.4 to 1.1; all Benjamini–Hochberg corrected p-values < 0.05) when compared to the type strain ATCC 15703 (Table S2 and Fig. 1d). These data demonstrate that the carbohydrate metabolizing abilities of *B. adolescentis* PRL2023 were more extensive when compared to the ATCC 15703 type strain, corroborating our *in silico* strain-based tracking analyses, which identified PRL2023 as the more prevalent strain among the healthy human gut (Table 2). Moreover, together with plant-derived glycans, PRL2023 shows appreciable growth on different hexose-containing sugars, such as lactulose, cellobiose, galactose, and N-Acetyl-D-glucosamine (final OD value from 0.3 to 1.0; all Benjamini–Hochberg corrected p-values < 0.05) (Table S2 and Fig. 1d), in accordance with the presence of GHs in its predicted glycobiome for the degradation of simple sugars, *i.e.*, GH26, GH35, GH36, GH38, and GH42. Moreover, both strains are rather limited in their ability to utilize mucin (Table S2 and Fig. 1d), in agreement with previous works showing that the ability to use mucin as a carbon source is a feature that has only been

described for *B. bifidum* and *B. longum* species (33, 38, 39). However, growth profiles indicated that the extensive repertoire of *B. adolescentis* PRL2023-encoded GHs provides this strain a clear advantage in metabolizing several carbon sources, suggesting that its dominance in most of the human population may at least be partially based on its expansive glycan degradation abilities.

Assessing the gastrointestinal resilience of PRL2023 *in vitro*. In order to colonize their natural ecological niche, bifidobacteria are expected to counteract the adverse and hostile environmental conditions that they encounter during transit toward the large intestine and in the colon, including exposure to biliary salts, osmotic stress, or the extreme acidic conditions encountered during their passage through the stomach (1, 40). In order to evaluate the ability to survive osmotic stress or exposure to bile salts, *B. adolescentis* PRL2023 was exposed to different concentrations of NaCl (2%, 6% and 10%) and Oxgall (0.5%, 1% and 2%) for 3h (41), after which cell viability was monitored through flow cytometry. Interestingly, PRL2023 showed a survival rate of 97.23% at 2% NaCl, which is in line with those observed for other gut commensal bifidobacterial strains (42), indicating that this strain is rather tolerant to osmotic stress (Table 3). Exposure to bile salts is another harsh condition that bifidobacteria have to cope with in the GIT. It is widely reported in literature that the physiological bile salt concentration in the human gut roughly varies from 0.3% to 0.4% (43). Interestingly, PRL2023 showed a survival rate of 30.9% at a concentration higher than the physiological one of Oxgall, *i.e.*, 0.5%. In addition, we also tested the ability of PRL2023 to survive in an acidic environment, simulating the human stomach. In general, bifidobacterial cells have been reported to display low viability toward acidic environment with values between 41.8% and 79.3% at the typical pH 2 of the stomach (41, 42, 44–46). Specifically, PRL2023 cells showed excellent survival of 78.3% following 2 h incubation at pH 4.

However, much lower survival rates were observed when the acid challenge assays were done at pH 2.0 and pH 3.0 (Table 3). Accordingly, *in vitro* survival assays of the selected *Bifidobacterium* prototype, mimicking harsh GIT conditions, highlighted how this strain can tolerate stressful conditions typical of the intestinal environment.

In addition, in order to investigate the ability of this strain to interact with the host, we evaluated the adhesive performance of *B. adolescentis* PRL2023 and those of the ATCC 15703 strain, which is the type strain of *B. adolescentis,* to the human intestinal mucosal cells by using a previously described methodology (47–49). Interestingly, both strains were able to adhere to HT29-MTX cell monolayers, as demonstrated by the adhesion index of 89,333 ± 4 and 86,400 ± 9 determined for PRL2023 and ATCC 15703 cells, respectively (Fig. S2). Several factors can influence the adhesion of a bacterial strain, including culture conditions and HT29-MTX cell characteristics, so there is not an "ideal value" of the adhesion index to be used as a reference. For this reason, the experimental approach was also applied to the *Bifidobacterium animalis* subsp. *lactis* Bb-12 strain (47), which is commonly used as a probiotic supplement. The comparison revealed an adhesion index of Bb-12 less than 20,000 (Fig. S2), highlighting a clear-cut superior adhesion of both *B. adolescentis* PRL2023 and the type strain ATCC 15703. In addition, an adhesion assay on mucin was performed (50), highlighting a higher relative adhesion to mucin of PRL2023 (61.5%) when compared to *B. adolescentis* ATCC 15703 (51.7%). These results are consistent with our other observations and indicate that the prototype PRL2023 strain is ecologically adapted to the human gut microbiota and likely expresses genes that are predicted to play a role in enhancing the colonization of the human intestinal mucosa. The improved capacity of the strain to withstand challenging gut environment conditions and its capacity to bind to the

human epithelial cells imply that PRL2023 is endowed with genetic features able to support its potential exploitation as a novel probiotic microorganism.

Table 3. Viability of *B. adolescentis* PRL2023 when exposed to human gastrointestinal challenges.

	Viable* PRL2023 cells	**Non-viable* PRL2023 cells**
MRS 3h	96.89%	3.11%
2% NaCl	97.23%	2.77%
6% NaCl	39.35%	60.65%
10% NaCl	50.67%	49.33%
0.5% Bile salts	30.94%	69.06%
1% Bile salts	9.42%	90.58%
2% Bile salts	11.14%	88.86%
MRS 2h	75.13%	24.87%
pH 2.0	12.21%	87.79%
pH 3.0	11.27%	88.73%
pH 4.0	78.26%	21.74%

*Data are expressed as the average of the obtained triplicates.

Investigation of the molecular interaction between PRL2023 and common members of the human gut microbiota. A correlation analysis among bacterial species inhabiting the human gut microbiota of adults allowed us to identify those species that are commonly sharing the same ecosystem with *B. adolescentis* taxon. Overall, 88 microbial species highlighted a positive and significant correlation with *B. adolescentis* (Benjamini-Hochberg, FDR *p*-values < 0.05) (Table S3). Among bifidobacteria, *B. adolescentis* species was found second only after *B. longum* species, which revealed a significant positive correlation with 91 human gut bacteria (Table S3). A Principal Coordinate Analysis (PCoA) analysis was performed to further investigate which bacterial species were most important in terms of their association with human gut microbiota variability (Fig. 2a). Normalized data highlighted that among the 88 bacterial species that positively correlated with the *B. adolescentis* taxon, only eight were found significant in defining the gut microbiome variability ($R^2 > 0.2$, FDR *p*-values < 0.005), *i.e.*, *Collinsella aerofaciens*, *Dorea longicatena*, *Anaerobutyricum hallii*, *Blautia massiliensis*, *Coprococcus comes*, *Blautia obeum*, *Dorea formicigenerans*, and *Blautia wexlerae*. Interestingly, the latter species is one of 14 species with the highest correlation values in respect to the *B. adolescentis* taxon (Fig. 2b), highlighting a clear interconnection between bacteria shaping the diversity of the human gut microbiota.

a)

1	2	3	
Bacillus luti *Prevotella copri*	*Alistipes putredinis* *Bacteroides uniformis* *Phocaeicola vulgatus*	*Anaerobutyricum hallii* *Blautia obeum* *Blautia massiliensis* *Blautia wexlerae*	*Collinsella aerofaciens* *Coprococcus comes* *Dorea formicigenerans* *Dorea longicatena*

b)

	Species name	correlation value	PCoA R2 value
1	*Collinsella aerofaciens*	0.4725	0.3257
2	*Bifidobacterium longum*	0.4700	NS
3	*Dorea longicatena*	0.3808	0.3034
4	*Anaerobutyricum hallii*	0.3792	0.3144
5	*Bifidobacterium bifidum*	0.3705	NS
6	*Blautia massiliensis*	0.3496	0.288
7	*Coprococcus comes*	0.3198	0.2821
8	*Bifidobacterium catenulatum*	0.2959	NS
9	*Blautia obeum*	0.2905	0.2514
10	*Gemmiger formicilis*	0.2719	NS
11	*Blautia luti*	0.2483	NS
12	*Dorea formicigenerans*	0.2437	0.3586
13	*Ruminococcus faecis*	0.2423	NS
14	*Blautia wexlerae*	0.2408	0.2602
15	*Blautia faecicola*	0.2275	NS
16	*Anaerostipes hadrus*	0.2217	NS
17	*Ruminococcus bicirculans*	0.2064	NS
18	*Collinsella bouchesdurhonensis*	0.2006	NS
19	*Coprococcus eutactus*	0.1973	NS
20	*Bifidobacterium angulatum*	0.1950	NS
21	*Senegalimassilia anaerobia*	0.1862	NS
22	*Eubacterium rectale*	0.1817	NS
23	*Lactobacillus ruminis*	0.1815	NS
24	*Bifidobacterium pseudocatenulatum*	0.1803	NS
25	*Eubacterium ramulus*	0.1800	NS
26	*Methanobrevibacter smithii*	0.1701	NS
27	*Ruminococcus callidus*	0.1700	NS
28	*Holdemanella biformis*	0.1662	NS
29	*Slackia isoflavoniconvertens*	0.1655	NS
30	*Asaccharobacter celatus*	0.1646	NS
31	*Ellagibacter isourolithinifaciens*	0.1634	NS
32	*Romboutsia timonensis*	0.1622	NS
33	*Ruminococcus bromii*	0.1556	NS
34	*Dialister invisus*	0.1519	NS
35	*Faecalibacterium prausnitzii*	0.1494	NS
36	*Eubacterium ventriosum*	0.1487	NS
37	*Agathobaculum butyriciproducens*	0.1473	NS
38	*Streptococcus thermophilus*	0.1371	NS
39	*Catenibacterium mitsuokai*	0.1339	NS
40	*Streptococcus salivarius*	0.1296	NS
41	*Evtepia gabavorous*	0.1174	NS
42	*Bifidobacterium ruminantium*	0.1112	NS
43	*Barnesiella intestinihominis*	0.1068	NS
44	*Parolsenella catena*	0.1021	NS
45	*Dialister hominis*	0.1004	NS
46	*Faecalibacillus intestinalis*	0.1003	NS
47	*Megasphaera elsdenii*	0.0993	NS
48	*Dialister massiliensis*	0.0988	NS
49	*Gordonibacter pamelaeae*	0.0930	NS
50	*Olsenella scatoligenes*	0.0904	NS
51	*Acidaminococcus intestini*	0.0883	NS
52	*Sutterella wadsworthensis*	0.0860	NS
53	*Alistipes communis*	0.0840	NS
54	*Mediterraneibacter butyricigenes*	0.0825	NS
55	*Streptococcus anginosus*	0.0745	NS
56	*Subdoligranulum variabile*	0.0732	NS
57	*Dialister succinatiphilus*	0.0728	NS
58	*Mitsuokella jalaludinii*	0.0699	NS
59	*Bifidobacterium scardovii*	0.0694	NS
60	*Intestinibacter bartlettii*	0.0686	NS

Figure 2

Figure 2. *B. adolescentis* and its role in shaping the human microbiome. Panel a) shows a Principal Coordinate Analysis (PCoA) where each yellow circle represents a human microbiome included in the analysis. Significant microorganisms in shaping the variability of the human gut are reported below the PCoA ($R2 > 0.2$, FDR p-values < 0.005). Panel b) displays the list of significant bacteria correlating with *B. adolescentis*. NS is the Not Significant acronym.

In order to evaluate interactions between the proposed prototype *B. adolescentis* PRL2023 strain and a selected number of bacterial species that shape the human gut microbiota of adults, we performed co-cultivation assays with PRL2023. In detail, we performed six co-cultivation assays where *B. adolescentis* PRL2023 cells were co-incubated with cells harboring *Collinsella aerofaciens* DSM 3979, *Dorea longicatena* DSM 13814, *Anaerobutyricum hallii* DSM 3353, *Blautia massiliensis* DSM 101187, *Blautia obeum* DSM 25238, and *Faecalibacterium prausnitzii* DSM 107838. The latter species was chosen in addition to the most relevant bacteria that co-occurred with *B. adolescentis* due to its notable scientific interest with respect to gut microbiota and associated host health (51–53). Furthermore, an additional co-cultivation assay involving all of the above mentioned species together with PRL2023 was performed. A quantitative PCR (qPCR) approach was used to quantify the bacterial DNA of each species to the total DNA extracted from co-cultivation experiments. This qPCR analysis highlighted that PRL2023 strain is able to grow in all the co-culture experiments with the different gut microbial commensals (Fig. S1). Thus, shotgun metatranscriptomic was carried out in each co-cultivation assay to investigate how PRL2023 interacts with other commensals. This analysis revealed that, compared to the reference condition (PRL2023 grown as a monoculture), the number of PRL2023 upregulated genes was always higher than downregulated genes, with a ratio ranging from 0.84 to 0.15 (down-/up-regulated genes), in *Anaerobutyricum hallii* and *Blautia obeum* co-cultivation, respectively (Table S4). Particularly, PRL2023 cells, when cultivated with other intestinal commensals, were shown to elicit increased transcription of genes belonging to a Tight Adherence (Tad) pilus locus (PRL2023_0134-0141), *i.e.*, seven genes encoding Type IV pilus assembly and secretion proteins (Fig. 3). In this context, it is well known that pili are considered one of the principal structures involved in microbe-host interactions (1, 54, 55) (Fig. 3). Thus, metatranscriptomics

data demonstrate that other human commensals directly enhanced the host interaction ability of PRL2023. Furthermore, expression of additional genes illustrated the synthesis of extracellular structures involved in interactions, such as a pilus assembly protein, a cell wall anchor domain-containing protein, and a sortase putatively involved in pilin biosynthesis (Table S4), corroborating the notion that PRL2023 is actively transcribing such complexes in co-culture.

Additionally, genes predicted to be involved in carbohydrate metabolism and their uptake were upregulated in co-cultivation assays with other microorganisms. Recently, it has been shown that the presence of other microbes allowed the development of cross-feeding strategies, in which bifidobacteria access a wider set of carbon sources compared to cells grown in monoculture (56, 57) (Fig. 3). Here, metatranscriptomics data revealed that two loci in particular increased their transcription under such co-cultivation circumstances, unveiling the activation of genes involved in the degradation of a complex polysaccharide, *i.e.*, xylan (PRL2023_0314-0323), and a simple monosaccharide, *i.e.*, xylose (PRL2023_0432-0442), that are typical indigestible pentose sugars found in the human diet (Fig. 3). The structure of both loci unveiled the presence of two dedicated uptake systems as well as degradative carbohydrate enzymes (Table S4), highlighting the activation of a metabolic machinery for the uptake and degradation of xylose and xylose-based oligosaccharides. As previously reported, xylose-containing glycans are metabolized in multiple associations due to the rapid depletion of simple substrates (58). Thus, resource competition in the bi-association assays, due to the combination of GH enzymes of other gut commensals, may have activated a plant-derived carbohydrate metabolism that was not expressed in mono-association cultures, a phenomenon that has also been observed previously (59, 60). Notably, the ability to switch carbohydrate metabolism between different substrates may represent successful strategies allowing this PRL2023 strain to colonize and/or

persist in the gut microbiota of adults. Furthermore, additional enzymes involved in the break-down of complex plant carbohydrates were identified to be upregulated in co-cultivation assays as well as other predicted β-xylanase-encoding genes scattered across the genome (Table S4). In this context, PRL2023 showed the enhanced transcription of genes predicted to encode α- and β-glucosidases, α- and β-galactosidases, β-mannosidases, and β-fructofuranosidases (Table S4), together with synthase-encoding genes for the conversion of glucose into glycogen for storage.

Interestingly, a gene encoding a hypothetical protein of PRL2023, *i.e.*, PRL2023_1457, was overexpressed in any of the bi-association experiments and when PRL2023 was co-cultivated with all these intestinal commensals (Fig. 3). Specifically, sequence homology analyses revealed the presence of a DUF4192 motif, suggesting its involvement in conjugation and DNA metabolism (61). The expression of this unknown gene may be pivotal in understanding the interaction between PRL2023 and (members of) the human microbiota.

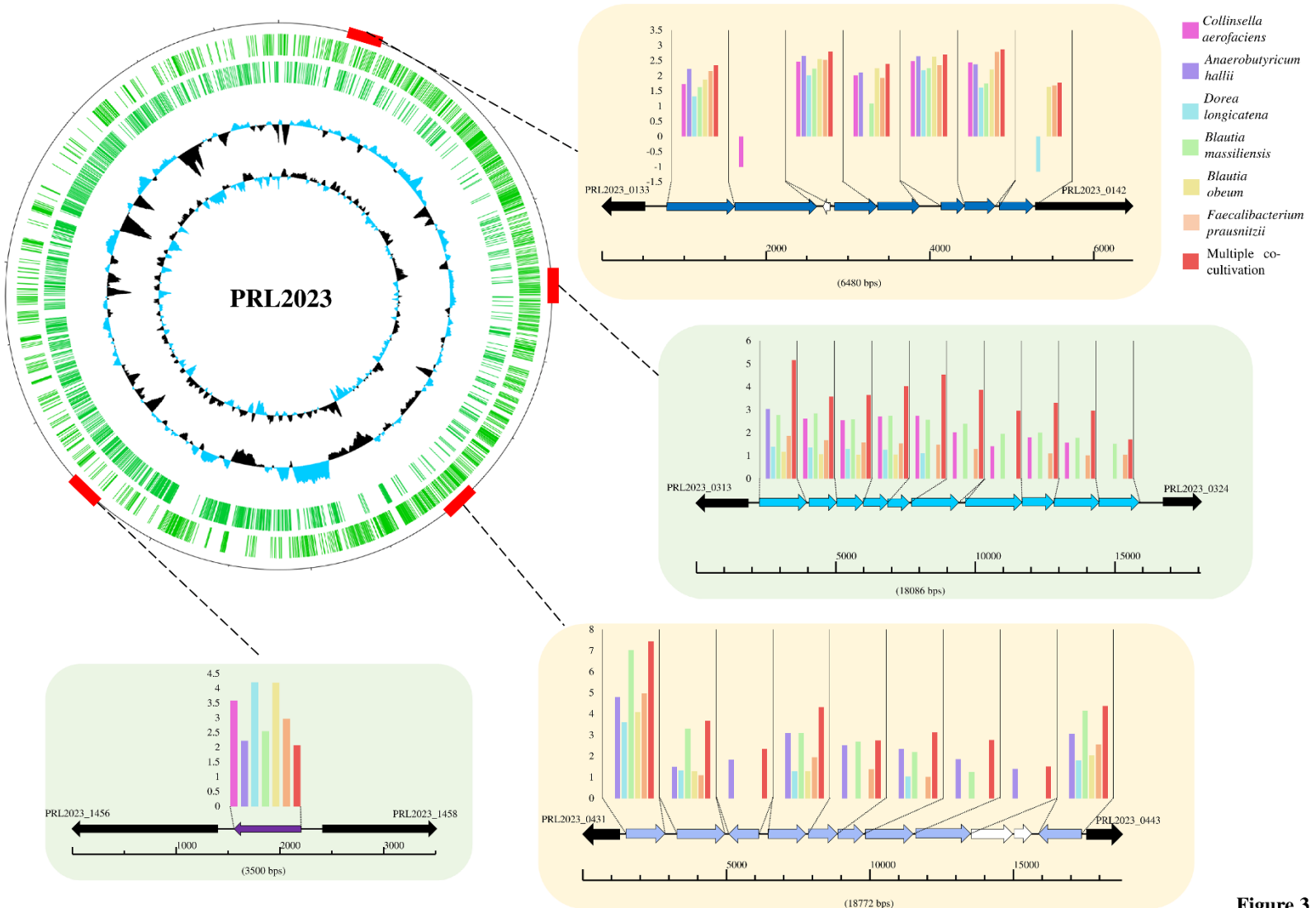
PRL2023
Collinsella aerofaciens
Anaerobutyricum hallii
Dorea longicatena
Blautia massiliensis
Blautia obeum
Faecalibacterium prausnitzii
Multiple co-cultivation
PRL2023_0133
PRL2023_0142
(6480 bps)
PRL2023_0313
PRL2023_0324
(18086 bps)
PRL2023_0431
PRL2023_0443
(18772 bps)
PRL2023_1456
PRL2023_1458
(3500 bps)

Figure 3

Figure 3. Transcriptome analyses of PRL2023 when co-cultivated with other human gut commensals. The circular genome atlas represents the collection of genes belonging to *B. adolescentis* PRL2023 (blue circles). Internal circles illustrate PRL2023 GC% deviation and GC skew (G − C/G + C), while the external maps highlight loci with increased transcription (compared to mono-cultivation control) as identified in co-cultivation assays. Each arrow indicates an ORF, whereas the length of the arrow is proportional to the length of the predicted ORF. Histograms upon each ORF report the expression in respect to the control condition, the Y axis reports normalized count reads (TMM).

Conclusions

To date, a large body of scientific literature indicates that bifidobacteria are well known for the benefits exerted on their host, such as promoting the intestinal barrier integrity, improving gut homeostasis, and supporting the development of the host immune system (16, 18, 62–64). In this context, *B. adolescentis* stands out among the most abundant species of bifidobacteria residing in the human adult intestine (11, 65). In the current study, an ecological and phylogenomic-driven system was applied allowing the identification of *B. adolescentis* PRL2023 strain as the most representative taxon of this species inhabiting the human gastrointestinal tract of adults. As recently reported, the use of a suitable strain for experimental research is necessary to manipulate bacteria sharing the most representative genomic features found in nature (20, 29).

Comparative genome analysis, involving the prototype PRL2023 and other *B. adolescentis* strains, revealed a high number of unique genes, including genes involved in the degradation of complex sugars typical of an adult host diet. Moreover, *in vitro* experiments involving models that simulate typical harsh gastrointestinal tract conditions as well as contact with human cell lines suggests that PRL2023 can survive well in the intestinal environment and efficiently adhere to the human epithelium. Thus, based on our data, the selected strain should be able to reach and colonize the intricate human intestinal ecological niche. In addition, metatranscriptomics analyses based on PRL2023 in correlation with other intestinal microbes, enhanced our understanding as to why this prototype is widely distributed among the population, highlighting the ability of PRL2023 to interact with its host as well as its ability to switch its carbohydrate metabolism to effectively compete with other members of the human microbiota. Ultimately, in order to further advance our scientific knowledge regarding PRL2023 and to validate our *in vitro* observations on its abilities to interact with the human host, a human clinical trial

involving this strain will be necessary. Nonetheless, our reported data suggests that this strain represents a very promising candidate whose potential health-promoting activities are worthy to be determined.

Materials and Methods

Metagenome dataset selection. In this project, 4,019 publicly available human gut microbiome datasets were retrieved from 82 cohorts of healthy adults (age from 18 to 80 years) (Table S1). Additionally, 76 samples belonging to healthy elderly (age > 80 years) were used to explore the aging-associated microbial diversity (Table S1).

Taxonomic classification of metagenomic reads. To analyze high-quality DNA sequence data, each dataset was subjected to a filtering step removing low-quality reads (minimum mean quality score 20, window size 5 nt, quality threshold 25, and minimum length 100 nt) using the fastq-mcf script (https://github.com/ExpressionAnalysis/ea-utils/blob/wiki/FastqMcf.md). Filtered reads were then collected and taxonomically classified through the METAnnotatorX2 pipeline (66), using the up-to-date RefSeq (genome) database retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/refseq/). Short-read sequences were taxonomically classified based on their sequence identity using megablast (67).

***B. adolescentis* prototype selection.** Complete and partial genomes of 113 *B. adolescentis* strains were retrieved from the RefSeq NCBI database representing the collection of validated publicly sequenced genomes of this taxon. By selecting strains for *in silico* analyses, we admittedly ignored metagenomic assembled genomes (MAGs), so as to investigate previously cultivated bacteria only. The

genome sequence of the reference strain ATCC 15703 was used to discard strains showing an average nucleotide identity (ANI) lower than 94% employing the software fastANI (68) to exclude taxonomically misclassified microorganisms. Furthermore, genome sequence quality was estimated for completeness and contamination using CheckM (69). High-quality genomes were then subjected to a de-replication-based analysis aiming to reduce strain redundancy among bifidobacterial genome sequences using dRep v2.0 (70). Among strains displaying sequence identity >99.8%, a single reference genome was selected for further analysis, representing putative prototypes of the *B. adolescentis* species. Then, a k-mer based analysis to explore the distribution of each putative prototype was investigated using InStrain software with a k-mer size of 23 (20). The selection of the *B. adolescentis* prototype of the healthy human gut was based on a previously validated index called AxP (20), defined as [the average ANI value of genomes constituting the same clade] * [prevalence score of the strain in the dataset] * [100].

Genome sequencing. The genome sequence of PRL2023 was determined by GenProbio Srl (Parma, Italy) using the MiSeq platform (Illumina, UK). Genome libraries were prepared using an Illumina Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA 92122, USA). Libraries were quantified using a fluorometric Qubit quantification system (Life Technologies, USA), loaded on a 2200 TapeStation instrument (Agilent Technologies, USA), and normalized to 4 nM. Sequencing was performed using the Illumina MiSeq platform with a 600-cycles flow cell version 3 (Illumina Inc., San Diego, CA 92122, USA). Additionally, PRL2023 extracted DNA was subjected to whole-genome sequencing using the Nanopore DNA sequencing platform according to the supplier's protocol (Oxford Nanopore, UK).

Genome assembly. Long reads were filtered by quality using the Filtlong tool (https://github.com/rrwick/Filtlong), while short reads were filtered through the fastq-mcf script (https://github.com/ExpressionAnalysis/ea-utils). Filtered fastq files of Nanopore long reads obtained from genome sequencing efforts were then used as input for genome assembly through CANU software (71). The resulting genome sequence has been polished through Polypolish (72) using Illumina paired-end reads. The whole process was managed by the MEGAnnotator2 pipeline (73)

Comparative genomics. Open reading frames (ORFs) of each *B. adolescentis* genome were predicted with Prodigal (74) and annotated utilizing the MEGAnnotator2 pipeline (73, 75).

Proteomes were employed for a pangenome calculation using the PGAP (76), to identify orthologues between analyzed *B. adolescentis* strains by means of BLAST analysis (cutoff E value, $<1*10^{-5}$; 50% identity over at least 80% of both protein sequences). The resulting output was then clustered into protein families named Clusters of Orthologous Groups (COGs) by means of MCL (graph theory-based Markov clustering algorithm), using the gene family (GF) method. Pangenome profiles were built using an optimized algorithm incorporated in the PGAP software, based on a presence/absence matrix that included all identified COGs in the 79 analyzed genomes.

Glycobiome profiling. The proteome of PRL2023 was screened for genes predicted to encode carbohydrate-active enzymes based on sequence similarity to genes classified in the carbohydrate-active enzyme (CAZy) database (37). Thus, each gene sequence was screened for orthologues against the dbCAN2 meta server (77) composed of 2,141,452 CDSs (coding sequences) using HMMER v3.3.2

(cutoff E-value of $1*10^{-15}$ and coverage >0.35) and DIAMOND (E-value $<1*10^{-102}$).

Bacterial cultivation conditions. *B. adolescentis* PRL2023 was cultivated in the de Man-Rogosa-Sharpe (MRS) medium (Sharlau Chemie, Spain) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (Merk, Germany) and incubated at 37°C in a chamber (Concept 400, Ruskinn) with an anaerobic atmosphere (2.99% H_2, 17.01% CO_2, and 80% N_2). For bi-association experiments, *Collinsella aerofaciens* DSM 3979, *Dorea longicatena* DSM 13814, *Anaerobutyricum hallii* DSM 3353, *Blautia massiliensis* DSM 101187, *Blautia obeum* DSM 25238 and *Faecalibacterium prausnitzii* DSM 107838 were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH. These microorganisms were cultivated anaerobically in yeast extract-casein hydrolysate-fatty acid (YCFA) medium in hungate tubes at 37° C for 48h.

pH, sodium chloride, and bile salts tolerance tests. To evaluate the ability of the selected strains to tolerate various pH levels, *B. adolescentis* PRL2023 was cultivated in 10 mL of MRS broth at 37°C under anaerobic conditions to reach a final concentration of 10^8 cells/ml. Subsequently, cells were centrifuged at 3,000 rpm for 8 min, washed with Phosphate Buffered Saline (PBS, pH 6.5) and resuspended in 10 mL of MRS broth whose pH was adjusted to pH 2.0, pH 3.0, or pH 4.0 with the addition of HCl. Cells were incubated under anaerobic conditions at 37°C for 2h, as previously described (41). The same procedure was performed to assess the ability of bifidobacteria to tolerate different concentrations/levels of NaCl (2%, 6%, and 10%) or bile salts (Sigma Aldrich, US) (0.5%, 1%, and 2%) with an exposure of 3h to these stressful conditions, as previously reported (41). All experiments were carried out in triplicate and a control sample was obtained by

inoculating bifidobacterial cells in MRS broth. After incubation, cell viability was evaluated by means of the LIVE/DEAD BacLight Bacterial Viability kit (ThermoFisher Scientific, USA) and an Attune NxT flow cytometer (ThermoFisher Scientific, USA).

Flow cytometry bacterial viability assay. Following exposure to acidic environment, or various bile salts or NaCl concentrations, a 10-fold serial dilution in PBS was obtained from each tested condition. The diluted cells were then used for a flow cytometry cell viability assay using the fluorescent dyes SYTO9 (3.34 mM) and PI (20 mM) of the LIVE/DEAD BacLight Bacterial Viability kit (ThermoFisher Scientific, USA), following the manufacturer's protocol (Manual of the LIVE/DEAD BacLight Bacterial Viability and counting kit, ThermoFisher Scientific, USA). Briefly, two aliquots of 1 mL of bacterial cell dilution (1:1000) were harvested by centrifugation at 3,000 rpm for 8 min and washed with PBS. Subsequently, one of the two aliquots of bacterial suspension was exposed to 70% isopropyl alcohol and kept on ice for 1h to permeabilize cell membranes and induce cell death, while the other 1 mL aliquot was maintained in PBS to preserve cell viability. Subsequently, 1.5 µl of a specific dye was added to samples for single staining assay, while for the double staining assay 1.5 µl of both dyes were added to samples. Once stained, samples were incubated in the dark for 15 min at room temperature. Furthermore, while single-stained controls were used for instrument parameter adjustment, non-stained cells were used as a background control. Cell viability assays were performed with the Attune NxT flow cytometer (ThermoFisher Scientific, USA), and obtained data sets were analyzed with the Attune NxT flow cytometer software.

Carbohydrate-dependent growth assays. To validate *in silico* findings, we performed growth assays on multiple carbon sources involving the prototype PRL2023 as well as the type strain *B. adolescentis* ATCC 15703 as reference. Notably, *in silico* analyses performed in this study generated predictions with regards to (carbohydrate) metabolic abilities of the above-mentioned strains and were further described in the *Results and Discussion* section. *B. adolescentis* strains were cultivated overnight on a semisynthetic MRS medium supplemented with 0.05% (w/vol) L-cysteine hydrochloride at 37 °C under anaerobic conditions. Subsequently, cells were diluted in MRS without glucose in order to obtain an $OD_{600\ nm}$~1 and 15 µl of the diluted cells were inoculated in 135 µl of MRS without glucose supplemented with 1% (wt/vol) of a particular sugar in a 96-well microtiter plate and incubated in an anaerobic cabinet. Specifically, each carbohydrate was dissolved in MRS without glucose previously sterilized by autoclaving at 121°C for 15 min. Subsequently, each obtained solution was filter sterilized using a 0.2 µm filter size prior to use. Cell growth was evaluated by monitoring the optical density at 600 nm using a plate reader (Biotek, VT, USA). The plate was read in discontinuous mode, with absorbance readings performed at 3 min intervals for three times after 48 h of growth, and each reading was ahead of 30 s of shaking at medium speed. Cultures were grown in triplicates, and the resulting growth data were expressed as the average of these replicates. Carbohydrates tested in this study were purchased from Merck (Germany) and were reported in Table S2.

***In vitro* evaluation of the interaction of PRL2023 cells with selected members of the human gut microbiota.** To evaluate how *B. adolescentis* PRL2023 interacts with other gut microbial players, batch cultures were set up to co-cultivate the selected strain with six different intestinal commensals, *i.e.*, *Collinsella aerofaciens* DSM 3979, *Dorea longicatena* DSM 13814, *Anaerobutyricum hallii* DSM 3353,

Blautia massiliensis DSM 101187, *Blautia obeum* DSM 25238 and *Faecalibacterium prausnitzii* DSM 107838. For bi-association experiments, overnight cultures of each microorganism were diluted in order to obtain an approximate OD value of 1.0, as previously described (78). Each culture was inoculated at 0.1% (vol/vol) into yeast extract-casein hydrolysate-fatty acid (YCFA) medium (79–81). We performed six different experiments in which *B. adolescentis* PRL2023 was inoculated respectively with six different intestinal players mentioned above and one experiment where all microorganisms were cultivated altogether. Batch cultures were performed in triplicate and incubated under anaerobic conditions and in Hungate tubes at 37°C. After 8h of incubation, cultures were centrifuged at 7000 rpm for 5 min, the supernatants were discarded, while the obtained bacterial pellets were used for RNA extraction (57, 82, 83). Moreover, pellets were subjected to DNA extraction using the GeneElute bacterial genomic DNA kit (Sigma, Germany) following the manufacturer's instructions. Each sample was subjected to a different cycle of quantitative PCR (qPCR) using strain specific primers: B0703_0097_FW(5'-TGCAATGATGAATCCACGCC-3') and B0703_0097_RV (5'-GCGGTTGAACTCGAACAGAT-3') for *B. adolescentis* PRL2023. qPCR was performed using qPCR green master mix (PowerUp™ SYBR™ Green Master Mix for qPCR, Thermo Fisher Scientific, US) on a CFX96 system (Bio-Rad, CA, USA) following previously described protocols (84, 85). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: one cycle of 50°C for 2 min, followed by one cycle of 95°C for 2, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The melting curve was 65°C to 95°C with increments of 0.5°C/s. In each run, negative controls (no DNA) were included. A standard curve was built using the CFX96 software (Bio-Rad).

RNA extraction. Total RNA from bacterial cells was isolated using a previously described method (58, 86). Briefly, bifidobacterial cell pellets were resuspended in 1 ml of QIAzol lysis reagent (Qiagen, Germany) in a sterile tube containing glass beads. Cells were lysed by alternating 2 min of stirring the mix on a bead beater with 2 min of static cooling on ice. These steps were repeated three times. Lysed cells were centrifuged at 12,000 rpm for 15 min, and the upper phase was recovered. Bacterial RNA was subsequently purified using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Then, the RNA concentration and purity were evaluated using a spectrophotometer (Eppendorf, Germany).

mRNA sequencing analysis. Total bacterial RNA (from 100 ng to 1 μg) was treated to remove rRNA by means of the QIAseq FastSelect – 5S/16S/23S following the manufacturer's instructions (Qiagen, Germany). The yield of rRNA depletion was checked by the use of a 2200 TapeStation (Agilent Technologies, USA). Then, a whole transcriptome library for prokaryotic RNA was constructed using the TruSeq Stranded mRNA Sample preparation kit (Illumina, San Diego, USA). Samples were then loaded onto a NextSeq high-output v2 kit (150 cycles) (Illumina) as indicated by the technical support guide. The obtained reads were filtered to remove low-quality reads using fastq-mcf tool (minimum mean quality 20, minimum length 100 bp) as well as any remaining ribosomal locus-encompassing reads (66). The retained reads were then aligned to the complete, closed PRL2023 genome sequence through Bowtie2 software (87). Subsequently, quantification of reads mapped to individual transcripts was achieved through htseq-counts script of HTSeq software in "union" mode (88). Raw counts were then normalized using the Trimmed Mean of M-Values (TMM) method implemented in the EdgeR package (version 3.6.1) ((89) and Log2 fold change (logFC) was used to evaluate the differences in gene expression of PRL2023 cultivated alone (reference

condition), and in bi- or multi-associations (test conditions). EdgeR package was also used to identify differentially expressed genes at a false discovery rate (FDR) of 5 % and minimal logFC 1.

Mucin adhesion assay of *B. adolescentis* PRL2023. The effect of bifidobacterial adhesion on mucin was assessed by adapting the protocol described by Valeriano *et al*. (50). Briefly, 100 µL of a 1 mg mL^{-1} sterile mucin dissolved in PBS (pH 7.4) was aliquoted into a 96-well microtiter plate (Sarstedt, Germany) and incubated overnight at 4°C. Subsequently, each well was washed with 200 µL of PBS, rinsed, filled with 100 µL of a 20 mg mL^{-1} sterile bovine serum albumin solution, and incubated at 4°C for 2 h. Two *B. adolescentis* strains, *i.e.*, PRL2023 and ATCC 15703, were grown at 37°C under anaerobic conditions (2.99% H_2, 17.01% CO_2 and 80% N_2) (Concept 400; Ruskin) in MRS broth (Sharlau Chemie, Barcelona, Spain) supplemented with 0.05% (wt/vol) L-cysteine HCl. Bifidobacterial growth was monitored until a concentration of 10^8 CFU mL^{-1} was reached. 100 µL of a corresponding bacterial suspension, previously washed and resuspended in PBS, was added in each well and incubated under anaerobic conditions at 37°C for 1 h. After incubation, each well was washed three times with 200 µL of PBS to remove unbound bacteria. Then, 200 µL of 0.5% (vol/vol) Triton X-100 was added and incubated at room temperature for 2 h, with gentle agitation to detach the adherent bacteria. The viable cell count expressed as CFU mL^{-1} was determined in all cases by plating on MRS medium. Each assay was performed in triplicate. Percentage adhesion was calculated as follows:

$$\textbf{\% relative adhesion} = (\textbf{logCFU}_{\textbf{N adhered}}/\textbf{logCFU}_{\textbf{N inoculum}}) \times 100$$

Adhesion of *B. adolescentis* PRL2023 to HT29-MTX cells. Bifidobacterial adhesion to HT29-MTX cells was assessed following the protocol described by

Serafini *et al.* (90, 91) Briefly, human colorectal adenocarcinoma HT29-MTX cells (kindly provided by Professor A. Baldi, University of Milan) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin and maintained in standard culture conditions. For the experiments, HT29-MTX cells were seeded on microscopy cover glasses previously settled into 10-cm^2 petri dishes. Confluent cells were carefully washed twice with PBS before bacterial cells were added. *B. adolescentis* strains, *i.e.*, PRL2023 and ATCC 15703, and *B. animalis* subsp. *lactis* BB12 were grown as previously described, until a concentration of 5×10^7 CFU mL^{-1} was reached. The strains were then centrifuged at 3,000 rpm for 8 min, resuspended in PBS (pH 7.3), and incubated with monolayers of HT29-MTX cells. After 1 h incubation at 37°C, cultures were washed twice with 2 mL of PBS to remove unbound bacteria. The cells were then fixed with 1 mL of methanol and incubated for 8 min at room temperature. Cells were then stained with 1.5 mL of Giemsa stain solution (1:20) (Sigma-Aldrich, Milan, Italy) and left in the dark for 30 min at room temperature. After two washes with 2 mL of PBS, the cover glasses were removed from the petri plate, mounted on a glass slide, and examined using a phase-contrast microscope Zeiss Axiovert 200 (objective, 100×/1.4 oil). Adherent bacteria in 20 randomly selected microscopic fields were counted and averaged. The proportion of bacterial cells that remained attached to the HT29-MTX monolayer was determined to reflect the extent of specific host-microbe interaction. The adhesion index represents the average number of bacterial cells attached to 100 HT29-MTX cells (47, 48, 91). A non-parametric Mann Whitney test was applied for the detection of statistically significant differences. All assays were performed at least in triplicate.

Statistical analysis. Similarities between samples (beta-diversity) were calculated by the Bray-Curtis dissimilarity matrix based on species abundance, using the "vegdist" function on RStudio (http://www.rstudio.com/). Beta-diversity was represented through Principal Coordinate Analysis (PCoA) using the function "ape" of the R suite package (92) Moreover, the various detected bacterial species were tested and plotted on the PCoA using the "envfit" and "plot" functions from vegan through R-studios (http://www.rstudio.com/). PERMANOVA analyses were performed on RStudio using 999 permutations to estimate p-values for population differences in PCoA analyses with adonis2 package. Furthermore, a correlation analysis between the available metadata and the various detected bacterial species of all samples was performed through Spearman's rank correlation coefficient using "rcorr" function (https://CRAN.R-project.org/package=Hmisc), and only results that were significantly different from a statistical perspective were retained. The False Discovery Rate (FDR) correction is applied to all statistical analyses based on Benjamini and Hochberg Correction through "p.adjust" function (93).

Data availability. Raw sequences of metatranscriptomics experiments were accessible through the SRA study BioProject PRJNA1011847. Furthermore, the updated genome sequence of *B. adolescentis* PRL2023 was deposited in the GenBank database with the NCBI RefSeq Accession code CP133648.

Competing interests

The authors declare that they have no competing interests.

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Chapter 8

Genomic and ecological approaches to identify the *Bifidobacterium breve* prototype of the healthy human gut microbiota

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Abstract

Members of the genus *Bifidobacterium* are among the first microorganisms colonizing the human gut. Among these species, strains of *Bifidobacterium breve* are known to be commonly transmitted from mother to her newborn, while this species has also been linked with activities supporting human well-being. In the current study, an *in silico* approach, guided by ecology- and phylogenome-based analyses, was employed to identify a representative strain of *B. breve* to be exploited as a novel health-promoting candidate. The selected strain, *i.e.*, *B. breve* PRL2012, was found to well represent the genetic content and functional genomic features of the *B. breve* taxon. We evaluated the ability of PRL2012 to survive in the gastrointestinal tract and to interact with other human gut commensal microbes. When co-cultivated with various human gut commensals, *B. breve* PRL2012 revealed an enhancement of its metabolic activity coupled with the activation of cellular defense mechanisms to apparently improve its survivability in a simulated ecosystem resembling the human microbiome.

Introduction

The human body harbours a multitude of microorganisms colonizing a given ecological niche, such as the mouth, pharynx, respiratory system, stomach, intestine, skin, and urogenital tract (Jandhyala et al., 2015). More specifically, with the term human microbiota, we refer to the set of microorganisms that colonize these various human body parts and which, through mutualistic relationships with the host, contribute to the maintenance of its health (Tojo et al., 2014; Bottacini et al., 2017; Hidalgo-Cantabrana et al., 2017; Alessandri et al., 2019). Among the human microbiotas, the intestinal microbiota comprises the most abundant and diverse microbial community of the human body. Bifidobacteria are commonly found as gut commensals throughout an individual's life (Turroni et al., 2011a, 2021), establishing with the host a multitude of trophic and immune interactions (Cebra, 1999; Hooper, 2004). Representatives of *Bifidobacterium bifidum*, *Bifidobacterium breve*, and *Bifidobacterium longum* are typically the first microbial colonizers of the infant gut microbiota (Turroni et al., 2011b, 2012).

Notably, *B. breve* strains are frequently isolated from stool samples of healthy breastfed infants (Collado et al., 2012) and milk samples of breastfeeding mothers (Khodayar-Pardo et al., 2014), highlighting events of vertical transmission between the mother and her newborn (Milani et al., 2015b). Furthermore, different studies have highlighted how this species is involved in protection against the development of allergies through its impact on the intestinal epithelial barrier, and in modulating the host's immune system (Inoue et al., 2009; Hougee et al., 2010; Bozzi Cionci et al., 2018). In this regard, a previous study showed the role of *B. breve* UCC2003 in the proliferative development of the intestinal epithelial cells during early life (Kiu et al., 2020).

Concerning the establishment of the infant gut microbiota, an *in silico* and *in vitro* analysis revealed the ability of *B. breve* to metabolize simple sugars that may be

encountered in the infant gut, such as lactulose, raffinose, maltose, lactose, and glucose (Bottacini et al., 2014). In addition, members of the *B. breve* species can metabolize (certain) Human Milk Oligosaccharides (HMOs), either directly or by cross-feeding, the latter involving other members of the bifidobacterial community such as *B. bifidum* (Egan et al., 2014). These activities clearly show how this species is specialized in colonizing the infant gut (Sela et al., 2008; Turroni et al., 2010; Egan et al., 2014; Lugli et al., 2020a). Interestingly, *B. breve* UCC2003 presents in its genome specific genes involved in the utilisation of particular HMOs, such as lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT) (James et al., 2016). Moreover, a comparative genomic analysis of the *B. breve* species (Bottacini et al., 2014) highlighted the presence of genes involved in the adaptation to the gastrointestinal environment, such as the production of extracellular structures like fimbria and exopolysaccharides (EPS) (Motherway et al., 2011). Particularly, an EPS produced by *B. breve* UCC2003 was shown to be involved in interactions with the host and protection from a pathogen (Fanning et al., 2012).

Previous research efforts have demonstrated the relevance of "omics" approaches in investigating the genetic makeup and activities of commensal bacteria (Fontana et al., 2022). Notably, analyses of bifidobacterial genomes have paved the way for a burgeoning field known as probiogenomics (Ventura et al., 2009). Specifically, research in the field enhanced our understanding of the diversity, evolutionary processes, and interaction with the human host as well as with other human gut commensals (Ventura et al., 2012; Turroni et al., 2018; Choi et al., 2021). More recently, an *in silico* analysis based on ecological and phylogenomic-driven approach allowed us to identify representative strains of the *Bifidobacterium* genus harbouring the human gut microbiota of healthy adults, *i.e.*, *B. longum* subsp. *longum* PRL2022 and *Bifidobacterium adolescentis* PRL2023 (Fontana et al., 2022; Alessandri et al., 2023).

Since *B. breve* has been incorporated as a functional ingredient in various probiotic supplements (Bozzi Cionci et al., 2018), we herein report a genome-based screening aimed at identifying a *B. breve* prototype of the adult gut. The identified representative *B. breve* strain, *i.e.*, PRL2012, was subjected to various omics-based evaluations by means of metatranscriptome analyses when co-cultivated with other bacterial species commonly found in the human microbiome, highlighting particular genetic features of PRL2012 that appear to respond to the presence of and/or sustain interactions with other commensal organisms.

Results and discussion

Ecological and phylogenomic-driven identification of the *B. breve* prototype. To evaluate the distribution of *B. breve* among the human gut microbiota, an InStrain-based profiling was applied to the genomes of 166 *B. breve* strains retrieved from the RefSeq NCBI database. First, to identify only autochthonous gut bacteria, *B. breve* strains formerly used as probiotics were removed from the analysis. Then, a de-replication procedure was applied using the dRep software among collected genome sequences, allowing the selection of 37 distinct genetic lineages of the *B. breve* species (Table 1 and Fig. 1). Finally, the distribution of these identified lineages across microbiomes of healthy individuals was investigated by means of a k-mer based analysis, employing 4,019 gut microbiomes of adults from 82 independent studies (Table S1), and an additional 9,505 gut microbiomes of infants (Lugli et al., 2023).

These analyses revealed that, when the *B. breve* species was detected, the associated lineage prevalence among infant microbiomes ranged from 7% to 13% for *B. breve* lineages 017W439 and 180W83, respectively (Table 1). Notably, the observed prevalence trend of the *B. breve* genetic lineages resembles a constant cumulative distribution without showing any overrepresented lineages in the infant gut. In contrast, among healthy adult microbiomes, when the *B. breve* species was detected, the lineage prevalence distribution ranged from 0% to 0.45% for *B. breve* 017W439 and *B. breve* NRBB09, respectively (Table 1). As expected, this data corroborates with a strong reduction of the overall distribution of members of the *B. breve* species during adulthood compared with infancy (Tarracchini et al., 2023).

Then, an Average x Prevalence index (AxP index) was generated, integrating genetic data produced as ANI values between de-replicated strains and ecological data based on the genetic lineage prevalence among metagenomes (Fontana et al., 2022). The procedure allowed the identification of *B. breve* prototypes in the gut of

adults and infants, represented by *B. breve* NRBB09 and 180W83, respectively (Table 1). Therefore, the RefBifSelector tool was employed to identify a representative *B. breve* strain among our local microbial collection (Table 2). The latter multi-omics approach was recently proposed as a tool to select reference strains of a particular (sub)species for *in vitro* analysis (Fontana et al., 2022). Thus, the genome sequence of 11 *B. breve* strains was compared to identify which one possesses the highest percentage of positive scoring matches (PPOS) in relation to the prototype of the adult human gut (Fontana et al., 2022). Accordingly, the genome sequence of the reference strain used as the prototype was *B. breve* NRBB09, whose AxP index was the highest (44.05) when exploring the distribution of the *B. breve* lineages among healthy adults (Table 1). As a result, *B. breve* PRL2012 showed a PPOS of 96.44, representing the optimal reference strain of our local microbial cell collection (Table 2). Interestingly, the selected *B. breve* strain was isolated from the milk sample of a lactating mother, representing a microorganism directly in contact with the newborn during the first days of its life (Mikami et al., 2009; Turroni et al., 2011a). This identified RefBif of the *B. breve* species was then further investigated through *in silico* genomic screenings and *in vitro* experiments to assess its interactions with other elements of the intestinal microbiota and with its host.

Table 1. *Bifidobacterium breve* strain distribution among publicly available datasets of the human gut microbiome.

			Adult (n=4,019)		Infant (n=9,505)	
NCBI code	**Strain**	**ANI[#]**	**Prevalence**	**AxP***	**Prevalence**	**AxP***
GCA_002838325.1	NRBB09	98.36	0.4%	44.05	9.6%	947.21
GCA_901212525.1	MC1	98.10	0.3%	34.17	13.0%	1270.70
GCA_902167875.1	B.breve_1_mod	98.08	0.3%	29.29	9.7%	948.86
GCA_020538685.1	MSK.23.130	98.10	0.3%	26.85	12.6%	1232.20
GCA_902167575.1	B.breve_2_mod	98.30	0.2%	22.01	10.8%	1057.37
GCA_002861455.1	UMB0915	98.10	0.2%	21.97	12.2%	1192.46
GCA_002838525.1	180W83	98.47	0.2%	19.60	13.1%	1285.29
GCA_003860285.1	lw01	98.10	0.2%	19.53	12.4%	1217.15
GCA_902167895.1	JG_Bg463	97.99	0.2%	19.51	11.3%	1105.42
GCA_014779815.1	142	98.14	0.2%	17.09	11.4%	1114.67
GCA_002838705.1	DRBB29	98.14	0.2%	17.09	10.1%	988.02
GCA_925285005.1	IM703	98.47	0.1%	14.70	10.7%	1058.10
GCA_002838365.1	NRBB50	98.46	0.1%	14.70	12.2%	1196.90
GCA_001990225.1	LMC520	98.32	0.1%	14.68	9.9%	972.65
GCA_000568955.1	PRL2012	98.52	0.1%	12.26	10.5%	1036.05
GCA_000247755.2	CECT 7263	98.47	0.1%	12.25	10.7%	1052.78
GCA_015547895.1	BSD2780061688	98.38	0.1%	12.24	10.6%	1038.89
GCA_013267755.1	JTL	98.62	0.1%	9.82	10.6%	1045.76
GCA_002838505.1	DRBB28	98.52	0.1%	9.81	10.2%	1001.59
GCA_000569015.1	JCM 7019	98.13	0.1%	9.77	7.7%	759.46
GCA_002914865.1	LMG S-29190	98.59	0.1%	7.36	10.7%	1051.87
GCA_002838645.1	NRBB20	98.55	0.1%	7.36	8.4%	832.74
GCA_902505445.1	LH_24	98.51	0.1%	7.35	9.9%	972.43
GCA_002838385.1	NRBB52	98.50	0.1%	7.35	8.5%	834.51
GCA_003813065.1	FDAARGOS_561	98.50	0.1%	7.35	9.2%	902.33
GCA_000220135.1	UCC2003	98.48	0.1%	7.35	8.6%	847.26
GCA_000226175.2	DPC 6330	98.46	0.1%	7.35	8.6%	850.29

GCA_002838425.1	NRBB56	98.40	0.1%	7.34	8.5%	836.82
GCA_002838345.1	NRBB57	98.59	0.0%	4.91	8.6%	848.17
GCA_009931415.1	JR01	98.56	0.0%	4.90	9.6%	949.16
GCA_000568975.1	JCM 7017	98.51	0.0%	4.90	8.5%	836.71
GCA_000411435.1	HPH0326	98.44	0.0%	4.90	8.7%	859.76
GCA_001189355.1	BBRI4	98.43	0.0%	4.90	8.5%	837.08
GCA_024760465.1	1101A	98.42	0.0%	4.90	8.6%	847.77
GCA_002838225.1	DRBB26	98.41	0.0%	4.90	8.7%	853.07
GCA_002838485.1	215W447a	98.50	0.0%	2.45	8.2%	807.57
GCA_002838465.1	017W439	98.57	0.0%	0.00	7.2%	713.28

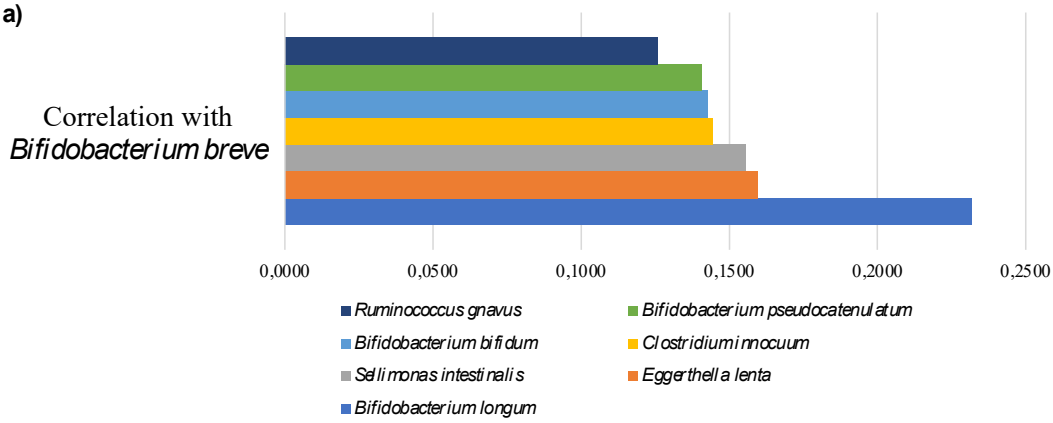
*Average x Prevalence index

[#]ANI between dereplicated genomes

Table 2. Identification of a genetically representative strain to the *B. breve* NRBB09 prototype.

Query (NCBI code)	*B. breve* Strain	ANI	Average PPOS (percentage of positive scoring matches)	ANI x Average PPOS
GCA_000568955.1	PRL2012	98.2	96.43	9469.6
GCA_000568895.1	2L	98.2	96.43	9468.8
GCA_000568875.1	31L	98.0	96.18	9422.1
GCA_002075865.1	1900B	98.2	95.62	9389.6
local	676B	98.0	95.65	9375.8
GCA_000569055.1	689b	98.0	95.64	9372.5
local	158B	98.1	95.51	9368.8
GCA_002076075.1	1889B	98.1	95.51	9367.9
GCA_002076055.1	1891B	97.7	95.31	9308.7
GCA_016648985.1	M1D	97.6	95.28	9300.5
GCA_016648955.1	PRL2020	97.7	95.23	9299.8

a)



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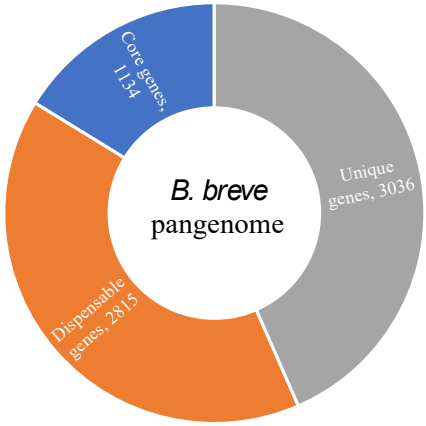


c)

Genome	Strain	TUGs
GCA_002838705.1	DRBB29	143
GCA_002838325.1	NRBB09	134
GCA_002861455.1	UMB0915	132
GCA_902167895.1	JG_Bg463	130
GCA_002838485.1	215W447a	130
GCA_020538685.1	MSK.23.130	127
GCA_000569015.1	JCM 7019	123
GCA_901212525.1	MC1	107
GCA_009931415.1	JR01	105
GCA_014779815.1	142	96
GCA_001990225.1	LMC520	93
GCA_003860285.1	lw01	91
GCA_001189355.1	BBRI4	85
GCA_002838505.1	DRBB28	83
GCA_002838345.1	NRBB57	83
GCA_002838425.1	NRBB56	81
GCA_002838365.1	NRBB50	81
GCA_002838645.1	NRBB20	79
GCA_902505445.1	LH_24	75
GCA_000226175.2	DPC 6330	75
GCA_002914865.1	LMG S-29190	74
GCA_925285005.1	IM703	72
GCA_002838385.1	NRBB52	72
GCA_000220135.1	UCC2003	68
GCA_000247755.2	CECT 7263	67
GCA_000411435.1	HPH0326	67
GCA_024760465.1	1101A	65
GCA_902167575.1	B.breve_2_mod	64
GCA_902167875.1	B.breve_1_mod	64
GCA_015547895.1	BSD2780061688	62
GCA_002838465.1	017W439	53
GCA_000568975.1	JCM 7017	48
GCA_002838225.1	DRBB26	47
GCA_002838525.1	180W83	46
GCA_013267755.1	JTL	41
GCA_003813065.1	FDAARGOS_561	40
GCA_000568955.1	PRL2012	33

d)

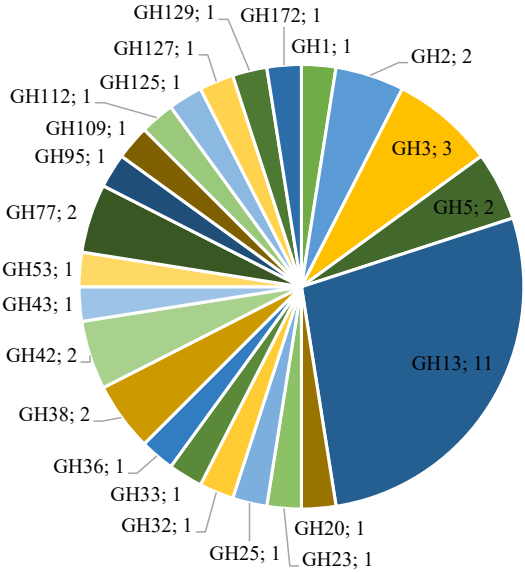


Figure 1

Figure 1. Genomic analyses of representative strains of the 37 identified *B. breve* lineages. Panel a) shows the correlation values of those bacterial species displaying a positive correlation with the *B. breve* taxon, which were statistically significant in shaping the variability of the gut microbiome. Panel b) exhibits the number of core genes (blue), unique genes (grey), and dispensable genes (orange) identified in the pangenome of the *B. breve* species. Panel c) displays the unique gene (TUG) distribution among representative strains of the *B. breve* lineages. Panel d) depicts the glycosyl hydrolase (GH) distribution in PRL2012.

Understanding the molecular cross-talk between gut commensals and *B. breve*.
As previously reported, the ability of *B. breve* PRL2012 to interact with other gut commensals, such as *B. bifidum* PRL2010, *B. adolescentis* 22L, and *B. longum* subsp. *infantis* ATCC15697, was investigated under *in vivo* conditions, analysing its transcriptome from murine cecum samples following its supplementation to conventional BALB/c mice (Turroni et al., 2016). Accordingly, the effects of bifidobacterial co-association between strains were explored by the administration of multiple strains, while their transcript expression was profiled by using custom-made arrays representing 98% of the genes harboured (Turroni et al., 2016). Among the four bifidobacterial species tested, PRL2012 showed the highest cross-talk index, *i.e.*, representing the total number of genes whose expression was modulated by the presence of PRL2012, suggesting that its transcriptome was highly affected by interactions with other bifidobacterial strains (Turroni et al., 2016). Interestingly, the re-analysis of the overexpressed genes unveiled that when PRL2012 was administered to mice at a daily dose of 10^9 colony-forming units, following the cluster orthologues gene (COG) classification, a large part of the up-regulated genes (≥2-fold change) was predicted to be associated with amino acid and carbohydrate transport and metabolism (>30% of the up-regulated genes). Similarly, administration of a combination of multiple bifidobacterial species to mice revealed a tendency of the PRL2012 strain in the up-regulation of COGs related to translation and replication, followed by amino acid and carbohydrate metabolism-related genes. Notably, cross-feeding interactions between members of the bifidobacterial species and the *B. breve* taxon, as well as with other gut commensals such as *Faecalibacterium prausnitzii* and *Eubacterium hallii* have previously been explored (Egan et al., 2014; Moens et al., 2016; Bunesova et al., 2018). Nonetheless, a complete understanding of the cross-talk among *B. breve* and other microbial taxa that coexist in the same intestinal environment is still lacking.

A correlation analysis between the commensals of the above reported 4,019 gut microbiomes of adults was performed to assess if *B. breve* PRL2012, as a proposed *B. breve* prototype of the adult human gut, is able to persist in the adult human gut and to exert benefits to the host not only during weaning but also in adulthood. This correlation analysis pinpointed those species that frequently coexist within the same ecosystem with the *B. breve* taxon. In total, 66 microbial species exhibited a statistically significant positive correlation with *B. breve* (Benjamini-Hochberg, FDR *p*-values < 0.05) (Table S2). Subsequently, a Principal Coordinate Analysis (PCoA) was performed to delve more deeply into the identification of those bacterial species that are significant in terms of their impact on the human gut microbiota variability. After normalizing the data, it became evident that out of the 66 bacterial species displaying a positive correlation with the *B. breve* taxon, only 20 of them were statistically significant in shaping the variability of the gut microbiome (R^2 > 0.2, FDR *p*-values < 0.01) (Table S2). Among them *Bifidobacterium longum*, *Eggerthella lenta*, *Sellimonas intestinalis*, *Clostridium innocuum*, *Bifidobacterium bifidum*, *Bifidobacterium pseudocatenulatum*, and *Ruminococcus gnavus* fit into the eight species with the highest correlation values with *B. breve*, illustrating a distinct interconnection between the bacteria influencing the diversity of the human gut and *B. breve* (Fig. 1).

Genetic features of *B. breve* PRL2012. To obtain a comprehensive view of the genetic traits of PRL2012, its genome sequence was decoded employing a combination of short- and long-read technologies (see Materials and Methods), resulting in a complete genome sequence (*i.e.* a single contig representing a circular chromosome). Then, a comparative genomic analysis of the *B. breve* species was performed, including the predicted encoded proteome of each representative strain of the above-identified 37 lineages (Table 1 and Fig. 1). In this manner, pangenome

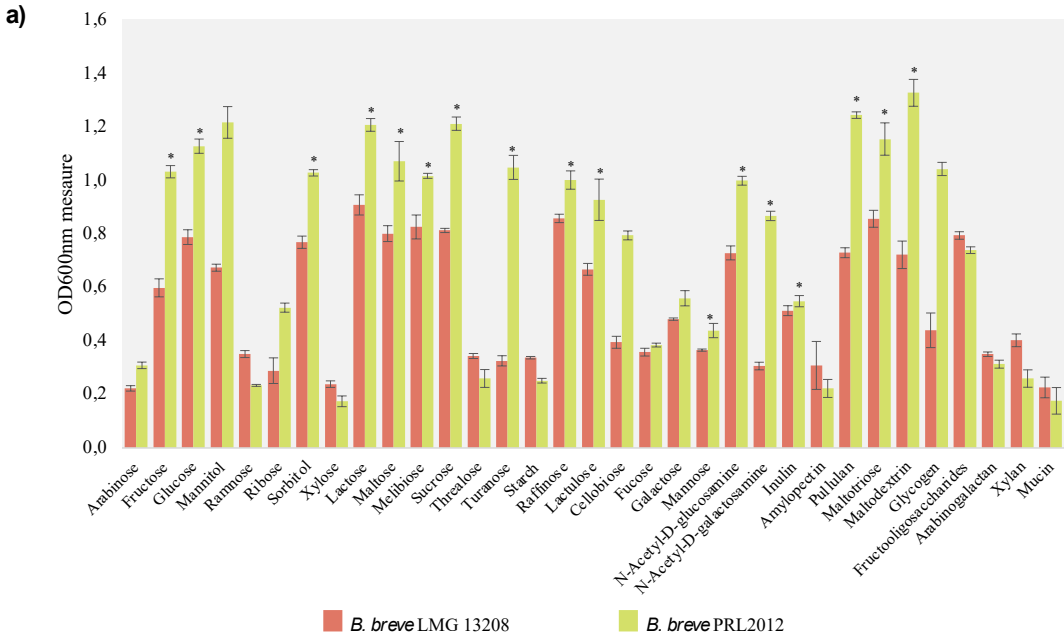
and core-genome analyses of this taxon were undertaken following a previously described method based on Clusters of Orthologous Groups (COGs) (Lugli et al., 2018, 2019, 2020b). The analysis resulted in the identification of 6,985 COGs, representing the pangenome of the representative strains covering all 37 lineages. Among them, 1,134 COGs were shared between the 37 *B. breve* genomes, thus representing their core genome (Fig. 1). Furthermore, truly unique genes (TUGs) of each strain were detected with an average of 82 TUGs per genome. Interestingly, *B. breve* PRL2012 was shown to be the strain with a lowest number of TUGs (n= 33) when compared to members of the other lineages (Fig. 1). Accordingly, the number of genes harboured by PRL2012 was shown to be lower than that of all other lineage-representative *B. breve* strains, suggesting a stable genome structure typical of microorganisms specialized in living in a defined ecological niche. In this context, two putative genes encoding carbohydrate-active enzymes were found among the identified PRL2012-specific TUGs.

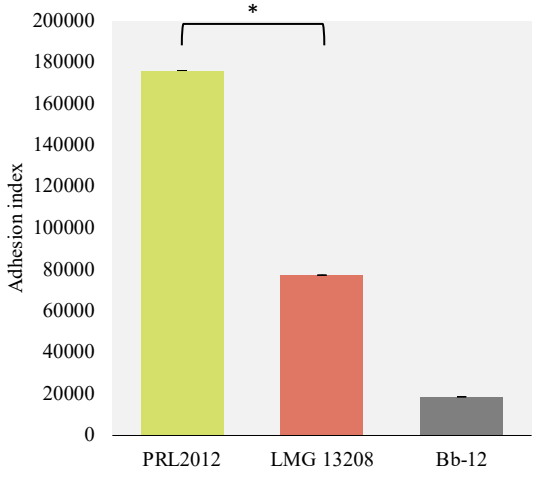
An *in silico* prediction of the PRL2012 genome, based on the Carbohydrate-Active enZYmes (CAZy) database (Drula et al., 2022), revealed that 40 genes were identified as glycosyl hydrolases (GHs) encompassing 23 different families (Fig. 1). Among them, most of the identified GHs (n =11) were predicted to encode members of the GH13 family, encompassing predicted a-glucosidase, amylase and pullulanase enzymes, which would be consistent with previously described starch- and pullulan-degrading activities of *B. breve* (Ryan et al., 2006). Notably such genes were also identified in the core genome of the species through comparative genomics analyses (Bottacini et al., 2014). On the other hand, additional GH family members encoded by the genome of PRL2012 were identified to process a wide variety of carbohydrates, such as α-galactosidase (GH36), β-galactosidase (GH2 and GH42), lacto-N-biosidase (GH20), α-mannosidase (GH38 and GH125), and β-L-arabinofuranosidase (GH127) (Fig. 1). The deduced glycobiome of PRL2012

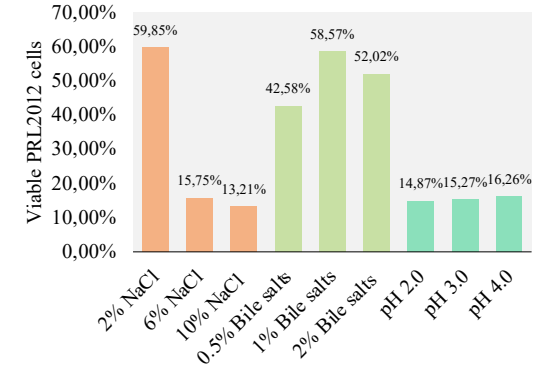
displays an overall glycan degradation capability, which is in line with that of other *B. breve* strains (Bottacini et al., 2014). To better understand *B. breve* PRL2012's actual ability in processing glycans, we set up *in vitro* growth experiments with various carbon sources.

Ability of *B. breve* PRL2012 to metabolize carbohydrates. Growth capabilities of *B. breve* PRL2012 on different carbohydrates were evaluated and compared with those obtained for the *B. breve* type strain, *i.e.*, LMG 13208 (Fig. 2). The choice of the latter strain was due to the results of *in silico* tracking analyses, which emphasized that the type strain of the species was less prevalent in healthy individuals compared to PRL2012 (Table 1). These carbohydrates (see Materials and Methods) include both plant- and host-derived glycans that are commonly found in the adult human gut microbiota (Chassard and Lacroix, 2013). To evaluate the carbohydrate growth capabilities of *B. breve* strains, we used a carbohydrate-free based MRS medium, which was supplemented with one of 33 different sugars, as the unique carbon source (Table S3 and Fig. 2). Results of each growth-profiling experiments were processed using a Mann-Whitney test with Benjamini–Hochberg correction (cut-off p-value < 0.05), highlighting statistically significant differences in growth performances between the two *B. breve* strains. In detail, PRL2012 shows broader metabolic capabilities on different monosaccharides and disaccharides, such as fructose, glucose, sorbitol, lactose, maltose, melibiose, sucrose, turanose, and raffinose (final OD value greater than 1.0, all Benjamini–Hochberg corrected p-values < 0.05) (Table S3). In addition, PRL2012 shows appreciable growth on different complex monosaccharides, disaccharides and polysaccharides, such as N-Acetyl-D-galactosamine, N-Acetyl-D-glucosamine, lactulose, pullulan and maltodextrin (final OD value from 0.4 to 1.3; all Benjamini–Hochberg corrected p-values < 0.05) (Table S3 and Fig. 2). These data demonstrate significantly broader

growth performances of *B. breve* PRL2012 compared with the type strain of this species, corroborating our *in silico* analyses on PRL2012.







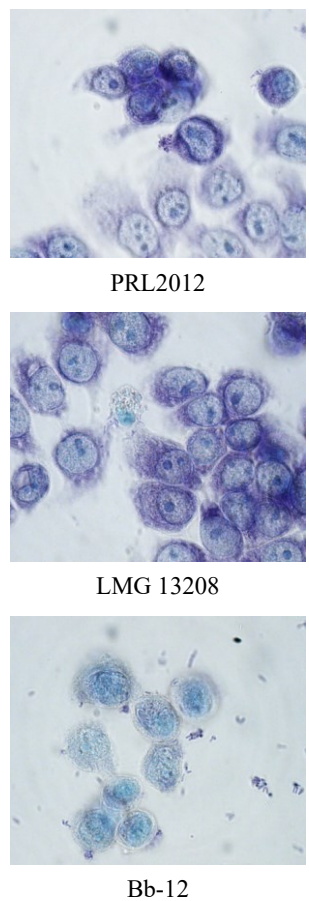


Figure 2

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Figure 2. *In vitro* evaluation of the capability of the PRL2012 prototype. Panel a) shows the growth performance of *B. breve* PRL2012 and LMG 13208 on different carbohydrates as measured by optical density at 600 nm (OD_{600nm}). Panel b) displays the adhesion index of *B. breve* PRL2012 and LMG 13208 and *B. animalis* subsp. *lactis* Bb-12 cells to HT29-MTX cells monolayers. The vertical bars indicate standard deviations, and the asterisks indicate Mann-Whitney test *p*-values < 0.05. Panel c) exhibits light microscopic images of HT29-MTX monolayer cells as observed with Giemsa staining of *B. breve* PRL2012 and LMG 13208 and *B. animalis* subsp. *lactis* Bb-12 cells. Panel d) Tolerance of *B. breve* PRL2012 strain toward human gut challenges. The Y axis represents the percentage of the viable PRL2012 cells.

Evaluation of the adhesion features of PRL2012 toward eukaryotic cells. To support PRL2012 strain resilience in the human gut, its adhesive performances to human intestinal mucosal cells were evaluated using a previously described methodology (Guglielmetti et al., 2008; Turroni et al., 2013; Rizzo et al., 2023), seeding cells of *B. breve* PRL2012 on human colon intestinal HT29-MTX monolayers. After multiple washes with PBS, the number of microbial cells attached to the HT29-MTX monolayer was assessed in order to calculate the adhesion index, which represents the number of bacterial cells adhering to 100 HT29-MTX cells (Guglielmetti et al., 2008). Interestingly, a higher adhesion index was observed for *B. breve* PRL2012 (adhesion index of $176{,}000 \pm 8$) compared to *B. breve* LMG 13208 (adhesion index of $77{,}333 \pm 6$) (Mann Whitney test p-value < 0.05) (Fig. 2). Furthermore, adhesion of the *Bifidobacterium animalis* subsp. *lactis* Bb-12 strain, commonly used as a health-promoting microorganism in many probiotic supplements (Garrigues et al., 2010; Jungersen et al., 2014), was assayed. Results indicated an adhesion index of Bb-12 less than 20,000 (Fig. 2), highlighting the markedly high adhesion yield of *B. breve* PRL2012 cells toward human cell monolayers. In addition, an adhesion assay on mucin was performed involving the two *B. breve* strains, highlighting an enhanced relative adhesion to mucin of *B. breve* PRL2012 when compared to the type strain LMG 13208, *i.e.*, 73.4% and 66.1%, respectively (Fig. 2). Additionally, PRL2012 demonstrates resilience toward typical adverse conditions that bacteria must confront during the passage through the gastrointestinal tract (Fig. 2) (see Supplementary Materials). These data confirm our previous observations, suggesting that *B. breve* PRL2012 is ecologically adapted to the human gut microbiota ecosystem and probably possesses genes involved in contributing to its (long term) colonization ability of the human intestinal mucosa.

Investigating the molecular interplay between PRL2012 and typical human gut microbiota members. To assess the interactions between PRL2012 and bacterial species that are typically associated with the adult human gut microbiota, we performed co-cultivation experiments of PRL2012 with those commensal bacteria previously identified to be significant in shaping the human adult gut microbiota and, at the same time, were significantly correlated with *B. breve* in the same environment (Table S2). In detail, a PCoA analysis was performed using the abundance data of the microbial species inhabiting the gut microbiomes of 4,019 healthy adults, allowing the identification of those bacterial species that drive the human gut microbiota biodiversity. Then, identified bacteria correlating with the *presence of B. breve* were selected (Table S2). Thus, six co-cultivation assays were performed using *B. breve* PRL2012 co-incubated with *B. bifidum* PRL2010, *B. longum* PRL2022, *B. pseudocatenulatum* LMG 10505, *Clostridium innocuum* 107F, *Eggerthella lenta* 180F, and *Ruminococcus gnavus* DSM 114966. Since exploring the molecular interplay between two bacterial strains is rather limited to understand the complex relationships occurring within the gut microbiota, we performed a co-cultivation experiment involving all the above-mentioned microbes, along with PRL2012. A quantitative PCR (qPCR) approach was applied to evaluate the bacterial load of each species from co-cultivation experiments. This qPCR analysis highlighted that PRL2012 can grow in all coculture experiments with various tested gut commensals (Fig. S1), since PRL2012 cells identified always yielded a genome copy number higher than $2x10^8$ per mL at the end of the co-cultivation experiments. Shotgun metatranscriptomics was then performed in each co-cultivation experiment to explore the interactions of PRL2012 with other bacterial commensals. Compared to the reference condition (PRL2012 grown in a monoculture), PRL2012 revealed a notable impact on its transcriptome (number of genes whose expression was significantly modified) only when it was grown in

combination with other bacteria (120 up-regulated genes vs. 42 down-regulated genes) (Table S4).

Transcriptomic data of PRL2012 co-cultivated with six different human gut commensals revealed an enhancement of the transcription of multiple genes related to bacterial metabolism, specifically those predicted to be involved in carbohydrate metabolism (Table S4), which is a common strategy of bacteria competing for the same resources (Khoroshkin et al., 2016). The identified up-regulated genes belong to various transporter systems encoded by genes that are scattered across the PRL2012 chromosome, including, among the most significant, a predicetd ABC transporter for rhamnose (3.A.1.2.9), as well as a putative ABC transporter for maltose (3.A.1.1.27) (Table S4). Furthermore, genes encoding putative multicomponent transporters composed and predicted to be involved in the uptake of methionine (3.A.1.24.4), glutathione (3.A.1.5.26), ascorbate (4.A.7.1.2) and branched chain hydrophobic amino acids (3.A.1.4.10) were shown to be up-regulated (Table S4). Additionally, transporters composed of a single subunit, which were predicted to be dedicated to the uptake of proline (2.A.21.2.5), manganese (2.A.55.3.3), fructose (2.A.1.7.17), and various metabolites (2.A.1.6.10) and amino acids (2.A.3.3.22), were shown to be up-regulated following the co-cultivation of PRL2012 with other gut commensals (Table S4). Overall, the ability to shift its carbohydrate and amino acid metabolism among various substrates may be reflective of an adaptive strategy enabling PRL2012 to successfully establish itself in the gut microbiota of adults.

Interestingly, genes directly linked to metabolic processes were not the only genes that underwent an upregulation upon co-cultivation of PRL2012 with multiple bacteria. A two-component multidrug efflux pump (3.A.1.135.6) of PRL2012 was also found to be up-regulated, probably as a defense system when sharing the same environment with multiple bacteria (Table S4). Also, a cluster of six genes

putatively encoding a biosynthetic machinery to produce an exopolysaccharide (EPS) were activated in co-culture, revealing a possible enhancement in production of these extracellular macromolecules that have previously been shown to play a key role in microbe-microbe interactions (Neville et al., 2013).

Conclusions

An extensive body of scientific literature supports the well-established benefits of bifidobacteria toward the human gut, which include promoting the integrity of the intestinal barrier, enhancing gut homeostasis, and aiding the development of the host's immune system (Alessandri et al., 2019). In this regard, *B. breve* stands out as one of the 17 most prevalent bacterial species residing in the gastrointestinal tract of infants (Lugli et al., 2023). Thus, we applied an innovative ecological and phylogenomic-driven approach to identify the most representative phylotype of this species within the human gut of infants and adults (Fontana et al., 2022; Alessandri et al., 2023). From our local microbial collection, *B. breve* PRL2012 was shown to exhibit the closest genetic resemblance to the phylotype of the species, while comparative genome analysis revealed a stable genome structure with a very small number of unique genes, two of which were predicted to be involved in carbohydrate metabolism. In this context, the metabolic capabilities of PRL2012 to swiftly adapt its carbohydrate metabolism to compete with other members of the human microbiota, as well as its ability to activate cellular defence mechanisms, were validated through a metatranscriptomic investigation in combination with other intestinal commensals. Similarly, its crosstalk ability was supported by the results of *in vitro* experiments demonstrating that PRL2012 has the capacity to survive and potentially colonize the intricate ecological niche of the human intestine, interacting with the epithelium, adhering to the host mucosa, and

withstanding stressful conditions commonly encountered in the intestinal environment.

Based on the results achieved from our *in silico* and *in vitro* analyses, PRL2012 represents a promising candidate to be used as health-promoting bacterium. Data suggest that its ecological adaptation to the human gut microbiota ecosystem contributes to its ability for long-term colonization of the human intestinal mucosa. Notably, the long-term impact of a bacterium that can persist in the human gut is superior to a transient one. However, to effectively study its ability to persist within the human gut, a clinical trial involving this strain will be essential to enhance our scientific understanding of PRL2012 and validate our *in vitro* findings regarding its interactions with the human host.

Materials and Methods

Metagenome dataset selection. In this project, 4,019 publicly available datasets of human gut microbiome were retrieved from 82 cohorts of healthy individuals (Table S1). Additionally, 9,505 samples belonging to infants (age < 3 years) were used to explore the infant-associated microbial diversity (Lugli et al., 2023).

***B. breve* prototype selection.** Complete and partial genome sequences of 166 *B. breve* strains were retrieved from the RefSeq NCBI database, representing a collection of publicly available genome sequences of this taxon. Then, the genome sequence of reference strain LMG 13208 was used to discard those strains showing an average nucleotide identity (ANI) lower than 94%, employing fastANI (Jain et al., 2018) to discard misclassified microorganisms. Furthermore, the quality of genomes was estimated for completeness and contamination using CheckM (Parks et al., 2015). High-quality genomes were then subjected to a de-replication based analysis aimed at reducing strain redundancy among bifidobacterial genome

sequences using dRep v2.0 (Olm et al., 2017). Among strains displaying sequence identity >99.8%, a single reference genome was selected for further analysis, representing putative prototypes of the *B. breve* species. Then, a k-mer based analysis to explore the distribution of each putative prototype was investigated using InStrain software with a k-mer size of 23 (Olm et al., 2021). The selection of the *B. breve* prototype of the human gut was chosen using a previously validated index called AxP (Fontana et al., 2022), defined as [the average ANI value of genomes constituting the same clade] * [prevalence score of the strain in the dataset] * [100].

Genome sequencing. The genome sequence of PRL2012 was determined by GenProbio Srl (Parma, Italy) using the MiSeq platform (Illumina, UK). Genome libraries were prepared using an Illumina
Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA 92122, USA). Libraries were quantified using a fluorometric Qubit quantification system (Life Technologies, USA), loaded on a 2200 TapeStation instrument (Agilent Technologies, USA), and normalized to 4 nM. Sequencing was performed using the Illumina MiSeq platform with a 600-cycles flow cell version 3 (Illumina Inc., San Diego, CA 92122, USA). PRL2012 extracted DNA was also subjected to whole-genome sequencing using the Nanopore DNA sequencing platform according to the supplier's protocol (Oxford Nanopore, UK).

Genome assembly. Long reads were filtered by quality using the Filtlong tool (https://github.com/rrwick/Filtlong), while short reads were filtered through the fastq-mcf script (https://github.com/ExpressionAnalysis/ea-utils). Filtered fastq files of Nanopore long reads obtained from genome sequencing efforts were then used as input for genome assembly through CANU software (Koren et al., 2017).

The resulting genome sequence has been polished through Polypolish (Wick and Holt, 2022) using Illumina paired-end reads. The whole process was managed by the MEGAnnotator2 pipeline (Lugli and Ventura, 2022).

Comparative genomics. Open reading frames (ORFs) of each *B. breve* genome were predicted with Prodigal (Hyatt et al., 2010) and annotated utilizing the MEGAnnotator2 pipeline (Lugli et al., 2016; Lugli and Ventura, 2022). Proteomes were employed for a pangenome calculation using the PGAP (Zhao et al., 2012), to identify orthologues among analyzed *B. breve* strains by means of BLAST analysis (cut-off E-value, $<1*10^{-5}$; 50% identity over at least 80% of both protein sequences). The resulting output was then clustered into protein families named Clusters of Orthologous Groups (COGs) by means of MCL (graph theory-based Markov clustering algorithm), using the gene family (GF) method. Pangenome profiles were built using an optimized algorithm incorporated in the PGAP software, based on a presence/absence matrix that included all identified COGs in the 37 analyzed genomes. Furthermore, genes encoding for transporters have been annotated using the Transport Classification (TC) system (Saier et al., 2021).

Glycobiome profiling. The proteome of PRL2012 was screened for genes predicted to encode carbohydrate-active enzymes based on sequence similarity to genes classified in the carbohydrate-active enzyme (CAZy) database (Drula et al., 2022). Thus, each gene sequence was screened for orthologues against the dbCAN2 meta server (Zhang et al., 2018) composed of 2,141,452 CDS using HMMER v3.3.2 (cut-off E-value of $1*10^{-15}$ and coverage >0.35) and DIAMOND (E-value $<1*10^{-102}$).

Cultivation conditions. *B. breve* PRL2012 was cultivated in de Man-Rogosa-Sharpe (MRS) medium (Sharlau Chemie, Spain) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (Merk, Germany) and incubated at 37°C in an anaerobic cabinet (Concept 400, Ruskinn) with an anaerobic atmosphere (2.99% H_2, 17.01% CO_2, and 80% N_2).

For bi-association experiments, *Bifidobacterium bifidum* PRL2010, *Bifidobacterium longum* PRL2022 and *Bifidobacterium pseudocatenulatum* LMG 10505 were cultivated as described above. In addition, *Clostridium innocuum* 107F, *Eggerthella lenta* 180F and *Ruminococcus gnavus* DSM 114966 (purchased from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) were cultivated anaerobically in yeast extract-casein hydrolysate-fatty acid (YCFA) medium in Hungate tubes at 37° C for 48h.

Carbohydrate growth assays. In order to extend *in silico* findings, we performed growth assays on selected carbon sources involving selected *B. breve* PRL2012 and the type strain LMG 13208. Notably, *in silico* analyses performed in this study generated predictions with regards to (carbohydrate) metabolic abilities of the above-mentioned strains and further discussed in the Results and discussion section. *B. breve* strains were cultivated overnight on a semisynthetic MRS medium supplemented with 0.05% (w/vol) L-cysteine hydrochloride at 37 °C under anaerobic conditions. Subsequently, cells were diluted in MRS without glucose to obtain an $OD_{600\ nm}$~1 and 15 µl of the diluted cells were inoculated in 135 µl of MRS without glucose supplemented with 1% (wt/vol) of a particular sugar in a 96-well microtiter plate and incubated in an anaerobic cabinet. Specifically, each carbohydrate was dissolved in MRS without glucose previously sterilized by autoclaving at 121°C for 15 min. Subsequently, each obtained solution was filter sterilized using a 0.2 µm filter size prior to use. Cell growth was evaluated by

monitoring the optical density at 600 nm with the use of a plate reader (Biotek, VT, USA). The plate was read in discontinuous mode, with absorbance readings performed at 3 min intervals for three times after 48 h of growth, and each reading was performed following 30 s of shaking at medium speed. Cultures were grown in triplicates, and the resulting growth data were expressed as the average of these replicates. Carbohydrates tested in this study were purchased from Merck (Germany) and include arabinose, fructose, glucose, mannitol, rhamnose, ribose, sorbitol, xylose, lactose, maltose, melibiose, sucrose, trehalose, turanose, soluble starch from potato, raffinose, lactulose, cellobiose, fucose, galactose, mannose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, inulin, amylopectin, pullulan, maltotriose, maltodextrin, glycogen, fructooligosaccharide (FOS), arabinogalactan, xylan and mucin from porcine stomach.

***In vitro* evaluation of the interaction of PRL2012 cells with other members of the human gut microbiota.** To evaluate if and how *B. breve* PRL2012 interacts with other gut microbial players, batch cultures were set up to co-cultivate the selected strain with each or a mix of six previously selected different intestinal commensals, *i.e.*, *B. bifidum* PRL2010, *B. longum* PRL2022, *B. pseudocatenulatum* LMG 10505, *C. innocuum* 107F, *E. lenta* 180F, and *R. gnavus* DSM 114966. For bi-association experiments, overnight cultures of each microorganism were diluted in order to obtain an OD value of 1.0, as previously described (Mancabelli et al., 2021). Each culture was inoculated at 0.1% (vol/vol) into yeast extract-casein hydrolysate-fatty acid (YCFA) medium (Dostal et al., 2015; Wylensek et al., 2020; Aranda-Díaz et al., 2022). We performed six different experiments in which *B. breve* PRL2012 was inoculated with each of six different intestinal players mentioned above and one experiment where all microorganisms were cultivated together. Batch cultures were performed in triplicate and incubated under anaerobic

conditions in Hungate tubes at 37°C. After 8h of incubation, cultures were centrifuged at 7000 rpm for 5 min, the supernatants were discarded, while the obtained bacterial pellets were used for RNA extraction (Scott et al., 2014; Schwab et al., 2017; Bunesova et al., 2018). Moreover, pellets were subjected to DNA extraction using the GeneElute bacterial genomic DNA kit (Sigma, Germany) following the manufacturer's instructions. Each sample was subjected to a different cycle of quantitative PCR (qPCR) using strain specific primers: Br12L_105Fw (5'-CGAAGTTCCAGTTCACCAT-3') and Br12L_105Rv (5'-GTTCTTGGCGTTCCAGATGT-3') for *B. breve* PRL2012. qPCR was performed using qPCR green master mix (PowerUp™ SYBR™ Green Master Mix for qPCR, Thermo Fisher Scientific, US) on a CFX96 system (Bio-Rad, CA, USA) following previously described protocols (Henriques et al., 2012; Milani et al., 2015a). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: one cycle of 50°C for 2 min, followed by one cycle of 95°C for 2, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The melting curve was 65°C to 95°C with increments of 0.5°C/s. In each run, negative controls (no DNA) were included. A standard curve was built using the CFX96 software (Bio-Rad).

RNA extraction. Total RNA from bacterial cells was isolated using a previously described method (Turroni et al., 2016; Milani et al., 2020). Briefly, bifidobacterial cell pellets were resuspended in 1 ml of QIAzol lysis reagent (Qiagen, Germany) in a sterile tube containing glass beads. Cells were lysed by alternating 2 min of stirring the mix on a bead beater with 2 min of static cooling on ice. These steps were repeated three times. Lysed cells were centrifuged at 12,000 rpm for 15 min, and the upper phase was recovered. Bacterial RNA was subsequently purified using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions.

Then, RNA concentration and purity were evaluated using a spectrophotometer (Eppendorf, Germany).

mRNA sequencing analysis. Total bacterial RNA (from 100 ng to 1 µg) was treated to remove rRNA by means of the QIAseq FastSelect – 5S/16S/23S following the manufacturer's instructions (Qiagen, Germany). The yield of rRNA depletion was checked using a 2200 TapeStation (Agilent Technologies, USA). Then, a whole transcriptome library for prokaryotic RNA was constructed using the TruSeq Stranded mRNA Sample preparation kit (Illumina, San Diego, USA). Samples were then loaded onto a NextSeq high output v2 kit (150 cycles) (Illumina) as indicated by the technical support guide. The obtained reads were filtered to remove low-quality reads using fastq-mcf tool (minimum mean quality 20, minimum length 100 bp) as well as any remaining ribosomal locus-encompassing reads (Milani et al., 2021). The retained reads were then aligned to the complete, closed PRL2012 genome sequence through Bowtie2 software (Langdon, 2015). Subsequently, quantification of reads mapped to individual transcripts was achieved through the htseq-counts script of HTSeq software in "union" mode (Anders et al., 2015). Raw counts were then normalized using the Trimmed Mean of M-Values (TMM) method implemented in the EdgeR package (version 3.6.1) (Robinson et al., 2010) and Log2 fold change (logFC) was used to evaluate the differences in gene expression of PRL2012 cultivated alone (reference condition), and in bi- or multi-associations (test conditions). EdgeR package was also used to identify differentially expressed genes at a false discovery rate (FDR) of 5 % and minimal logFC 1.

Mucin adhesion assay of *B. breve* PRL2012. The effect of bifidobacterial adhesion on mucin was performed by adapting the protocol described by Valeriano

et al. (Valeriano et al., 2014). Briefly, 100 µL of a 1 mg mL^{-1} sterile mucin solution dissolved in a buffer saline (PBS, pH 7.4) was aliquoted into 96-well microtiters (Sarstedt, Germany) and incubated overnight at 4°C. Subsequently, each well was washed with 200 µL of PBS, rinsed, filled with 100 µL of a 20 mg mL^{-1} sterile bovine serum albumin solution, and incubated at 4°C for 2 h. Bifidobacterial growth was monitored until a concentration of 10^8 CFU mL^{-1} was reached, after which 100 µL of the resulting bacterial suspension, previously washed and resuspended in PBS, was added in each well and incubated under anaerobic condition at 37°C for 1 h. After incubation, each well was washed two times with 200 µL of PBS to remove unbound bacteria. Then, 200 µL of 0.5% (vol/vol) Triton X-100 was added and incubated at room temperature for 2 h, under slight agitation to detach the adherent bacteria. The viable cell count expressed as CFU mL^{-1} was determined in all cases by plating on MRS medium. Each assay was performed in triplicate. Percentage adhesion was calculated as follows:

$$\textbf{\% relative adhesion} = (\log CFU_{N\ adhered} / \log CFU_{N\ inoculum}) \times 100$$

Adhesion of *B. breve* PRL2012 to HT29-MTX cells. Bifidobacterial adhesion to HT29-MTX cells was assessed following the protocol described by Serafini *et al.* (Guglielmetti et al., 2008; Serafini et al., 2013). Briefly, human colorectal adenocarcinoma HT29-MTX cells (kindly provided by Professor A. Baldi, University of Milan) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin and maintained in standard culture conditions. For the experiments, HT29-MTX cells were seeded on microscopy cover glasses previously settled into 10-cm^2 petri dishes. Confluent cells were carefully washed twice with PBS before bacterial cells were added. *B. breve* strains, *i.e.*, PRL2012 and LMG 13208, and *B. animalis* subsp. *lactis* Bb-12 were grown as

previously described, until a concentration of 5×10^7 CFU mL^{-1} was reached. The strains were then centrifuged at 3,000 rpm for 8 min, resuspended in PBS (pH 7.3), and incubated with monolayers of HT29-MTX cells. After 1 h incubation at 37°C, cultures were washed twice with 2 mL of PBS to remove unbound bacteria. The cells were then fixed with 1 mL of methanol, incubated for 8 min at room temperature, stained with 1.5 mL of Giemsa stain solution (1:20) (Sigma-Aldrich, Milan, Italy) and left in the dark for 30 min at room temperature. After two washes with 2 mL of PBS, the cover glasses were removed from the petri plate, mounted on a glass slide, and examined using a phase-contrast microscope Zeiss Axiovert 200 (objective, 100×/1.4 oil). Adherent bacteria in 20 randomly selected microscopic fields were counted and averaged. The proportion of bacterial cells that remained attached to the HT29-MTX monolayer was determined to reflect the extent of specific host-microbe interaction. The adhesion index represents the average number of bacterial cells attached to 100 HT29-MTX cells (Guglielmetti et al., 2008; Turroni et al., 2013; Rizzo et al., 2023). A non-parametric Mann Whitney test was applied for the detection of statistically significant differences. All assays were performed at least in triplicate.

Statistical analysis. Similarities between samples (beta-diversity) were calculated by the Bray-Curtis dissimilarity matrix based on species abundance, using the "vegdist" function on RStudio (http://www.rstudio.com/). Beta-diversity was represented through Principal Coordinate Analysis (PCoA) using the function "ape" of the R suite package (Paradis and Schliep, 2019). Moreover, the various detected bacterial species were tested and plotted on the PCoA using the "envfit" and "plot" functions from vegan through R-studios (http://www.rstudio.com/). PERMANOVA analyses were performed on RStudio using 999 permutations to estimate *p*-values for population differences in PCoA analyses with adonis2

package. Furthermore, a correlation analysis between the available metadata and the various detected bacterial species of all samples was performed through Spearman's rank correlation coefficient using "rcorr" function (https://CRAN.R-project.org/package=Hmisc), and only results that were significantly different from a statistical perspective were retained. The False Discovery Rate (FDR) correction was applied to all statistical analyses based on Benjamini and Hochberg Correction (Benjamini et al., 2001), using RStudio through "p.adjust" function.

Data availability. Raw sequences of metatranscriptomics experiments were accessible through the SRA study BioProject PRJNA1045385. Furthermore, the updated genome sequence of *B. breve* PRL2012 was deposited in the GenBank database with the NCBI RefSeq Accession code CP006711.2.

Competing interests

The authors declare that they have no competing interests.

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Chapter 9

General conclusion

Advances in the exploration of the dissection of the functionality of the human microbiota in different body districts

In recent decades, the scientific community has shown considerable interest in studying the human gut microbiota and vaginal microbiota [9,31]. The biological importance of these microbiomes in terms of crucial drivers and natural regulators of human health is representing a new frontier of molecular biology research. However, several gaps of knowledge still exist, especially in the way how the members of these human microbiotas interact with each other as well as with the human body. Thus, the current research is rapidly evolving from the well-established "who is there" to the novel paradigm "what they are doing". Thus, it is of paramount importance to move to the next step of knowledge represented by the disentangling of the functional roles exploited by the gut and vaginal microbes toward the human host.

In this context, we have in this Ph.D. thesis provided novel insights about the functional roles exploited by lactobacilli and in particular *L. crispatus* in the vaginal tract of human beings. Remarkably, *L. crispatus* demonstrated to possess a higher ecological fitness respect to other members of the vaginal microbiome, which are represented by the most advanced colonization performances. In this thesis, we have emphasized some fascinating new features about the great genetic variability of *L. crispatus* taxon concerning its ability to produce bacteriocins and other antimicrobial compounds like lactic acid, as well as its antibacterial effectiveness against human vaginal infections (Chapter 3 and 4).

Concerning the gut microbiota, this can be influenced by different factors. Among the most recognized stressors of the gut microbiota, it is worth mentioning the

antimicrobial agents such as antibiotics, which are recognized for their dramatic impact on the composition and complexity of the human gut microbiota [135,136]. A very commonly prescribed antibiotic combination during infancy and adolescence is amoxicillin and clavulanic acid (AMC), leading to significant effects on reducing the complexity of the gut microbiota composition and thus fostering the development of gut dysbiosis. Our hypothesis postulates that fortifying the gut microbiota with naturally AMC-resistant strains during AMC treatment can prevent or reverse dysbiosis and restore the innate resilience of the gut microbiota. This assumption was tested using three AMC-resistant bifidobacterial strains, *i.e.*, *Bifidobacterium longum* subsp. *longum* 1898B and *Bifidobacterium breve* 1891B and *Bifidobacterium breve* M1D, which have been evaluated through *in vitro* and *in vivo* models. In this context, intriguing outcomes related to the restoration of the original microbial diversity were observed for all these strains, albeit with distinct and strain-specific consequences. The biological rationale supporting these significant ecological effects of these bifidobacterial strains is represented by their capacity to support the growth of other members of the gut microbiota through the establishment of metabolic trophic interactions like those previously documented for other bifidobacterial strains [137,138] (Chapter 5).

In this framework, exopolysaccharides (EPS) are synthesized by different bifidobacterial taxa commonly identified in the human gut microbiota, which have been documented to act as a key extracellular structure in the modulation of the interactions of bifidobacteria with the host as well as with other members of the human gut microbiota. However, nothing is known about the potential effects of EPS produced by bifidobacteria in terms of enhancing their tolerance toward various antibiotics commonly used in antibiotic therapies. In this thesis, we examined whether the production of EPS by four selected EPS-producing bifidobacterial strains is linked to increased resistance against antibiotic treatments.

Our findings revealed that stimulating the EPS production, achieved by altering the growth medium with different carbon sources, and/or submitting the bacterial cultures to stressful conditions, resulted in an enhanced tolerance of bifidobacterial cells to various beta-lactam antibiotics including AMC (Chapter 6).

In this context, the identification of bacterial strains, *i.e.*, bacterial prototypes, that represent the genetic features of a specific microbial taxon occurring in a particular environment is of paramount importance. In this context, in this Ph.D. thesis, we have focused our interest on the identification of bacterial prototypes for the *Bifidobacterium adolescentis* and *Bifidobacterium breve* species, which are two key microbial species of the human gut microbiota [103,138–140]. In detail, the selection of a *B. adolescentis* prototype, *i.e.*, PRL2023, through an ecological-driven approach was confirmed with laboratory experiments featuring models that replicate typical rigorous conditions of the GIT, alongside interaction with human cell lines, indicating that PRL2023 can endure adeptly in the intestinal milieu and effectively adhere to the human epithelium. Additionally, meta transcriptomics analyses, involving PRL2023 strain in combination with other intestinal microbes, enriched our understanding of the crosstalk features of this strain with the other members of the human gut microbiota and in general of the microbe-microbe interactions occurring in the human gut (Chapter 7).

Furthermore, we identified a *B. breve* PRL2012 prototype that from our local microbial collection, was revealed to showcase the closest genetic similarities with the phylotype of the species. At the same time, a comparative genome analysis unveiled a genome structure of *B. breve* species with a minimal number of distinctive genes, two of which have been previously identified to be implicated in carbohydrate metabolism [138,140]. In this setting, the metabolic performances of PRL2012 strain toward carbohydrates, alongside its capacity to survive in the human gut have been explored. Furthermore, the abilities of *B. breve* PRL2012 to

interact with the human host as well as with the other members of the gut microbiota were untangled by using omics approaches. Altogether such experiments showed that the *B. breve* prototype PRL2012 strain possesses the capability to establish itself in the intricate ecological niche of the human intestine, attaching to the host mucosa as well as establishing a molecular interaction with this host's surface, and enduring the demanding conditions commonly encountered in the intestinal milieu (Chapter 8).

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