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Insights into factors influencing the colonization and composition of the human intestinal microbiota

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Summary

The entangled and heterogeneous community of microorganisms inhabiting the human gastrointestinal tract (GIT) is the so-called gut microbiota. This intricated ecosystem has evolved over time to establish a symbiotic relationship with the human host, providing mutual advantages to both. Given the continuous molecular interactions between bacteria and the human being, which play a crucial role in maintaining overall health, substantial research efforts have been directed toward deciphering the microbial composition and its functional implication of the gastrointestinal microbiome.

This Ph.D. thesis aims to investigate and elucidate some of the factors influencing the colonization and composition of the gut microbiota throughout life and how these influence the modulation of this complex ecosystem.

In particular, the first section of this Ph.D. thesis focuses on examining how bifidobacterial intrinsic genetic factors, such as specific genes involved in the interaction's mechanisms, impact the colonization and persistence of this species within the intestinal environment. Specifically, the bifidobacterial intricate molecular mechanisms involved in their survival within a competitive intestinal niche are investigated.

Additionally, the second section of this thesis highlights the influence of extrinsic factors, such as insulin, a hormone typically presents in healthy women's breast milk, on the colonization mechanisms of *Bifidobacterium bifidum* PRL2010 in the gut.

Finally, we have provided novel insights into the emerging field of pharmacomicrobiomics, where the effects of six different oral corticosteroids on the

composition of ten artificial gut microbiota (AGM) were examined. Such study explores how the diverse compositions of AGMs influence the metabolism of the same drugs.

Sommario

La complessa popolazione di microrganismi che risiedono nel tratto gastrointestinale umano viene definito microbiota intestinale. Questo intricato ecosistema si è evoluto nel tempo stabilendo un'interazione simbiotica con l'ospite, fornendo vantaggi reciproci ad entrambe le parti. Evidenze scientifiche mostrano come l'interazione molecolare tra batteri ed esseri umani, svolge un ruolo cruciale nel mantenimento della salute generale. Per questo motivo la comunità scientifica ha volto la propria attenzione nel decifrare la composizione microbica e le implicazioni funzionali del microbioma gastrointestinale.

Questa tesi di dottorato si propone di investigare e chiarire alcuni dei fattori che influenzano la colonizzazione e la composizione del microbiota intestinale nel corso della vita e come questi siano in grado di modularla. In particolare, la prima parte si concentra nell'esaminare come i fattori genetici intrinseci dei bifidobatteri, come alcuni geni specifici coinvolti nei meccanismi di interazione, possano influenzare la colonizzazione e la persistenza di questa specie nell'ambiente intestinale. In particolare, vengono esaminati i complessi meccanismi molecolari che garantiscono la sopravvivenza di questi batteri all'interno di un ambiente intestinale competitivo.

La seconda parte di questa tesi evidenzia l'influenza di fattori estrinseci, come l'insulina — un ormone tipicamente presente nel latte materno di donne sane — sui meccanismi di colonizzazione di *Bifidobacterium bifidum* PRL2010 nell'intestino. Infine, sono stati esaminati gli effetti di sei diversi corticosteroidi sul microbiota intestinale utilizzando microbioti intestinali artificiali (AGM), studio che si innesta nella branca ancora poco esplorata degli studi di farmacomicrobiomica. Lo studio

esplora inoltre come le diverse composizioni degli AGM influenzino il metabolismo degli stessi farmaci.

Chapter 1

General Introduction

A. The human gut microbiota

The term human microbiota identifies the complex and intricate communities of microorganisms residing in the human body [1, 2]. This heterogeneous ecosystem of viruses, bacteria, fungi, and other microbes inhabits the human organism, playing a crucial role in the health and well-being of its host [3, 4]. Notably, these microorganisms colonize multiple body regions, including the skin, oral cavity, respiratory system, vaginal tract, and most notably, the gastrointestinal tract, where they establish a complex microbial ecosystem [5] (Figure 1).

Millions of years of co-evolution between the human host and intestinal bacterial players have led to the development of numerous trophic interactions. In these mutualistic relationships, the host provides nutrients, protection, and a suitable environment for the growth of the intestinal microbes, which, in turn, carry out various physiological and metabolic functions [6, 7]. Indeed, in recent decades, particular emphasis and attention have been given to the profound and extensive influence that the microorganisms colonizing the human body have on the host's physiology, mental health, metabolism, endocrine, and immune systems [8-10]. Among these, the gut microbiota is one of the main directly and indirectly implicated in human health, in fact, it is committed to various physiological and biological functions, including the digestion and fermentation of foods and the metabolism of non-digestible carbohydrates for the human enzymatic repertoire [11]. Additionally, it has been demonstrated that the gut microbiota impacts the development and

regulation of the immune system through the downregulation of an excessive immune reaction [12].

Furthermore, the gut microbiota plays a crucial role in metabolic pathways responsible for synthesizing vitamins and metabolites while regulating hormones that impact overall health [10, 13]. Notably, recent studies have revealed bidirectional interactions between the gut microbiota and other body systems, including the gut-brain, gut-lung, and gut-skin axis. These connections, primarily mediated by metabolites and the immune system, emphasize the wide-ranging influence of the gut microbiota in supporting overall human health [14-16].

The ability of the gut microbiota to exploit these functions is strictly correlated to its composition, which varies from individual to individual and is subject to unceasing changes throughout human life. Firstly, the colonization of the human intestine occurs immediately after birth, and it is influenced by the type of delivery (natural or C section) and the feeding mode (breast feeding or formula) [17], underlining how the gut microbiota is undoubtedly shaped and imprinted by the crucial factors occurring from birth to weaning.

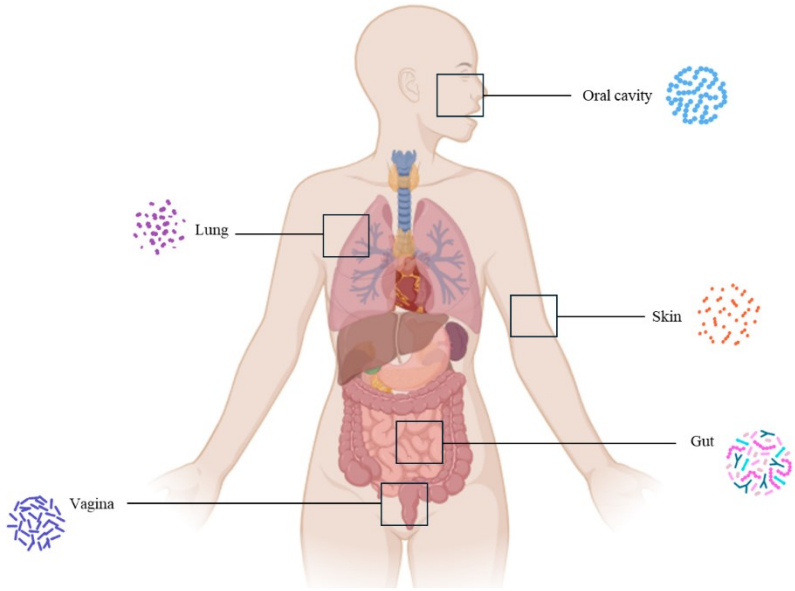

Figure 1: Graphical representation of different microbiota colonizing the human body.

Subsequently, fluctuations in the gut microbiota then occur with diet, physical and mental health status, medication intake, and the living environment [18]. The complexity of the microbiota increases considerably in the first months of life, reaching a composition made up, for almost 97% of adults, of Bacteroidetes (20-25%), Firmicutes (60-65%), Proteobacteria (5-10%) and Actinobacteria (1-3%) [19], focusing the attention of the scientific community on these phyla and their functional role.

B. Onset and development of the human gut microbiota

The colonization and development of the gut is an intricated and continuous process that establishes immediately postpartum and persists until old age [20, 21]. Despite the scientific community's long-held belief that the uterine cavity is sterile, recent studies have suggested that the placenta may harbor its own microbiota, closely resembling that of the newborn's meconium. This implies that pre-colonization of the intestine might occur prior to birth [22]. The scientific community is therefore wondering whether prenatal factors can also have a certain importance in defining the composition of the microbiota of the adult. However, the factor that most significantly influences the establishment of the gut microbiota occurs at the moment of birth is represented by the type of delivery [23, 24]. Indeed, infants born via natural childbirth typically acquire their gut microbiota through vertical transmission from the mother [25], i.e., microorganisms from the mother urogenital tract such as *Bifidobacterium* genera [26, 27].

In contrast, babies born via cesarean section are mainly colonized by bacteria from external niches, such as mother's skin and the hospital environment, leading to *Staphylococcus*, *Corynebacteria*, and *Propionibacterium* spp predominance. [28-30]. Additionally, another major factor in shaping the gut microbiota of infants is undoubtedly the weaning strategy [31]. Considerable scientific evidence emphasizes that breastfeeding is preferable to formula feeding whenever possible

[32, 33]. In fact, it is recognized that breast milk provides numerous essential nutrients for the appropriate neurological and physiological development of the baby, including lipids, vitamins, hormones (i.e., insulin, melatonin, leptin), lactose, immune system components such as Immunoglobulin A, and oligosaccharides like Human Milk Oligosaccharides (HMOs) [34]. Indeed, it has been observed that hormones in breast milk regulate the newborn's sense of satiety and the onset of obesity. However, there is limited information on the direct involvement of hormones in the gut microbiota modulation [35, 36]. HMOs, along with lactose, are the most abundant carbohydrates in breast milk that are involved in the epithelial barrier and immune system development and have been demonstrated to promote gut colonization by members of the genus *Bifidobacterium,* which are valuable microbial biomarkers indicative of a healthy human host [37].

Consequently, the gut microbiota composition of breastfed infants displays a high abundance of bifidobacteria. In contrast, formula-fed infants exhibit a microbiota dominated by bacteria from the genera *Bacteroides*, *Clostridium*, and *Staphylococcus*, as well as members of the *Enterobacteriaceae* family [31, 38, 39]. These alterations can significantly impact host physiology and immunology, potentially increasing vulnerability to diseases such as asthma and atopic illnesses in adulthood [40, 41].

Subsequently, after the initial colonization of the intestine, the gut microbiota undergoes variations and changes following weaning and until around three years of age, when it reaches near-complete maturation and stability [1]. It has been observed that microorganisms belonging to Actinobacteria and Proteobacteria

phyla are predominant in the intestine during the first months of life, later being replaced by bacteria from Bacteroidetes and Firmicutes phyla as age progresses [42]. Notably, only a subset of bacteria, referred to as the core microbiota, is consistently found in most individuals within a population [42]. The core microbiota refers to the stable elements that persist over time and across individuals, in contrast to the transient microbiota components, which change in response to varying environmental and individual conditions such as diet, host genetics, and other primarily external factors [4, 43].

C. Postnatal factors influencing the gut microbiota composition

As previously mentioned, the composition of the gut microbiota undergoes different changes in the first months of life. Following an initial maturation and stabilization, which occurs around three years of age with complete weaning, numerous factors throughout life influence the stability and variability of the microbial composition (Figure 2) until elderly when the composition of the microbiota tends to decrease in terms of species richness gradually [40].

Among the various factors, diet is undoubtedly one of the most significant, in fact different types of nutrients can directly promote or inhibit the growth of specific microorganisms [44]. Different studies have shown how various types of diets have a significant impact on both the composition of the gut microbiota and, consequently human health [45]. It has been widely demonstrated that the 'Western' diet, characterized by high-fats, high-sugars and low amounts of fiber, is closely linked to the onset of obesity and the development of type II diabetes [46]. This type of diet, with frequent consumption of processed foods and red meats, leads to a shift in gut microbial composition, with an increased presence of members of the genera *Bacteroides*, *Alistipes*, *Klebsiella*, and *Shigella* [47, 48]. These bacterial species contribute to an increase in the intestinal environment of metabolites such as ammonia, secondary bile acids and hydrogen sulfide; an uplift in lipopolysaccharides (LPS), trimethylamine-N-oxide (TMAO), and a decrease in short-chain fatty acids (SCFAs) [49]. Together, these changes generate a state

of low chronic inflammation, which provides fertile ground for the development of metabolic diseases [49-51]. Conversely, adopting a plant-based or Mediterranean diet, characterized by high fiber intake, low sugars, and low fats, leads to a significant increase in genera as *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium prausnitzii*, and *Roseburia* [52, 53]. The presence of these microorganisms results in a reduction of pro-inflammatory cytokines (e.g., IL-12 and IL-17), an increase in anti-inflammatory cytokines (e.g., IL-10), and a rise in SCFAs [54, 55]. The latter are associated with high fiber intake and contribute to a reduction in inflammation [56]. Since human beings do not possess specific genes in their enzymatic arsenal to degrade fibers, this function is carried out by certain gut microbial groups [57]. The ability of gut bacterial species to extract energy from specific dietary constituents provides them with an evolutionary advantage upon other intestinal microbial members [57]. For instance, strains belonging to the genera *Ruminococcus*, *Bacteroides*, and *Bifidobacterium* possess enzymes in their glycobiomes capable of metabolizing and degrading these complex dietary glycans [58]. These specific microorganisms not only have a higher survival capacity than other gut bacterial species, which are unable to metabolize those complex components, but they also release in the intestinal environment simpler glycans that can be utilized by other gut microorganisms, creating a complex mechanism of cross-feeding [59].

Another factor that significantly impacts the composition of the gut microbiota is lifestyle, specifically the overall level of well-being maintained i.e., exercise, limited alcohol consumption and the absence of smoking [60, 61]. It has been shown that regular physical activity, as opposed to a predominantly sedentary

lifestyle, reduces inflammation and modulates the gut microbiota [62]. In particular, it decreases the Firmicutes/Bacteroidetes ratio, which is an indicator of obesity, as well as the abundance of the genus *Blautia*, which is associated with inflammation [63]. Physical exercise has been found to influence the gut microbiota in two primary ways: first, by affecting the autonomic nervous system, specifically through the vagal tone, which is part of the "gut-brain axis", and second, by enhancing immune function [64].

Exercise has long been recognized for its ability to enhance vagal tone, the neural connection to the gut, which exerts anti-inflammatory and immune-modulatory effects. This may represent an indirect mechanism through which physical activity influences the composition of the gut microbiota [65].

Another aspect to consider in the shift of gut microbiota composition is the onset of diseases. In recent years, there has been considerable interest not only in conditions more directly linked to the gut, such as inflammatory bowel diseases (IBDs), but also in other diseases that are less obviously connected but equally impactful, including depression, psychiatric disorders, and Alzheimer's disease [66-68].

Moreover, it is essential to consider one of the most influential factors impacting gut microbiota composition: the widespread use of medications and antibiotics [69]. Incorrect use of antibiotics, i.e., multiple therapies reduce species diversity, disrupts metabolic activity, and leads to the selection of antibiotic-resistant microorganisms [70]. Notably, the extensive use of antibiotics has been linked to infections caused by *Clostridium difficile*, which is an opportunistic pathogen that is difficult to eradicate and can cause severe, potentially fatal colitis [71, 72].

Studies have shown that even short-term antibiotic use (i.e., less than a week) can produce long-lasting effects on the gut microbiota, with alterations persisting for six months to two years after treatment. These changes may predispose the host to various diseases, many of which are not yet fully understood [73]. Recently, it has been understood that the influence of drugs on the gut microbiota is not limited to antibiotics but also extends to non-antibiotic drugs [74]. For example, paracetamol has been demonstrated to decrease the abundance of the genus *Dorea* while increasing the prevalence of *Bifidobacterium dentium* and *Staphylococcus salivarius* species [75]. Given the significant implications for global health, there has been a growing emphasis in recent years on the field of pharmacomicrobiomics [76, 77]. This scientific discipline investigates not only the effects of drugs on gut microbiota—specifically their impact on bacterial growth, composition, and function—but also how the gut microbiota can modulate an individual's response to specific medications. The microbial enzymatic repertoire has the capacity to transform the active ingredients of drugs, thus altering their bioavailability, bioactivity and toxicity [78]. The bidirectional relationship between drugs and the gut microbiota has been recognized, highlighting the importance of further investigation into this interaction. Understanding these dynamics is crucial for advancing the development of more precise, targeted, and personalized medical therapies.

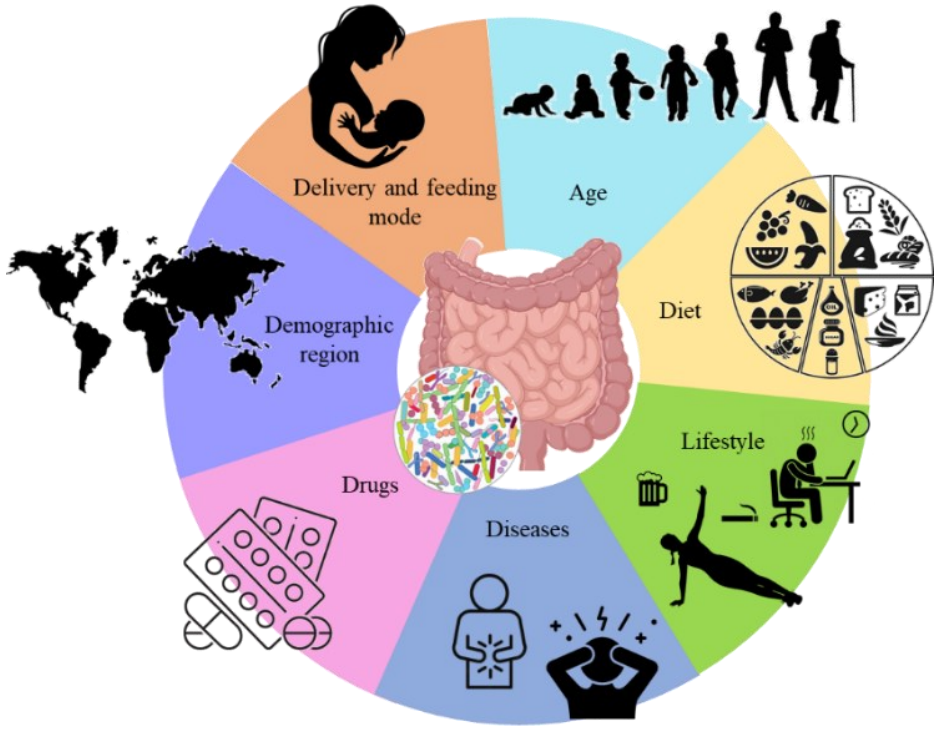

Figure 2. Schematic illustration of different factors impacting the gut microbiota.

D. The connection between human gut microbiota composition and overall health

As extensively discussed, the composition of the gut microbiota is in constant flux, influenced by a multitude of factors, including age, diet, lifestyle, and medication use [79]. These factors can disrupt the balanced equilibrium among the resident microorganisms of the intestine, a state known as eubiosis [80]. In eubiotic conditions, the gut microbiota is characterized by a predominance of beneficial species, while potential pathogenic species (i.e., opportunistic pathogens) are present in very low percentages [81]. This balance is essential for maintaining the overall health and well-being of the host. However, irregular dietary habits, unhealthy lifestyles, diseases, and medication use can disturb this equilibrium, leading to a disruption of the gut microbiota, known as dysbiosis [79]. Dysbiotic conditions typically involve the depletion of health-associated microorganisms, particularly those producing SCFAs, which can contribute to the development of various diseases [8] (Figure 3). At the same time, there is an increase in opportunistic pathogens, including some mucolytic bacteria that could produce hydrogen, methane, and hydrogen sulfide, along with a rise in Proteobacteria and elevated levels of LPS endotoxin [42].

These changes can negatively impact the host health by compromising the integrity of the intestinal mucosa, triggering acute inflammation, and facilitating bacterial translocation. Moreover, these disruptions can exert toxic effects on

colonocytes, lead to oxidative damage, alter cytokine profiles, and cause various systemic repercussions [82, 83].

Numerous studies investigating the human gut microbiota have yielded crucial insights into the composition and functional capacity of the microbial communities inhabiting the human intestine [84, 85]. These studies reveal significant interindividual variability alongside notable stability, allowing researchers to distinguish between states of eubiosis and dysbiosis. Dysbiotic profiles have explicitly been identified in association with gastrointestinal pathologies such as Crohn's disease, ulcerative colitis, non-alcoholic liver disease, irritable bowel syndrome (IBS), as well as metabolic disorders conditions like obesity, and both type 1 and type 2 diabetes [86-89].

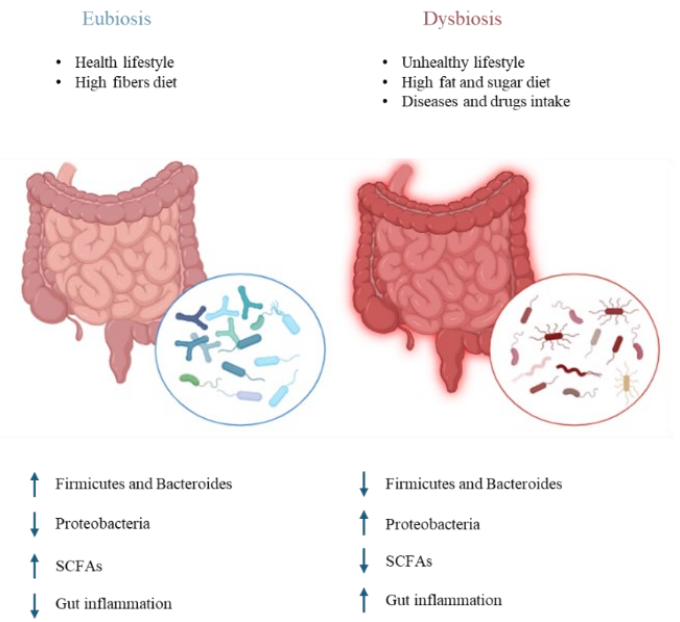

Figure 3: Schematic illustration of factors influencing eubiosis and those causing alteration of the human microbiota composition.

Additionally, ongoing investigations are probing the correlation between altered gut microbiota composition and other pathological conditions, including autism spectrum disorders, multiple sclerosis, neurodegenerative diseases, and cancer [90-92]. Recent meta-analyses suggest that intestinal microbial dysbiosis is dynamic and evolves over time, underscoring the need of understanding its progression to develop personalized therapeutic strategies [93, 94]. A reduction in microbial diversity—quantified by assessing the variety and abundance of bacterial taxa—emerges as a key marker of potential deviations from a balanced eubiotic state. In dysbiosis, this decreased diversity is marked by the decline of dominant commensal taxa [95]. Notably, a diminished microbial richness is identified as a biomarker for metabolic disorders, and it is insufficient on its own to definitively diagnose an active pathological condition [1].

E. Health- promoting bacteria and their impact on human well-being

In the context where the well-being of the gut microbiota is intricately connected to human health, the significance of restoring the gut microbiota from a dysbiotic state becomes crucial. In recent years, numerous studies have increasingly focused on the development of health-promoting bacteria, i.e., probiotics, based formulations to address this necessity [96].

In 2001, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) provided a definition for probiotics, characterizing them as “live microorganisms that confer a health benefit on the host when administered in adequate amounts” [97, 98]. Probiotic bacteria, encompassing a few species predominantly belonging to genera *Bifidobacterium, Lactobacillus*, *Enterococcus*, *Bacillus*, *Escherichia*, and *Saccharomyces*, which are widely recognized for their role in preventing various gastrointestinal disorders. These microorganisms operate on multiple fronts to maintain and restore the gastrointestinal barrier and promote eubiosis [99]. To be considered for use in food products and pharmaceutical supplements, probiotic microorganisms must follow strict guidelines set by the National Ministry of Health and the European Food Safety Authority (EFSA), which ensure their safety, efficacy, and technological viability [100, 101]. Safety assessments require that these bacteria do not possess virulence factors and antibiotic resistance genes while also demonstrating the ability to survive the diverse

conditions of the gastrointestinal tract and adhere to the intestinal mucosa [102, 103]. Additionally, an ideal probiotic candidate should originate from human sources, aligning with the autochthonous human microbiota. Probiotic bacteria contribute to health through various mechanisms, including modulating microbiota composition, enhancing mucin production, strengthening intestinal mucosa tight junctions, regulating immune responses, and synthesizing beneficial compounds [104-106]. Due to their health-promoting properties, probiotic products are frequently integrated into the daily diets of healthy subjects [106, 107]. Different studies into human body ecosystems and genetic analysis of microorganisms have led to the identification of new probiotic strains with unique characteristics and specific mechanisms of action, termed next-generation probiotics. These are identified and characterized through advanced sequencing and bioinformatics, offering new avenues for preventing and treating gut microbiota dysbiosis [102, 108].

Future next-generation probiotics may include not only traditional probiotic bacteria but also other microbial groups from the core human gut microbiota which are lacking [109, 110]. However, for an accurate analysis of the microbiota, innovative methodologies are needed, such as the "omics" technologies that have been pivotal in analyzing the composition and functions of commensal bacteria, particularly at the genomic level, leading to the emergence of probiogenomics [111, 112]. This field, in conjunction with functional genomic data, enhances the understanding of the diversity, evolution, and beneficial effects of commensal bacteria. The foundations of probiogenomics were laid with the study and characterization of strains belonging to the *Lactobacillus* and *Bifidobacterium* genera, among which the most renowned probiotic microorganisms are present.

The *Bifidobacterium* genus

Bifidobacteria, classified under the genus *Bifidobacterium* within the phylum Actinobacteria, represents a significant group of commensal microorganisms [113]. These bacteria are characterized as Gram-positive, non-motile, non-sporulating, non-gas-producing, and anaerobic (with some species exhibiting facultative aerobic properties). They are also catalase-negative, saccharolytic, and possess a high Guanine + Cytosine (G+C) content [114]. Notably, bifidobacteria display pleomorphism, adopting either Y-shaped or rod-shaped morphologies depending on the growth conditions [115]. These mesophilic bacteria thrive optimally at temperatures between 36°C and 38°C, with species isolated from animal sources requiring slightly higher temperatures, ranging from 41°C to 43°C. Furthermore, they exhibit moderate acid tolerance, with optimal growth occurring at a pH range of 6.5 to 7.0 [116]. Originally isolated by Tissier in 1899 from the fecal sample of a breastfed infant, bifidobacteria have since been found in a wide range of ecological niches, including the gastrointestinal tracts of mammals and insects, oral cavities, wastewater, human blood, and fermented dairy products. The genus *Bifidobacterium* now comprises 107 taxa, including 87 species and 7 subspecies [117]. The adaptability of bifidobacteria to diverse ecological environments is species-dependent; for example, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium pseudolongum*, *Bifidobacterium breve* and *Bifidobacterium bifidum* are considered cosmopolitan species, whereas others are more specialized, such as *Bifidobacterium angulatum*

in cows, *Bifidobacterium cuniculi* in rabbits, and *Bifidobacterium gallinarum* in chickens [113, 118].

The ecological success of bifidobacteria is intricately linked to their genetic repertoire. Comparative genomic studies have shed light on the core and pan-genomes of bifidobacteria, revealing that the core genome, shared across different species, primarily encodes functions related to carbohydrate metabolism [119]. This finding underscores the importance of saccharolytic activity for the ecological fitness of bifidobacteria. In contrast, the pan-genome encompasses unique genes specific to particular bifidobacterial species. While the function of many genes remains unclear, some are implicated in host-microbe and microbe-microbe interactions, including sortase-dependent pili and exopolysaccharide structures [120]. A crucial physiological trait of bifidobacteria is their ability to degrade complex carbohydrates that are indigestible by the host [121, 122]. Interestingly, some species belonging to *Bifidobacterium* genus can break down dietary polysaccharides such as glucans, xylans, pectins, fructans, cellulose, and resistant starch, as well as host-derived glycans like mucins—glycoproteins that line the intestinal epithelium and are secreted by goblet cells in the gastrointestinal tract. Bifidobacteria are also capable of metabolizing oligosaccharides present in breast milk, such as human milk oligosaccharides (HMOs) [121]. The genes responsible for these carbohydrate degradation processes include glycoside hydrolases (GHs), ATP-dependent sugar-specific transporters, permeases, import pumps, and components of the phosphoenolpyruvate phosphotransferase system [120, 121].

Relationship between bifidobacteria and the human gut

The gut microbiota, as anticipated, is a complex ecosystem characterized by intricate relationships and interactions among bacteria, collectively contributing to various metabolic and physiological activities. Specifically, numerous studies have begun to explore the mechanisms by which bifidobacteria interact with each other, with other microbial species, and with the host [6]. These mechanisms imply the presence of distinct clusters of genes coding for extracellular structures (i.e., pili, exopolysaccharides, teichoic acids) and glycoside hydrolases (GHs) involved in cross-feeding activities, genes encoding for bacteriocins, and other metabolites involved in the interactions between microorganisms [123-126]. Notably, extracellular structures such as pili, i.e., hair-like protein appendages extending from the extracellular surface, are crucial for the successful colonization of a specific environment. Their functions include adhesion to the intestinal epithelium, biofilm formation, and inter-microbial aggregation [127-130]. Teichoic acids, negatively charged extracellular polymers exposed on the cell wall of Gram-positive bacteria, are also involved in host-microbe interactions. These structures promote bacterial adhesion to intestinal epithelial cells, thus facilitating colonization [131, 132]. Exopolysaccharides (EPS), which are glycan-based layers forming an external envelope around various bacterial species, represent another crucial extracellular structure. These macromolecules not only facilitate host colonization and persistence but also provide protection

against hostile conditions, such as sudden pH fluctuations, excessive dryness and exposure to bile acids. Moreover, EPS structures play a pivotal role in shielding bacterial cells from environmental stressors, including pancreatin, acidic environments, and antimicrobial agents such as antibiotics [133-135].

Among the factors promoting bifidobacterial growth and colonization of the intestine are GHs. These enzymes represent a large class responsible for catalyzing the hydrolysis of glycosidic bonds in complex sugars such as starch, cellulose, pullulan, and amylopectin, which constitute most dietary carbohydrates. For instance, the GH enzyme β-galactosidase enables bifidobacteria to grow on saccharide substrates containing beta-galactosidic bonds, which are abundant in human breast milk [125, 136]. The extensive diversity of enzymes within these families provides significant potential for research focused on elucidating their roles in bifidobacteria and their interactions with the host [123]. Additionally, the less-explored area of synergy and complex interactions between different bifidobacterial species, such as cross-feeding mechanisms, should be considered. These specific trophic interactions allow different bifidobacteria species not only to support each other's growth and survival in the gut environment but also to confer health benefits to the host’s intestinal health [137]. This underscores the importance of studying such interactions, which may hold the key to modulating gut microbial composition, particularly in the context of probiotic treatments following dysbiosis.

Chapter 2

Outline of the thesis

The aim of this Ph.D. thesis is to investigate the various factors involved in the modulation of the human gut microbiota, using model microorganisms representative of typically human gut species such as *Bifidobacterium bifidum* PRL2010, *Bifidobacterium breve* PRL2012 and *Bifidobacterium longum* PRL2022. Specifically, the first section of this thesis focuses on intrinsic/molecular factors, such as the role of a specific gene, i.e., *per*B, in the colonization and persistence of *B. bifidum* PRL2010 in the intestinal environment. Furthermore, following the investigation of genetic and molecular involvements in colonization, the genetic interactions between different species of bifidobacteria that inhabit the gut and their implications for the host health have been evaluated. In the second section of this thesis, attention is directed towards analyzing the role of extrinsic factors/chemical molecules, such as the hormone insulin, present in breast milk and its impact on the colonization of *B. bifidum* PRL2010. Moreover, the final section explores the emerging field of pharmacomicrobiomics, where an in-depth analysis is conducted on how drug intake can influence the composition of the gut microbiota and how different microbiota can affect the metabolism of the drugs themselves. The overarching goal of this research is to provide insights and various perspectives on the multitude of factors involved in gut microbiota modulation over the course of life.

Chapter 3 of this thesis examines the role of the *per*B gene, typical of certain bifidobacterial strains, in host interaction and colonization through the use of a mutant strain, *B. bifidum per*B::pFREM30.

Chapter 4 focuses on the molecular interactions between different strains of bifidobacteria, typical colonizers of the human gut. Using a human gut model,

this section investigates cooperative relationships among these strains and how their association contributes to the overall health of the host's epithelial cells. Chapter 5 shifts the focus to the role of insulin present in breast milk on B. *bifidum* PRL2010, demonstrating how this hormone, which is found in lower concentrations in the breast milk of mothers with gestational diabetes, plays a crucial role in regulating genes involved in the colonization of the intestinal environment by this strain.

Chapter 6 delves into the bidirectional interaction between drugs and the gut microbiota. Specifically, it examines the effects of several commonly used oral corticosteroids on ten different synthetic microbiotas (AGM). This investigation not only analyzes how these corticosteroids alter the microbial composition of the various AGMs, but also conducts a metabolomic analysis, highlighting how different AGMs influence the generation of metabolites derived from corticosteroids.

Chapter 3

GH136-encoding gene (*per*B) is involved in gut colonization and persistence by *Bifidobacterium bifidum* PRL2010

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For Supplementary Materials see the article published in Microbial Biotechnology.

Abstract

Bifidobacteria are commensal microorganisms that typically inhabit the mammalian gut, including that of humans. As they may be vertically transmitted, they commonly colonize the human intestine from the very first day following birth and may persist until adulthood and old age, although generally at a reduced relative abundance and prevalence compared to infancy. The ability of bifidobacteria to persist in the human intestinal environment has been attributed to genes involved in adhesion to epithelial cells and the encoding of complex carbohydrate-degrading enzymes. Recently, a putative mucin-degrading glycosyl hydrolase belonging to the GH136 family and encoded by the *per*B gene has been implicated in gut persistence of certain bifidobacterial strains. In the current study, to better characterize the function of this gene, a comparative genomic analysis was performed, revealing the presence of *per*B homologues in just eight bifidobacterial species known to colonize the human gut, including *Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *longum* strains, or in non-human primates. Mucin-mediated growth and adhesion to human intestinal cells, in addition to a rodent model colonization assay, were performed using *B. bifidum* PRL2010 as a *per*B prototype and its isogenic *per*B-insertion mutant. These results demonstrate that *per*B inactivation reduces the ability of *B. bifidum* PRL2010 to grow on and adhere to mucin, as well as to persist in the rodent gut niche. These results corroborate the notion that the *per*B gene is one of the genetic determinants involved in the persistence of *B. bifidum* PRL2010 in the human gut.

Introduction

In recent decades, scientific interest has focused on the characterization of members of the genus *Bifidobacterium* since they are not only recognized as dominant and symbiotic inhabitants of the human gut, particularly in the early stages of life, but also as microorganisms purported to exert various health-promoting effects upon their host (Bottacini, et al., 2017, Hidalgo-Cantabrana, et al., 2017, Alessandri, et al., 2021). In this context, convincing scientific evidence has accumulated, highlighting that the presence of bifidobacteria in the human intestine supports host immune system development, promotes intestinal barrier integrity, and contributes to maintain intestinal eubiosis, limiting pathogen proliferation, and preventing the onset of inflammatory bowel disease, ulcerative colitis, and celiac disease (Duranti, et al., 2016, Fujimura, et al., 2016, Milani, et al., 2017, Longhi, et al., 2020, Luck, et al., 2020, Shang, et al., 2022). At the same time, bifidobacteria can produce bioactive metabolites, including vitamins, polyphenols, and short-chain fatty acids, which may elicit beneficial effects on both intestinal epithelial host cells and may also affect growth and abundance of other gut commensals (Bottacini, et al., 2014, Bunesova, et al., 2018, Khromova, et al., 2022).

Being vertically transmitted and, therefore, among the first colonizers of the human gut, bifidobacteria are believed to elicit their health-promoting effects soon

after birth (Duranti, et al., 2017, Milani, et al., 2017, Kumar, et al., 2020, Henrick, et al., 2021). Indeed, during the first months of life, the highest relative abundance and prevalence of bifidobacteria have been recorded in the human intestinal tract (Milani, et al., 2017). However, despite a decline in their levels when the gut microbiota evolves from an "infant-" to an "adult-like" gut microbial ecosystem, bifidobacteria remain relatively stable and persist over time until old age (Arboleya, et al., 2016, Milani, et al., 2017, Alessandri, et al., 2019).

The ability of members of the genus *Bifidobacterium* to persist in the host throughout life may be attributed to specific genomic traits. Indeed, bifidobacterial genomes contain genes involved in the production of certain extracellular structures such as exopolysaccharides, teichoic acids, and pili, which contribute to bifidobacterial persistence in the human gut by promoting their interaction with the host and other commensal microorganisms (Fanning, et al., 2012, Turroni, et al., 2013, Milani, et al., 2017, Alessandri, et al., 2021). In parallel, bifidobacteria possess a set of genes, which encodes a large enzyme arsenal required for carbohydrate metabolism, providing bifidobacteria with a selective advantage to colonize and persist in the competitive human intestinal environment, possibly throughout host life (Turroni, et al., 2018, Arzamasov and Osterman, 2022). In this context, bifidobacteria are not only able to degrade complex, diet-derived carbohydrates, but may also be able to access host-associated complex glycans,

including Human Milk Oligosaccharides (HMOs) and mucin (Egan, et al., 2014, Sakanaka, et al., 2019, Nishiyama, et al., 2020, Arzamasov and Osterman, 2022). In particular, since the ability to degrade HMOs and mucin is restricted to a limited number of intestinal microbes, this feature has been proposed to represent a crucial advantage in ensuring colonization and persistence of bifidobacteria in the human intestine (Turroni, et al., 2010, Turroni, et al., 2011, Katoh, et al., 2017, Alessandri, et al., 2021). Indeed, it has been demonstrated that bifidobacterial strains possessing a genetic repertoire for mucin metabolism, including *Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *longum*, show higher resilience and long-term colonization in the human gut than strains lacking such genetically predicted ability (Katoh, et al., 2023, Tarracchini, et al., 2023). Specifically, the more efficient persistence of strains of *B. bifidum* and *B. longum* subsp. *longum* was attributed to *per*B, a gene encoding an enzyme belonging to the glycosyl hydrolase family 136 (GH136), which is proposed to facilitate mucin degradation (Tarracchini, et al., 2023).

Based on these observations, the aim of this study was to investigate and validate the involvement of PerB in mucin breakdown and enhancement of gut persistence. A comparative genomic survey was performed, revealing that the presence of a *per*B homologue is a specific genetic trait harbored by just eight bifidobacterial species, all originating from the intestinal tract of primates. Furthermore, *in vitro*

and *in vivo* experiments confirmed the involvement of *per*B in mucin degradation and enhancement of bifidobacterial cell persistence in the gut environment.

Results and Discussion

Assessing the prevalence of *per*B homologues among members of the genus *Bifidobacterium*. A previous study had identified *per*B (for Persistence of Bifidobacteria), predicted to encode a glycosyl hydrolase (GH), i.e., GH136 (Yamada, et al., 2017), as a key genetic determinant involved in bifidobacterial gut persistence across human life (Tarracchini, et al., 2023). A very similar enzyme, designated LnbX, originally identified in a *B. longum* subsp. *longum* strain had been shown to act as an extracellular lacto-N-biosidase (Sakurama, et al., 2013, Gotoh, et al., 2015, Yamada, et al., 2022). Transcription of *lnbX* is co-regulated by the global regulator NagR, which controls transcription of all genes involved in the utilization of N-acetyl-glucosamine-containing host glycans (Arzamasov, et al., 2022). However, despite the role of *per*B/*lnb*X in host-derived glycan utilization, an analysis of all complete and well-annotated publicly available genome sequences of bifidobacterial species commonly found in the infant gut microbiota, that is, *B. bifidum*, *Bifidobacterium breve*, *B. longum* subsp. *longum*, and *Bifidobacterium pseudocatenulatum*, highlighted the non-ubiquitous nature of *per*B/*lnb*X in bifidobacterial genomes (Tarracchini, et al., 2023). While all examined *B. bifidum* and certain *B. longum* subsp. *longum* strains were shown to possess a *per*B homologue, none of the *B. breve* and *B. pseudocatenulatum* genomes appeared to contain a homologue of this sequence (Tarracchini, et al.,

2023). Based on these results, to further investigate the presence of *per*B among all currently recognized species of the genus *Bifidobacterium,* a BlastP analysis was performed between a custom database containing the amino acid sequences deduced from *per*B homologues previously identified in *B. longum* subsp. *longum* and *B. bifidum* (Tarracchini, et al., 2023) and the derived amino acid sequences from genes identified in publicly available bifidobacterial genomes. Genomes exhibiting an average nucleotide identity (ANI) of ≥ 99% were excluded from the BlastP analysis to reduce genetic redundancy, generating a custom-database containing a total of 877 non-redundant bifidobacterial genomes (Table S1). Interestingly, only 201 of these 877 genomes, covering 27 different bifidobacterial (sub)species, harbored a *per*B homologue (Figure 1 and Table S2). However, in-depth insights into the amino acid length match coupled with a protein domain check for all genes with a sequence homology with *per*B, revealed that the genomes of only eight bifidobacterial species, i.e., *Bifidobacterium aerophilum*, *B. bifidum, Bifidobacterium colobi, Bifidobacterium imperatoris, Bifidobacterium leontopitheci, Bifidobacterium saguini,* and *Bifidobacterium samirii* together with a portion (111 out of the 312 tested genomes) of the *B. longum* subsp. *longum* strains (Table S1 and Table S2) encompassed the GH136 catalytic region corresponding to the FIVAR domain (Tarracchini, et al., 2023), and a full-length *per*B homologue in their genomes. Conversely, all other genomes with a *per*B

homologue did not encode the FIVAR domain or the other domain identified in the *per*B gene, i.e., a secretion signal peptide and various beta-helix and Rib domains (Tarracchini, et al., 2023), except for the perB homologue of *Bifidobacterium jacchi* which encodes multiple Rib domains (Figure 2 and Figure S1). The *per*B gene is predicted to be involved in mucin degradation, a metabolic feature exclusive to the species *B. bifidum* among the genus *Bifidobacterium* with certain *B. longum* subsp. *longum, B. longum* subsp. *infantis*, and *B. breve* strains that, despite lacking the complete genetic arsenal dedicated to mucin breakdown, can take advantage of the extracellular release of mucin components through cross-feeding events (Egan, et al., 2016, Katoh, et al., 2017, Katoh, et al., 2020, Alessandri, et al., 2021). Furthermore, a small number of other intestinal bacterial members are also capable of mucin degradation, i.e., *Akkermansia muciniphila*, *Ruminococcus gnavus*, *Ruminococcus torques, Phocaeicola vulgatus,* and certain members of the genus *Bacteroides,* including *Bacteroides caccae, Bacteroides fragilis* and *Bacteroides thetaiotaomicron.* Such bacteria require various genes encoding for the degradation of mucin-associated glycans, such as neuraminidases/sialidases (GH33), fucosidases (GH29 and GH95), exo- and endo-β-N-acetylglucosaminidases (GH84 and GH85), β-galactosidases (GH2, GH20, and GH42), α-N-acetylglucosaminidases (GH89), and α-N-acetylgalactosaminidases (GH101, GH129), sulfatase and GlcNAc phosphate-

deacetylase (Tailford, et al., 2015, Kim, et al., 2021, Hayase, et al., 2022, Kim, et al., 2023). Interestingly, the above-mentioned species corresponded to bifidobacterial taxa were exclusively isolated from the feces of either humans or non-human primates (Endo, et al., 2012, Michelini, et al., 2016, Bottacini, et al., 2017, Lugli, et al., 2018, Duranti, et al., 2019, Modesto, et al., 2019, Duranti, et al., 2020, Lugli, et al., 2021). These results suggest that species of bifidobacteria adapted to colonize the intestinal niche of humans or non-human primates have specifically acquired or evolved *per*B to support their colonization and persistence in the associated competitive intestinal environment.

In addition, since it has been demonstrated that the expression of the *lnb*X gene in *B. longum* subsp. *longum* requires the chaperone molecule LnbY (Sakurama, et al., 2013), the presence of conserved *lnb*Y homologues in the other bifidobacterial genomes with a *per*B gene was investigated through a BlastP analysis. Interestingly, only *B. aerophilum*, *B. imperatoris*, and *B. saguini* showed a *lnb*Y homologue in their genomes (Table S3). This suggests that this chaperone-encoding gene is not conserved in all *per*B-containing bifidobacterial genomes, leading to suggest that *per*B expression and activation may be subject to a different control.

Furthermore, to evaluate whether *per*B is an exclusive genetic feature of some members of the genus *Bifidobacterium*, BlastP analysis was performed against the

deduced amino acid sequences corresponding to all bacterial genomes contained in the National Center for Biotechnology Information NCBI by excluding bifidobacteria. Interestingly, this analysis highlighted the presence of *per*B homologues in bacterial species other than bifidobacteria (Table S4). However, evaluation of the protein domains presents in the deduced protein sequences of such *per*B homologues indicated that only a small proportion of the *Bifidobacteriaceae* family, excluding *Gardnerella vaginalis*, shared all identified PerB domains, while putative PerB homologues found in other species lack the functional domain or possess a fibronectin type III domain (Table S4). Assuming that the fibronectin type III domain is involved in adhesion to and interaction with human intestinal epithelial cells (Alessandri, et al., 2023), these results suggest that only certain members of the *Bifidobacteriaceae* family, represented by particular bifidobacterial species/strains, possess the *per*B gene, and that *per*B provides them with a selective and competitive advantage to colonize and persist in the human gut environment.

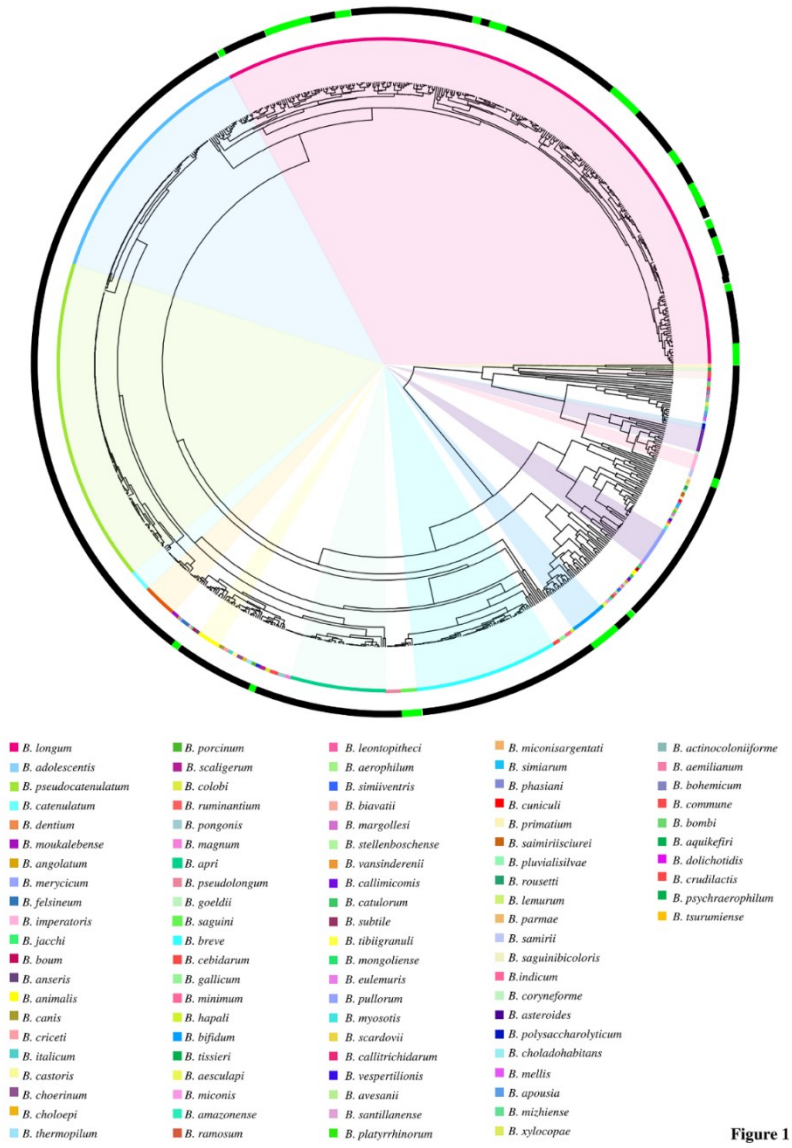


Figure 1. Prevalence of *per*B among the genus *Bifidobacterium*. The panel reports a circular cladogram generated through a comparative genomic analysis based on the Average Nucleotide Identity (ANI) of all publicly available genome sequences of the genus *Bifidobacterium*. The inner circle provides a division of bifidobacteria into species. The outer circle around the cladogram depicts the presence, indicated in green, and the absence marked in black, of *per*B among bifidobacterial strains.

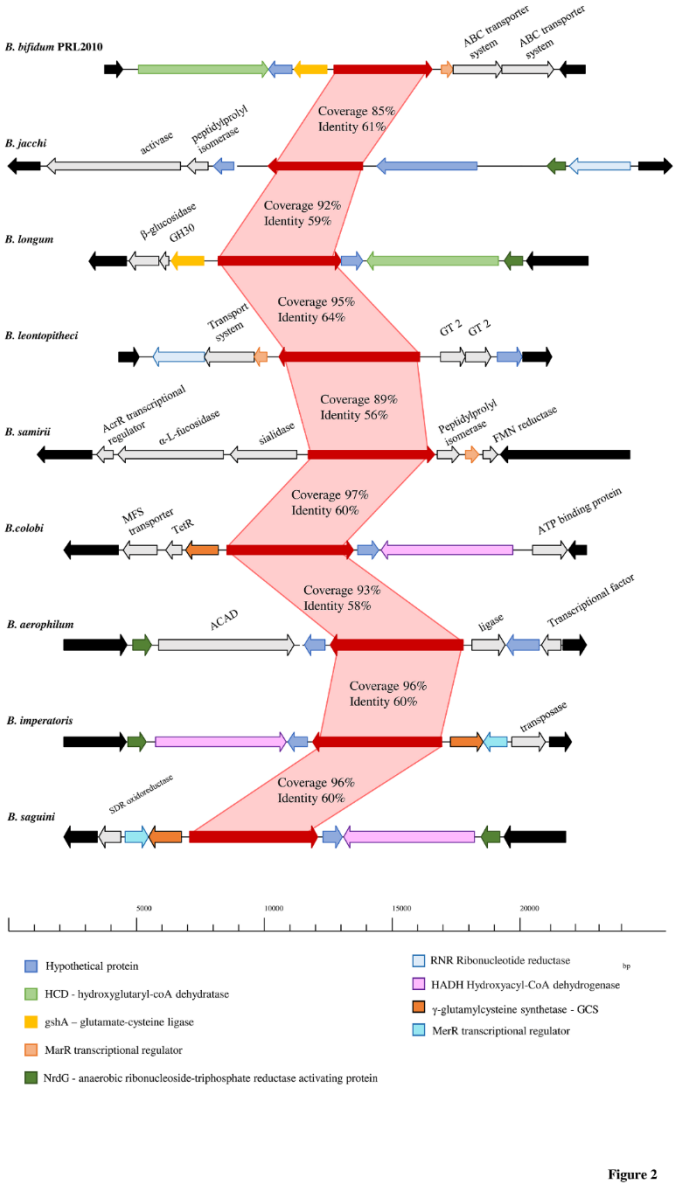


Figure 2

Figure 2. Locus map of the nine species of *Bifidobacterium* containing a full-length *per*B gene. The image shows the genomic region belonging to *Bifidobacterium*

aerophilum, *Bifidobacterium bifidum, Bifidobacterium colobi, Bifidobacterium imperatoris, Bifidobacterium leontopitheci, Bifidobacterium saguini, Bifidobacterium samirii*, *Bifidobacterium longum* subsp. *longum* and *Bifidobacterium jacchi,* in which a *per*B homologue has been identified. The amino acid sequence coverage and identity percentage between *per*B of *B. bifidum* PRL2010 and the other strains are reported.

Impact of *perB* inactivation on *B. bifidum* PRL2010 ability to grow on and adhere to mucin.

Based on the transcriptome results of a recent study in which *B. longum* PRL2022 exposed to human intestinal cells revealed an up-regulation of the *per*B gene compared to the control (Tarracchini, et al., 2023), other mucin-related experiments were carried out. To validate the involvement of *per*B in enhancing the gut persistence of bifidobacterial strains, the ability of a *B. bifidum* prototype, that is, *B. bifidum* PRL2010, to grow on and adhere to mucin and to persist in a rodent-model gut environment was compared to that of the isogenic *B. bifidum* PRL2010 *per*B mutant.

Since bifidobacteria, especially *B. bifidum*, have been reported to be recalcitrant to transformation due to the cell wall composition and thickness and the action of restriction-modification (R/M) systems (Sakaguchi, et al., 2012, O'Connell Motherway, et al., 2009), an optimized protocol for high-efficiency transformation

was developed. First, the R/M recognition sites of *B. bifidum* PRL2010 were identified based on methylome sequencing (Pacbio) and methylated sites prediction from REBASE. The methylome analysis was performed to identify sequence motifs recognized by the strain endonucleases which cut any exogenous DNA that contain such motifs if they are non-methylated, thereby decreasing the efficiency of transformation. Analysis of PacBio sequencing data allowed identification of two Type I (CGAYNNNNNGGT, CAAYNNNNNCTC) and one Type II (CTGCAG) R/M recognition motifs, while the bioinformatic prediction based on REBASE highlighted the presence of different Type II sites (GTCGAC, GGCGCC, GCSGC, GAATTC), allowing the delineation of the *B. bifidum* PRL2010 methylome. Subsequently, these recognition motifs, when present, were removed from the plasmids of interest, pNZ003 and pFREM30, to increase the transformation efficiency of the strain (O'Callaghan, et al., 2015). The first plasmid, which is a derivative of pNZ44 (McGrath, et al., 2001), was used to optimize the transformation protocol until an efficiency of 10E+06 CFU (Colony Forming Unit)/µg of pNZ003 DNA was reached. Optimization was achieved by assessing various critical transformation parameters, such as electroporation voltage and growth/washing/recovery media to be used for growth, transformation and plating of the strain, as based on a previous study (Serafini, et al., 2012). Following optimization, the integration vector pFREM30, which is a derivative of

plasmid pFREM28 (Hoedt, et al., 2021), was employed to target and successfully disrupt *per*B in *B. bifidum* PRL2010 to generate *B. bifidum per*B::pFREM30.

*B. bifidum per*B::pFREM30 was then tested for its ability to use mucin. For this purpose, two *in vitro* growth assays were performed using mucin as the sole carbon source. Firstly, *B. bifidum* PRL2010 wild-type (wt) and *B. bifidum per*B::pFREM30 were grown in mMRS without lactose and supplemented with 0.5% mucin to evaluate possible differences in growth performance between the mutant and wt strains (using mMRS plus lactose as control). Additionally, to assess if the presence of chloramphenicol influences the ability of *per*B::pFREM30 to grow on mucin, the mutant strain was cultivated both in the presence and absence of this antibiotic. Subsequently, growth of each strain was monitored over time (0 h, 5 h, 10 h, 24 h, 36 h and 48 h) by plating on mMRS agar and in parallel by a viability assay and total bacterial cell count through flow cytometry (Figure 3a-b and Figure S2a).

Interestingly, no statistically significant difference was observed of the mutant when grown in the presence or absence of antibiotic neither in the medium supplemented with mucin nor in mMRS (Bonferroni Post Hoc test p-value > 0.05), except for the 24 h time point when grown in mMRS where the mutant strain in chloramphenicol showed a significantly higher growth performance compared to the condition without antibiotic (Figure 3a-b). Therefore, since the presence of

chloramphenicol did not appear to affect mutant growth performance, to maintain the selective pressure of the integrated plasmid, all subsequent experiments involving growth and adhesion to mucin were performed by growing the mutant in the presence of the antibiotic.

Furthermore, in-depth insight into the mucin growth assay data obtained through the plating method revealed no significant differences between *B. bifidum* PRL2010 wt and perB::pFREM30 (Bonferroni Post Hoc test *p*-value >0.05) for the 5 h, 10 h and 24 h time points (Figure 3a). Conversely, at 36 h and 48 h, a significantly higher CFU value was observed for *B. bifidum* PRL2010 wt growing on mucin as the sole carbon source (2.08E+08 CFU/ml ± 1.83E+07 and 1.19E+08 ± 5.74E+07, respectively) when compared to the mutant (5.69E+06 CFU/ml ± 1.79E+06 and 4.42E+06 ± 3.39E+06, respectively) (Bonferroni Post Hoc p-value <0.001 and p-value = 0.008 for the 36 h and 48 h, respectively). Therefore, despite an initial equal ability of the mutant to grow when compared to the wt strain, probably due to other genetic determinants involved in mucin degradation that are present in both strains (Turroni, et al., 2010, Turroni, et al., 2011, Egan, et al., 2014) and/or to the presence of contaminating carbohydrates in the partially purified mucin, these data suggest that the presence of the *per*B gene enhances and supports growth/persistence of *B. bifidum* PRL2010 wt on mucin at 36 h and 48 h.

In addition, the data obtained by plating *B. bifidum* PRL2010 wt and perB::pFREM30 strains grown on mucin as the unique carbon source were further confirmed by analyzing the normalized number of viable cells obtained by combining the viability assay and total bacterial cell count via flow cytometry. Indeed, although the mutant displayed a slight yet significantly higher growth performance at 5 h when compared to the wt strain with a total number of viable cells of 1.71E+07 and 1.13E+07, respectively, for all other time points except for 10 h, a significant higher number of viable cells was recorded for the *B. bifidum* PRL2010 wt strain when compared to the mutant (Figure S2). These observations support the notion that *per*B plays a role in improving the growth performance of *B. bifidum* PRL2010 in the presence of mucin.

Therefore, since the ability to access complex carbohydrates, such as mucin, is limited to a small number of intestinal microbial players, especially bifidobacterial species, and is considered to be a crucial feature for the survival and successful colonization of the intestinal environment, where high levels of competition for nutrients occur (Paone and Cani, 2020, Alessandri, et al., 2021), these results support the hypothesis that *per*B plays a role in *B. bifidum* PRL2010 colonization in the human gut.

Furthermore, to corroborate the impact of *per*B gene in promoting *B. bifidum* PRL2010 persistence in the human intestine via mucin anchoring, the adhesion

abilities of *B. bifidum* PRL2010 wt and perB::pFREM30 to human mucin-secreting HT29-MTX cells were assessed. Interestingly, a statistically significant (Student's t-test p-value of < 0.001) reduction in the adhesion index, calculated as the average number of bacterial cells/100*HT29-MTX cells, to HT29-MTX was observed for *B. bifidum* perB::pFREM30 (adhesion index 62,001 ± 260) when compared to *B. bifidum* PRL2010 wt (adhesion index 146,499 ± 577) (Figure 3c-d). These results demonstrate that *per*B gene inactivation not only appears to affect the ability of *per*B::pFREM30 to utilize mucin, but that it also diminishes its ability to adhere to human intestinal cells.

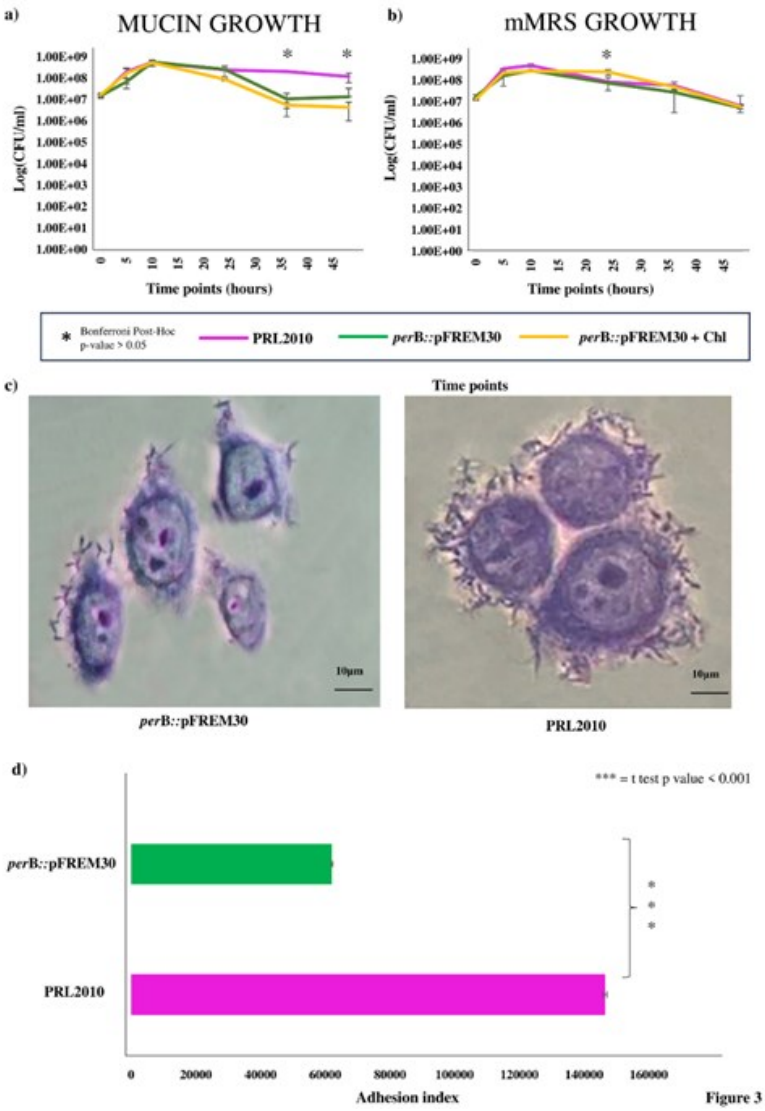


Figure 3. Evaluation of *B. bifidum* PRL2010 and *B. bifidum per*B::pFREM30 ability to grow on mucin. Panels a and b depict growth performances of *B. bifidum* PRL2010 and *B. bifidum per*B::pFREM30 (grown with or wthout chloramphenicol) on mucin as the sole carbon source at different time points (5 h, 10 h, 24 h, 36 h and 48 h) and on mMRS, respectively. The x-axis reports the examined time points, while the y-axis displays the logarithmic total bacterial count obtained by plating an aliquot for each

time point and expressed as CFU/ml. The vertical bars indicate standard deviations. The experiments were carried out in triplicates and the Bonferroni Post-hoc test was used to statistically analyzed data. Panel c shows light microscope images of HT29-MTX cells with *B. bifidum* PRL2010 and *B. bifidum per*B::pFREM30 and colored with the Giemsa staining. Bar, 10 μm. Panel d displays the quantification of the ability of *B. bifidum per*B::pFREM30 and *B. bifidum* PRL2010 to adhere to HT29-MTX secreting-mucin cells. The horizontal bars indicate standard deviations, the three asterisks indicate a t-test *p*-value < 0.001.

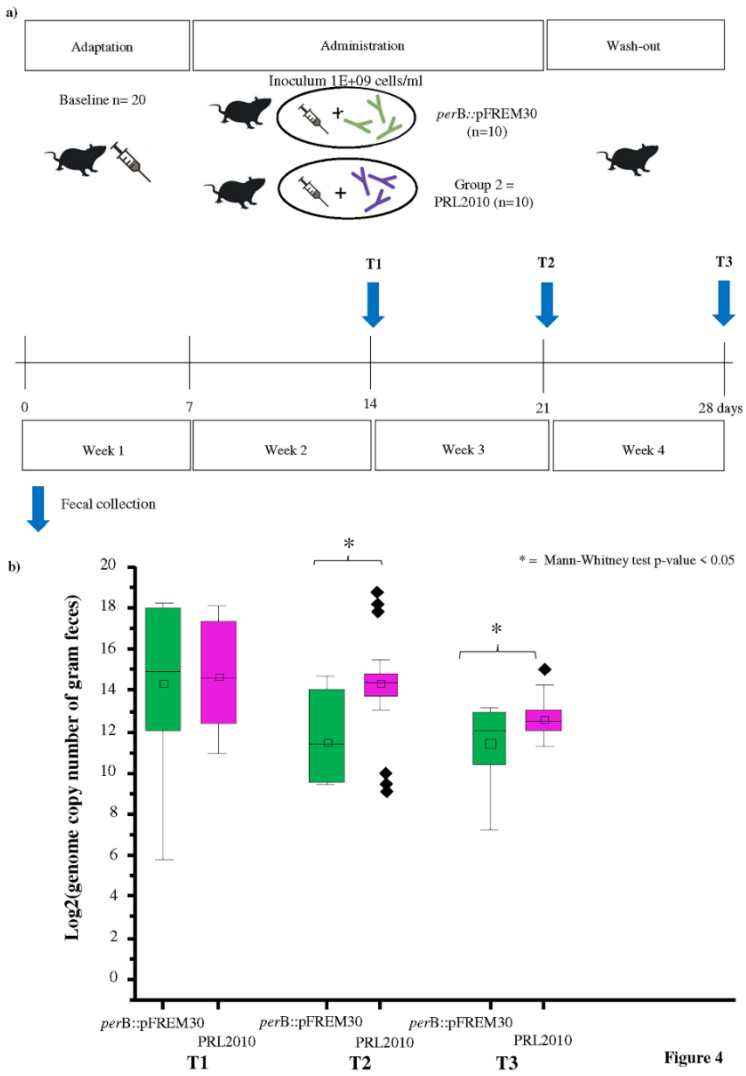
A rodent model-based assessment of *per*B involvement in intestinal colonization. To assess whether a functional *per*B gene contributes to a successful gut colonization and persistence in a competitive environment, the ability of *B. bifidum* PRL2010 wt and *B. bifidum per*B::pFREM30 to colonize the intestinal tract was tested in a rodent model. We first assessed the stability of the integrated pFREM30 plasmid used to create *B. bifidum* PRL2010 *per*B::pFREM30, as it is not possible to select for chloramphenicol resistance in the context of a murine model. For this purpose, *B. bifidum* PRL2010 *per*B::pFREM30 was grown in the presence or absence of 5 μg/mL chloramphenicol and sub-cultivated daily for a total of two weeks. Furthermore, every two days of sub-culturing, cells were spotted on mMRS plates with or without antibiotics, and after 48h of incubation, the presence of the integrated plasmid was confirmed by colony PCR. This analysis not only confirmed the

stability of the integrated plasmid over time in the absence of antibiotic selection but also that the number of colony forming units (CFUs) in both tested conditions remained stable (10E+08 CFU/mL) for the full duration of assessment. After plasmid stability confirmation, a murine model-based *in vivo* trial was performed. The latter consisted of two groups of animals: one receiving a 1 ml daily inoculum of approximately 10E+09 cells/ml of *B. bifidum* PRL2010 *per*B::pFREM30 (Group 1), while the second group received the same amount of *B. bifidum* PRL2010 wt (Group 2) (Figure 4a). The abundance of *B. bifidum* PRL2010 in the fecal samples of the animals enrolled in this study was evaluated using qPCR (Figure 4b). Interestingly, analysis of data collected from qPCR revealed no significant differences in the abundance of *B. bifidum* PRL2010 between the two groups after the first week of strain administration (T1) (Mann-Whitney test *p*-value = 0.694), even if a slight increment in the genome copy number (GCN) of *B. bifidum per*B::pFREM30 was observed when compared to the wild-type. In contrast, a statistically significant increase in GCN (Mann-Whitney test *p*-value < 0.01) of *B. bifidum* PRL2010 *wt* (7.23E+04 GCN/g ± 1.52E+05) was observed after two weeks of strain administration (T2) compared to *B. bifidum per*B::pFREM30 (7.11E+03 GCN/g ± 8.34E+03) (Figure 4b). These data suggest that, despite the initial similar ability of the two strains to colonize the intestinal environment, over a prolonged intervention period, the wild-type strain showed a

more efficient gut colonization and a higher persistence performance when compared to *B. bifidum per*B::pFREM30, with a 10-fold higher average load in the rodent fecal samples. This finding was further confirmed by the observation that after the washout period (T3), the wild-type strain showed a significantly higher abundance (Mann-Whitney test p-value < 0.05) than *B. bifidum per*B::pFREM30 (7.56E+03 ± 5.31E+03 GCN/g and 4.41E+03 ± 3.01E+03 GCN/g for wt and *per*B::pFREM30 strains, respectively) (Figure 4b). In addition, we previously demonstrated that *perB* is involved in favoring bifidobacterial persistence in the human intestinal tract throughout life, yet also that this gene appears to contribute to a more efficient colonization of bifidobacteria in females compared to males (Tarracchini, et al., 2023). We therefore also assessed possible differences in the ability of *B. bifidum* PRL2010 wt and *per*B::pFREM30 strains to colonize the intestine of female and male rats. No statistically significant differences were observed in *B. bifidum* GCN between female and male rats for either of the strains at T1 and T2 (Bonferroni Post Hoc p-value > 0.05) (Figure S2), even if a slight increase of the average *B. bifidum* GCN/g was observed at T2 for the female group administered with *B. bifidum* PRL2010 wt (average GCN/g of 5.98E+04) (Figure S2). Thus, suggesting that, after two weeks of treatment, *per*B presence may play a role in favoring *B. bifidum* PRL2010 colonization performance in female hosts as previously observed in the human

host (Tarracchini, et al., 2023). The limit of detection is 95% (based on the Ct of the NTC). In contrast, at T3, a significantly higher average of *B. bifidum* GCN/g was recorded for the fecal samples of male rats when compared to the female counterpart for both the wt and *per*B::pFREM30 strains (Bonferroni Post-Hoc p-value > 0.05) (Figure S2). These seemingly conflicting results should be contextualized knowing that there are differences in intestinal mucous composition between humans and rodents (Hugenholtz and de Vos, 2018, Robinson, et al., 2019) and that, therefore, PerB is specialized to mucin present in human females as opposed to that of rodents. Overall, these results suggest that *per*B support *B. bifidum* PRL2010 host colonization, allowing enhanced persistence of the strain in the intestinal environment.

Figure 4. Timeline and experimental design of the *in vivo* study. Panel a shows the timeline of the experimental procedures in rats. Panel b reports qPCR data associated with the load of *B. bifidum perB*::pFREM30 and *B. bifidum* PRL2010 in the fecal samples of rats at T1, T2 and T3. The x-axis represents the different time points for each group, while the y axis indicates the genome copy numbers of *B. bifidum* PRL2010 per gram of feces. The boxes represent the 25th and 75th percentiles, and the whiskers represent the standard deviations. The lines in the boxes represent the medians, while the squares represent the average. The rhombi indicate outliers. * = Mann-Whitney test *p*-value < 0.05.



Conclusions

To investigate the contribution of the previously identified mucin-degrading glycosyl hydrolase PerB to bifidobacterial gut persistence, we first investigated the distribution of *per*B homologues in all currently recognized bifidobacterial species, demonstrating that only eight bifidobacterial taxa possessed in their genomes a full-length *per*B homologue, which encodes the FIVAR domain, corresponding to the GH136 catalytic domain. These eight species correspond to bifidobacterial taxa that typically colonize the human or non-human primate intestinal environment, suggesting that only these bifidobacterial species have specifically acquired or evolved *per*B to support their colonization and persistence in the associated competitive intestinal environment.

This notion was confirmed by *in vitro* experiments with human mucin-producing cells, involving a strain with a *per*B gene in its genome, that is, *B. bifidum* PRL2010, and its isogenic *per*B negative variant, emphasizing the role of this gene in enhancing growth on and adherence to mucin. In addition, our *in vivo* rodent experiment highlighted how *per*B inactivation results in a reduced ability of *B. bifidum* PRL2010 cells to persist in the gut environment with a consequent decrease in its colonization capacity when compared to the wild-type strain. Our results, therefore, suggest that, beyond previously characterized bifidobacterial genetic determinants involved in bifidobacterial colonization of and persistence in the human gut environment, also *per*B is implicated in these specific functions (Alessandri, et al., 2019, Alessandri, et al., 2021, Turroni, et al., 2022).

Nonetheless, biochemical assays and mechanistic studies involving PerB are required to better characterize and confirm the involvement of this enzyme in mucin degradation as well as its multifaceted nature since this enzyme not only seems to degrade mucin but also HMOs, as previously reported (Sakurama, et al., 2013, Tarracchini, et al., 2023). A limitation of the present study is that a murine model was used to evaluate *per*B involvement in *B. bifidum* PRL2010 gut colonization although this gene is exclusively present in bifidobacterial species that typically colonize the human or non-human primate gut. However, the murine model has been and continues to be the gold standard model organism for human-related studies to avoid *in vivo* trials that do not allow the administration of *in vitro*-obtained mutants. In addition, the application of the protocols used in this study on other *B. bifidum* strains and on the other bifidobacterial species that possess the *per*B gene would be necessary to confirm the role of the GH136 across bifidobacteria.

Materials and methods

Bifidobacterial genome sequences. Publicly available genomes (complete and draft genome sequences) encompassing all currently characterized bifidobacterial species were retrieved in July 2023 from the NCBI public database. Duplicate bacterial genomes (ANI value > 99%) were removed to avoid genetic redundancy, resulting in a final dataset consisting of 813 bifidobacterial genome sequences.

Presence of *per*B homologues in bifidobacterial species. The deduced proteome of each publicly available bifidobacterial genome was screened for the presence of *per*B homologues, based on sequence similarity to a custom reference database. The latter was obtained by considering all PerB protein sequences previously identified in *B. bifidum* and *B. longum* subsp. *longum* strains. The latter comprised four non-redundant protein sequences (sequence similarity < 90%) of the *per*B gene, previously identified in *B. bifidum* and *B. longum* subsp. *longum* strains(Tarracchini, et al., 2023). Subsequently, all redundant sequences (sequence similarity > 90%) were removed, resulting in a custom database of four (three from *B. bifidum* strains and one from a *B. longum* subsp. *longum* strain) non-redundant *per*B protein sequences, covering the genetic variability of this gene in these species. BlastP analysis was performed employing DIAMOND software(Buchfink, et al., 2015). In addition, Pfam v34.0 (https://pfam.xfam.org/), InterPro 86.0, (https://www.ebi.ac.uk/interpro/), and HMMER (http://hmmer.org/)

were employed to identify protein domains. The deduced proteome of each publicly available bifidobacterial genome was also screened for the presence of *lnb*X and *lnb*Y homologues.

Bacterial strains, plasmids and cultivation conditions. Plasmid pNZ003, which is a derivative of pNZ44 plasmid(McGrath, et al., 2001), was used as a positive control, whereas plasmid pFREM30 is a derivative of pFREM28(Hoedt, et al., 2021), a suicide vector used to target *per*B for mutagenesis by gene disruption. Construction of these two plasmids and their derivatives are described below. *Escherichia coli* EC101 w(Law, et al., 1995)as used as a host strain for the propagation of the aforementioned plasmids, and was cultivated at 37°C in LB medium (Luria Bertani, Scharlab, Spain) supplemented with chloramphenicol at a final concentration of 25 µg/mL. *B. bifidum* PRL2010 was cultivated in modified de Man-Rogosa-Sharpe (MRS) medium without glucose supplemented with 0.05% cysteine-HCl and 2% lactose (mMRS) in an anaerobic chamber (Davidson and Hardy; Belfast; United Kingdom) at 37˚C for 24 h when in broth, and 48 h on agar plates. For the cultivation of bifidobacterial transformants or mutants, mMRS medium was supplemented with 5 µg/mL chloramphenicol. The *B. bifidum* PRL2010 strain used in this study is a bifidobacterial strain isolated from the fecal sample of a breast-fed infant(Turroni, et al., 2010).

Prediction of the methylome of *B. bifidum* PRL2010. To remove the restriction and modification (R/M) sites present on certain plasmids and targeted by endogenous R/M systems encoded by *B. bifidum* PRL2010, the methylome of *B. bifidum* PRL2010 was predicted by assessing the *B. bifidum* PRL2010 genome using the REBASE database (http://rebase.neb.com/rebase/rebase.html) and sequencing its genome using the Pacific Biosciences (PacBio) sequencing platform. For PacBio sequencing, *B. bifidum* PRL2010 was grown in MRS broth supplemented with 0.05% cysteine-HCl until it reached an Optical Density at 600 nm (OD_{600}) of approximately 0.6. Genomic DNA was extracted from harvested PRL2010 cells using the GenElute Bacterial Genomic DNA kit and then subjected to sequencing using PacBio Sequel I technology and SMRT cells (Macrogen service). The PacBio sequencing reads were processed and mapped to the *B. bifidum* PRL2010 sequence. The interpulse durations were measured as previously described (Murray, et al., 2012). To identify methylated positions, the PacBio SMRTPortal analysis platform was adopted, employing an *in silico* kinetic reference and a t-test based kinetic score detection of modified base positions.

Plasmid manipulation and construction. Plasmid pNZ003 was obtained by removing three different R/M sites present in its predecessor, pNZ44 (McGrath, et al., 2001), by PCR using the primers listed in Table 1. Primers pNEW_002, pNEW_003, pNEW_001, and pNEW_004 were used to amplify the pNZ44

plasmid with Q5 polymerase (New England Biolabs). After purification (GeneJet Gel Extraction Kit – Thermo Fisher Scientific), the amplicons obtained were digested with ApaLI and Esp3I (New England Biolabs), ligated (T4 DNA ligase, New England Biolabs), and used to transform *E. coli* EC101 prepared using a modified protocol for chemically competent cells (Hanahan, et al., 1991). Transformants were selected on LB supplemented with 25 µg/mL chloramphenicol, and individual transformants were screened for the presence of the expected plasmid by colony PCR. Plasmid pFREM30 was obtained by amplifying the chloramphenicol gene (Cm^R) with primers CLMC_207 and CLMC_208 and the backbone of pFREM28 (Hoedt, et al., 2021) with primers CLMC_009 and CLMC_010. Following DNA fragment purification (GeneJet Gel Extraction Kit, Thermo Fisher Scientific) the generated amplicons were used to obtain plasmid pFREM30 using the Golden Gate Cloning approach (Engler and Marillonnet, 2014), utilizing the Type IIS enzyme SapI (New England Biolabs). The product of the reaction was introduced into *E. coli* EC101, following the protocol for chemically competent cells. Subsequently, the colonies grown on LB supplemented with 25 µg/mL chloramphenicol were screened by colony PCR for the presence of the expected recombinant plasmid pFREM30, the genetic integrity of which was then validated by sequencing (Genewiz, Leipzig, Germany).

For the construction of plasmid pFREM30-*per*B, the to be targeted, internal region of the gene was amplified by PCR from chromosomal *B. bifidum* PRL2010 DNA (GenElute Bacterial Genomic DNA kit, Sigma, Germany) using the Q5 polymerase and the primers LMV_011 and LMV_012. Plasmid DNA was isolated from *E. coli* using the GeneJET Plasmid Maxiprep Kit (Thermo Fisher Scientific, USA). The amplicon and plasmid were digested with ApaLI and XbaI, ligated, and introduced into *E. coli* EC101 as previously reported (Hanahan, et al., 1991). To select for transformants, the manipulated cells were plated on LB supplemented with 25 μg/mL chloramphenicol, and the colonies were screened for the presence of the expected plasmid construct by colony PCR.

Preparation of bifidobacterial cells for DNA electroporation. An overnight culture of PRL2010 was inoculated into fresh mMRS broth supplemented with 7% (v/w) sucrose (sMRS) and cultivated at 37°C until the exponential growth phase was reached, i.e., an OD_{600} between 0.5 and 0.6. Subsequent steps of the protocol were performed by maintaining the cells refrigerated on ice. Specifically, cells were harvested by centrifugation (4500 × *g* for 10 min at 4°C), washed twice with an ice-cold citrate-sucrose buffer (0.5 M sucrose, pH5.8) and resuspended in 250 μL of the same buffer before electroporation.

Electroporation and selection of PRL2010 mutants. 100 μL of concentrated bacterial cells resuspended in citrate-sucrose buffer (pH 5.8) were mixed with 1.5

µg of the plasmid in a precooled disposable electroporation cuvette with an interelectrode distance of 0.2 cm (Cell project, Kent, United Kingdom). A resistance of 200 Ω, capacity of 25 µF, and voltage of 2.5 kV were applied using a Gene Pulser apparatus (BioRad, UK). After cell electroporation, bacteria were resuspended in 950 µL of sMRS and incubated for 3 h at 37°C in an anaerobic cabinet. Following this, cells were plated on sMRS agar supplemented with 5 µg/mL of chloramphenicol and incubated anaerobically at 37°C for 48 h. Potential mutants were screened by colony PCR using the primers LMV_015 and LMV_016, which annealed to the chromosomal gene outside of the gene target region, and CLMC_02, which annealed to the (integrated) pFREM30 plasmid. The expected amplicons sizes are approximately 2000 bp and 800 bp, respectively. The amplicons obtained were sent for sequencing (Sanger) to further confirm the expected sequence following the expected homologous recombination-mediated integration event. All primer sequences are listed in Table 1.

Mutant stability. To assess the stability of the integrated pFREM plasmid, a spot assay was performed, and the presence of the plasmid was checked by colony PCR. For this assay, *B. bifidum per*B::pFREM30 was cultivated in mMRS broth, with or without 5 µg/mL chloramphenicol. Every day for a total of 14 days, an aliquot of the culture was sub-cultured in fresh medium, and every second day, the overnight inoculum of the two growth conditions was serially diluted (1.00E-04,

1.00E-05, 1.00E-06) and spotted on mMRS plates with or without 5 µg/mL of chloramphenicol. After the anaerobic incubation of the plates at 37°C for 48 h, a colony PCR was performed on one colony per replicate with primers LMV_015 and LMV_016. The experiments were performed in triplicate.

Mucin growth assay. To evaluate possible differences in mucin utilization, *B. bifidum* PRL2010 wild-type (wt) and *B. bifidum per*B::pFREM30 were grown overnight until the exponential growth phase was reached in mMRS, with and without the addition of 5 µg/ml of chloramphenicol to the mutant. Subsequently, strains were washed twice with phosphate-buffered saline (PBS) (Sigma, Germany), and inoculated in a final volume of 20 mL of both mMRS and mMRS with 0.5% mucin (from porcine stomach - Type III, Sigma, Germany) as the sole carbon source (with and without 5 µg/ml of chloramphenicol for the mutant for both culture media) to reach a final inoculum with an OD_{600} of ~0.1. Cells were grown in biologically independent triplicates. Cells were incubated under anaerobic conditions at 37°C for 48 h. Aliquots of the bacterial cultures were collected at different time points (0 h, 5 h, 10 h, 24 h, 36 h and 48 h) and subjected to a viability assay and total bacterial cell count using flow cytometry, as described below. Each aliquot was in parallel plated on mMRS agar. Plates were incubated under anaerobic conditions at 37°C for 48 h. Bacterial growth was assessed by colony forming unit counting.

Evaluation of bacterial cell density and viability assay by flow cytometry. For total cell counts, each culture replicate was 100,000-fold diluted in physiological solution (PBS). Subsequently, 1 mL of the obtained bacterial cell suspension was stained with 1 µl of SYBR®Green I (ThermoFisher Scientific, USA) (1:100 dilution in dimethylsulfoxide; Sigma, Germany), vortexed, and incubated at 37 °C in the dark for at least 15 min before measurement, as previously described (Vandeputte, et al., 2017, Alessandri, et al., 2022). For the bacterial cell viability assay, the fluorescent dyes SYTO9 (3.34 mM) and Propidium Iodide (PI; 20 mM) of the LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher Scientific, USA) were used, following the manufacturer's instructions. Specifically, five different tubes of the diluted cells (final volume of 1 mL each) were obtained per sample. One of the tubes was subjected to centrifugation at 3,000 rpm for 8 min, the supernatant was discarded, and the microbial cell pellet was resuspended in 70% isopropyl alcohol for 1h to allow the permeabilization of microbial cell membrane to induce cell death. The treated cells were then centrifuged, resuspended in PBS and dyed with the addition of 1.5 µl of PI. Other two tubes were stained with 1.5 µl of one of the two dyes, while a fourth tube was not stained. Finally, the last tube was stained with both SYTO9 and PI. Immediately following staining, samples were vortex-mixed and incubated in the dark for 15 min at room temperature. For instrument parameter adjustment, single-dyed samples and the

sample exposed to isopropyl alcohol were used as controls, while the non-stained cells were used as a background control.

Both count and viability experiments were performed with an Attune NxT flow cytometry (ThermoFisher Scientific, USA) 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, that is, forward scatter (FSC) and side scatter (SSC), while fluorescence was detected on a BL1 530/30 nm optical detector. Cell debris were excluded from the acquisition analysis by setting the BL1 threshold. Furthermore, gated fluorescence events were evaluated on the forward-sideways density plot to exclude remaining background events and to obtain an accurate microbial cell count, as previously described. All data were analysed with the Attune NxT flow cytometry software.

Adhesion of *B. bifidum* PRL2010 to HT29-MTX cells. Bifidobacterial adhesion to HT29-MTX cells was assessed by slightly modifying a previously described protocol (Guglielmetti, et al., 2008, Serafini, et al., 2013, Rizzo, et al., 2023). Briefly, human colon carcinoma-derived mucin-secreting goblet HT29-MTX cells (kindly provided by Prof. Antonietta Baldi, University of Milan) were cultured in Minimum Essential Medium (MEM) with high glucose (4.5 g/L) as previously described (Bianchi, et al., 2019). The medium was supplemented with 10% Fetal Bovine Serum (FBS), 4 mM glutamine, 100 µg/mL streptomycin, 100 U/mL

penicillin, and 10 mM HEPES. For the experiment, HT29-MTX cells were seeded on microscope cover glasses previously settled in 10 cm^2 Petri dishes. The cells were carefully washed twice with PBS before the addition of bacterial cells. *B. bifidum* PRL2010 wt and *B. bifidum per*B::pFREM30 were grown as previously described until a concentration of 1.00E+08 cells mL^{-1} was reached. The two strains were then centrifuged at 5,000 x *g* for 8 min, resuspended in PBS, and incubated with HT29-MTX cells. After 1 h incubation at 37°C, cells 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. Finally, the cells were stained with 1.5 ml of Giemsa stain solution (1:20 in PBS) (Sigma Aldrich, Milan, Italy) and incubated in the dark for 30 min at room temperature. After two washes with 2 mL of PBS, cover glasses were removed from the Petri plate, mounted on a glass slide, and examined using a Leica DM 1000 phase contrast microscope (objective: 100X/1.4 oil). The adherent bacteria in 20 randomly selected microscopic fields were counted and averaged, as previously described (Rizzo, et al., 2023). The proportion of bacterial cells that remained attached to HT29-MTX was determined to reflect the extent of specific host-microbe interactions. The adhesion index is calculated as the average number of bacterial cells counted on 20 random spots/(100 the number of HT29-MTX cells) (Guglielmetti, et al., 2008).

Ethics Statement. All experimental procedures and protocols involving animals were approved by the Italian legislation on animal experimentation (D.L. 04/04/2014, n. 26, authorization n° 370/2018-PR) and conducted in accordance with the European Community Council Directives dated September 22, 2010 (2010/63/UE).

Animal housing and design of the *in vivo* experiment. The *in vivo* rodent experiments involved 10 male and 10 female 5-week-old Wistar rats bred at the University of Parma. After weaning, rats were individually housed in polymethyl methacrylate cages in a room under controlled humidity (50±10%) and temperature (22±2°C) conditions, maintained on a 12 h light-dark cycle (lights on at 7 a.m.), and with food and water available *ad libitum*. The first week of the *in vivo* trial corresponded to the acclimatization period, during which rats were fed a standard chow diet supplemented with an oral administration of 500 µL of sucrose solution (2%) through a syringe to adapt them to this form of administration (Figure 4). For the following two weeks (intervention period), rats were randomly and equally divided into a control (administration of *B. bifidum* PRL2010 wt) and treatment (administration *B. bifidum per*B::pFREM30) group, both orally administered once a day with 1.00E+09 cells/mL of the specific strain (Figure 4). For each day of the intervention period, bifidobacterial cells were harvested by

centrifugation, washed in PBS, and resuspended in 1 ml of sucrose solution (2%) for oral administration to rats (10E+09 cells/ml).

Five females and five males were included in each group (Kiss, et al., 2019). Finally, the last week corresponded to the washout period (Figure 4).

During the *in vivo* experiment, fresh fecal samples were collected at three different time points. Fecal samples were collected after the first week of bifidobacterial strain administration (T1), after the second week of treatment, and at the end of the washout (T2 and T3, respectively) (Figure 4). The cage litter was changed 1 h prior to each fecal collection. Afterward, Fresh fecal samples were collected in the morning and stored at −20°C until analysis.

DNA extraction and qPCR analysis. 0.2 g of the collected rat fecal samples were individually subjected to DNA extraction using the QIAmp DNA Stool Mini kit, following the manufacturer's instructions (Qiagen, Germany). Subsequently, quantitative PCR (qPCR) was performed using *B. bifidum* PRL2010-specific primers: BBPR_0282_FW and BBPR_0282_RV (Table 1). These primers had previously been designed and tested to specifically identify *B. bifidum* PRL2010 cells under *in vitro* and/or *in vivo* conditions (Turroni, et al., 2013, Turroni, et al., 2015, Duranti, et al., 2016, Turroni, et al., 2016). qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, USA) on a CFX96 system (BioRad, CA, USA) following previously described protocols (Milani, et

al., 2015). PCR products were detected using SYBR green fluorescent dye and amplified according to the following protocol: one cycle at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 1 min. Negative controls (no DNA) were included for each run. A standard curve was generated through CFX96 software (BioRad) using *B. bifidum* PRL2010 chromosomal DNA extracted with the GenElute bacterial genomic DNA kit (Sigma-Aldrich) following the manufacturer's guide.

Table 1: List of all primers used.

Target	Primer	Primer sequence 5' - 3'
pNZ003	PNEW_001	AACAATGTGCACGACGCGGATTATGCGACGCGTGCATGCGGTACCACTAGTTC
	PNEW_002	CGCGTCGTGCACATTGTTAGATCTGGAGCTGTAATATAAAAACCTTCTTC
	PNEW_003	GATCGACGTCTCAGCTGCGTTAGCTATAGAAGAATATGCAAGAAAG
	PNEW_004	GATCGACGTCTCACAGCAACCGCAGATTTTGAAAAACC
pFREM30	CLMC_009	GATCGCTCTTCTCCCCACCAAAACCGAAATCCAC
	CLMC_010	GATCGCTCTTCTAAGGTGTGCTCCTTTCCCTCAC
	CLMC_207	GATCGCTCTTCGGGGATTATAAAAGCCAGTCATTAGGCCTATCTGAC
	CLMC_208	GATCGCTCTTCACTTATGAACTTTAATAAAATTGATTTAGACAATTGGAAGAG
	PFREM30 MCST	ATATATCCATGGTACCCGGGGTGCACGAAGCTTACGCGTAGACGTCATATGG
		ATATCGCCGTGCTCGAGT
	PFREM30 MCSB	ATATATTCTAGACTCGAGCACGGCGATATCCATATGACGTCTACGCGTAAG
		CTTCGTGCACCCCGGGTAC
Colony PCR-screening	LMV_015	CGCAGCAGCGGTCGCTATG
	LMV_016	GAAGTTGAGGTACGCGGTGTAGC
	CLMC_022	GCCAACGTTTTCGCCAACG
per B gene	LMV_011	TATTGGGTGCACGGCGAACGGCGTCAAGG
	LMV_012	GGTATGTCTAGACCGCCGTTGGCCATGTGC
qPCR	BBPR_0282_FW	GCGAACAATGATGGCACCTA
	BBPR_0282_RV	GTCGAACACCACGACGATGT

Statistical analyses. SPSS software (www.ibm.com/software/it/analytics/spss) was employed to compute statistical analyses, including ANOVA with Bonferroni Post Hoc Multiple Comparison, Student's t-test and the non-parametric Mann-Whitney test.

Data availability. Raw sequences of PacBio sequencing data are available in the SRA database with accession number PRJNA999613.

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Chapter 4

Molecular cross-talk among human intestinal bifidobacteria as explored by a human gut model

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Abstract

Bifidobacteria are well known as common and abundant colonizers of the human gut and are able to exert multiple beneficial effects on their host, although the cooperative and competitive relationships that may occur among bifidobacterial strains are still poorly investigated. Therefore, to dissect possible molecular interactions among bifidobacterial species that typically colonize the human gut, three previously identified bifidobacterial prototypes, i.e., *B. bifidum* PRL2010, *B. breve* PRL2012, and *B. longum* PRL2022 were cultivated individually as well as in bi- and tri-association in a human gut-simulating medium. Transcriptomic analyses of these co-associations revealed up-regulation of genes predicted to be involved in the production of extracellular structures including pili (i.e., flp pilus assembly TadE protein gene), exopolysaccharides (i.e., GtrA family protein gene) and teichoic acids (i.e., ABC transporter permease), along with carbohydrate, amino acid and vitamin metabolism-related genes (i.e., exo-alpha-sialidase; beta-galactosidase and pyridoxamine kinase), suggesting that co-cultivation of bifidobacteria induces a response, in individual bifidobacterial strains, aimed at enhancing their proliferation and survival, as well as their ability to cooperate with their host to promote their persistence. Furthermore, exposure of the selected prototypes to human cell line monolayers unveiled the ability of the bifidobacterial tri-association to communicate with their host by increasing the

expression of genes involved in adherence to/interaction with intestinal human cells. Lastly, bifidobacterial tri-association promoted the transcriptional upregulation of genes responsible for maintaining the integrity and homeostasis of the intestinal epithelial barrier.

Introduction

The interactions among bacteria inhabiting the human gut impact host health by altering human metabolism and influencing the presence and virulence of pathogens (Tojo et al., 2014;Milani et al., 2017). Notably, metabolic interactions between particular gut microbiota members may encompass synergistic activities or antagonistic occurrences, such as those observed between transient and indigenous gut microorganisms (Robles Alonso and Guarner, 2013;Rescigno, 2014). In this context, recent scientific research has included various molecular investigations pertaining to members of the genus *Bifidobacterium* (Margolles et al., 2003;Turroni et al., 2009;Bottacini et al., 2010;Turroni et al., 2012;Laursen et al., 2021;Altaib et al., 2022), which are considered early colonizers of the human gut. Bifidobacteria appear to have a symbiotic relationship with their host, where the latter profits through various purported health benefits, including defense against pathogens, reduction of gut inflammation, immune system modulation, and strengthening the protective mucus layer that coats the intestinal lining. Additionally, bifidobacteria metabolize a large variety of non-digestible glycans and produce various health-related metabolites, including short-chain fatty acids, vitamins, and polyphenols (Ventura et al., 2012;Schiavi et al., 2016;Turroni et al., 2018a;Blanco et al., 2020;Choi et al., 2021;Quintanilha et al., 2022). Bifidobacteria are highly prevalent and abundant inhabitants of the infant

intestine, though their presence decreases after weaning; nonetheless, they persist in the adult gut (at 1-5% relative abundance), levels that are believed to be sufficient to provide their host with the aforementioned advantages (Avershina et al., 2013;Milani et al., 2015b;Avershina et al., 2016). Specifically, host sex-related persistence of strains belonging to common, maternally inherited bifidobacterial species, including *Bifidobacterium longum* subsp. *longum* and *Bifidobacterium bifidum*, was observed based on their ability to degrade host-derived glycans (Tarracchini et al., 2023;Rizzo et al., 2024). Members of the species *B. bifidum*, *B. longum*, and *Bifidobacterium breve* are not only the first commonly encountered species colonizing the gut, but they also exert beneficial influence on the gut microbial ecosystem and toward the epithelial host cells (Hougee et al., 2010;Bozzi Cionci et al., 2018;Tanno et al., 2019;Yan et al., 2019;Turroni et al., 2020). In this context, it has been demonstrated that bifidobacteria interact with each other through cross-feeding relationships, thereby enhancing their fitness within the human intestinal environment and exerting beneficial effects on their host (Turroni et al., 2014b;Riviere et al., 2016;Turroni et al., 2016;Turroni et al., 2018b).

Thus, to dissect and investigate putative molecular synergies between bifidobacterial strains and to determine whether their interactions may enhance their host colonization and promote beneficial health effects, three bifidobacterial

strains, i.e., *B. bifidum* PRL2010, *B. longum* PRL2022, *B. breve* PRL2012 were selected. These strains were previously identified as prototypes of the human gut microbiota based on functional and genomic surveys (Fontana et al., 2022;Alessandri et al., 2023;Argentini et al., 2024). These strains were grown in bi- and tri-association in a human intestinal environment-simulating medium (Alessandri et al., 2022) and their molecular cross-talk activities were investigated through a transcriptomic analysis. Moreover, an *in vitro* experiment involving Caco-2/HT-29-MTX cells was employed to evaluate whether the association of the three selected bifidobacterial strains when in contact with human cell intestinal monolayer may impact both bifidobacterial and human cell line transcriptomes.

Materials and Methods

Bifidobacterial bi- and tri-association. To evaluate whether or not the co-association of the selected bifidobacterial strains (*B. bifidum* PRL2010 and *B. longum* PRL2022 were isolated from human infant/adult feces respectively, while *B. breve* PRL2012 was isolated from human milk) impacts their gene expression, the three strains were cultivated in mono-, bi- and tri-associations in a human colon environment-simulating growth medium (IGSM) (Alessandri et al., 2022) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride under anaerobic conditions (2.99% H_2, 17.01% CO_2, and 80% N_2). The IGSM contains various sources of carbohydrates i.e., inulin (0.2 g/L), pectin (0.2 g/L), arabinogalactan (0.2 g/L), xylan (0.2 g/L), lactose (0.2 g/L), mucin (3 g/L) and starch (3 g/L). Specifically, viable cells were inoculated in 30 ml of freshly prepared IGSM medium. The bifidobacterial cells were enumerated by using the Thoma cell counting chamber (Herka). Cells were inoculated approximately to $1x10^6$ cells /mL. After inoculation, growth was monitored until the exponential phase was reached (around $1x10^8$ cells /mL), then cells were harvested by centrifugation at 6000 rpm for 5 min. Bacterial cells were stored at -80°C until they were processed for RNA extraction. Growth assays were carried out in triplicate.

DNA extraction and qPCR analysis. An aliquot of each condition i.e., mono-culture, bi-association and tri-association was subjected to DNA extraction using

the QIAmp DNA Stool Mini kit following the manufacturer's instructions (Qiagen, Germany). Subsequently, to evaluate bifidobacterial cell numbers in each sample, a quantitative PCR (qPCR) was performed using *B. bifidum* PRL2010, *B. breve* PRL2012 and *B. longum* PRL2022-specific primers listed in the Supplementary Tables (Table S14). qPCR was performed using the PowerUp SYBR Green Master Mix (Applied Biosystem, USA) on a CFX96 system (BioRad, CA, USA) following previously described protocols (Milani et al., 2015a). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: one cycle of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 58°C for 15 s, and 72°C for 1 min. The melting temperature was 56°C for *B. longum* PRL2022. In each run, negative controls (no DNA) were included. A standard curve was generated using the CFX96 software (BioRad). In detail, a known DNA standard sample for each species was selected, quantified, and serially diluted to obtain the number of copies of double-stranded DNA per µL ranging from 10^3 to 10^9. The standard curve was then automatically generated by the software.

Human cell line trials. Caco-2 cells, derived from a colorectal adenocarcinoma of a human male donor (purchased from ATCC), and HT-29-MTX, a human colon carcinoma-derived mucin-secreting goblet cell line from a female donor (kindly provided by prof. Antonietta Baldi, University of Milan) were cultured in

Minimum Essential Medium (MEM) and Dulbecco's Modified Eagle's medium (DMEM) with high glucose (4.5 g/L) and sodium pyruvate (10 mM), as previously described (Bianchi et al., 2019). Both media were supplemented with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere in 10-cm dishes and passaged three times a week. Subsequently, a mixed suspension of Caco-2 and HT-29-MTX cells (7:3) was seeded in DMEM + FBS at a density of $\approx 10^5$ cells/cm^2 into cell culture inserts with membrane filters (pore size 0.4 µm) for Falcon 24-well-multitrays (Becton, Dickinson & Company, Franklin Lakes, NJ, USA). Cells were grown for 21 days until a tight monolayer was formed (TEER > 600 Ω x cm^2) with medium replacement every three days.

Co-cultures of human cell monolayers and bifidobacteria. After 21 days from seeding, the culture medium of the 24-well plates was replaced with fresh, antibiotic-free DMEM. Subsequently, 400 µL of bifidobacterial cells with a final concentration of $\approx 10^8$ cells/ml were inoculated on the Caco-2/HT-29-MTX cell monolayers, as previously described (Serafini et al., 2013;Rizzo et al., 2023). The 24-well plates were then incubated at 5% CO_2 at 37°C. After 4h of incubation, the non-adherent bacterial cells were washed using 500 µL of PBS (Phosphate Buffered Saline), then the adherent bacterial cells were gently detached from the human cells using a pipette with 500 µL of RNA later and stored at -80°C until

processing. For this experiment, the three bifidobacterial strains were grown in the IGSM medium in anaerobic conditions at 37 °C. Once the exponential growth phase was reached, bifidobacterial cells were enumerated by using the Thoma cell counting chamber (Herka). The bacterial cells were diluted to reach a final concentration of 10^8 cells/mL in 400 µL, washed in the same volume of PBS, and then resuspended in 400 µl of antibiotic-free DMEM. At the end the bacterial cells were added to the cell monolayers. Bacterial studies were performed either with single strains (used as sample control) or with a mixture of the three strains and added to the human cell monolayers. All experiments were performed in triplicate, with three technical replicates for each biological replicate.

Prokaryotic RNA extraction and sequencing. Total RNA from each bifidobacterial culture was isolated as previously described (Turroni et al., 2010b). Briefly, cell pellets were resuspended in 1 ml of QIAZOL (Qiagen, United Kingdom) and placed in a tube containing 0.8 g of glass beads (diameter, 106 µm; Sigma). Cells were lysed by alternating 2 min of agitating the mix on a bead beater with 2 min of static cooling on ice. The mixture was then centrifuged at 12,000 rpm for 15 min, and the RNA-containing sample was recovered from the upper phase. The latter was further processed using the RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. The quality of the RNA was verified employing a Tape station 2200 (Agilent Technologies,

USA). RNA concentration and purity were evaluated using a spectrophotometer (Eppendorf, Germany). For RNA sequencing, from 100 ng to 1 μg of extracted RNA was treated to remove rRNA by employing QIAseq FastSelect – 5S/16S/23S following the manufacturer's instructions (Qiagen, Germany). RNA yield following rRNA depletion was checked by the use of a Tape station 2200 (Agilent Technologies, USA). Subsequently, a whole transcriptome library was constructed using the TruSeq Standard mRNA preparation kit (Illumina, San Diego, USA). Samples were processed using a NextSeq high output v2.5 kit (150 cycles, single end) and sequenced (Illumina) according to the technical support guide. The obtained reads were filtered to remove low-quality reads (minimum mean quality 20 and minimum length 150 bp) as well as any remaining ribosomal locus-encompassing reads using the METAnnotatorX2 (Milani et al., 2021). Subsequently, the retained reads were aligned to the specific reference genome of each employed bifidobacterial strain through Bowtie2 software (Langdon, 2015). Quantification of reads mapped to individual transcripts was achieved through htseq-counts script of HTSeq software in "union" mode (Anders et al., 2015). Raw counts were then normalized using CPM (Counts per million mapped reads) for filtering genes with low counts (CPM <1) and TMM (Trimmed Mean of M-Values) for statistically robust differential gene expression analysis through the EdgeR package (Robinson et al., 2010). Evaluation of expression differences

was calculated for each gene as log2 fold change (logFC) of average expression between the control (the single strain) and "treated" samples (strains all together and in pairs). Additionally, for each comparison, a Volcano plot was created to simultaneously visualize expression changes (log fold change) and their statistical significance (*p*-value).

Eukaryotic RNA extraction and analyses. Total RNA from human cells was isolated using a previously described method (Alessandri et al., 2023). Briefly, total RNA from human cell lines was extracted by adding 350 µL of RLT buffer from the RNeasy Mini Kit (Qiagen, Germany) and following the manufacturer's instructions, then the RNA concentration and purity were evaluated using a spectrophotometer (Eppendorf, Germany). Reverse transcription to cDNA was conducted utilizing the iScript Select cDNA synthesis kit (Bio-Rad Laboratories), employing the following thermal cycle: 5 minutes at 25°C, followed by 30 minutes at 45°C, and concluding with 8 minutes at 85°C. The mRNA expression levels were assessed using SYBR green technology in quantitative Real-Time PCR (Biorad) executed on a Bio-RAD CFX96 system. Gene expression was standardized in relation to a housekeeping gene (*atp*D), as previously outlined (Turroni et al., 2011a). The primers used are listed in the Supplementary Material Table S14.

Statistical analysis. For differential gene expression analysis, EdgeR package was used to estimate the statistical significance of differences between fold changes as the False Discovery Rate (FDR).

Data availability. Raw sequences of RNA sequencing data are available in the SRA database with accession number PRJNA1108206.

Results and Discussion

Effects of bifidobacterial prototype co-associations on transcriptomes. To evaluate whether particular co-associations of *B. bifidum* PRL2010, *B. longum* subsp. *longum* PRL2022, and *B. breve* PRL2012 strains induce a modification in the corresponding bifidobacterial transcriptomes, these bacteria were grown individually (mono-association), as well as in bi- and tri-associations, in a human gut environment-simulating culture medium. Alterations in gene expression in the bi-associations and tri-association compared to the single strain cultivation were evaluated through RNA sequencing. The latter generated a total of 2,379,454 quality-filtered reads, with an average of 67,984 reads per sample (Table S1). In this context, only genes showing a fold-change in transcription of ≥ 2 in combination with a p-value of ≤ 0.05 calculated through correction for multiple comparisons using the False Discovery Rate (FDR) procedure were considered as significantly differentially transcribed between the bi- and tri-associations when compared to mono-associations. Specifically, the above-mentioned species were chosen due to the fact that they colonize the host throughout their entire lifespan. These specific strains were selected as prototype of the species they belong to as previously demonstrated through ecological and phylogenetic approaches (Turroni et al., 2011b;Turroni et al., 2012;Derrien et al., 2022;Turroni et al., 2022;Argentini et al., 2024).

Among the considered co-associations, in-depth insights into those genes with significantly altered transcription revealed that the highest number of up-regulated genes in *B. breve* PRL2012 and *B. longum* subsp. *longum* PRL2022 was observed when both were cultured in tri-associations. Analysis of *B. breve* PRL2012 demonstrated an up-regulation of 410 genes, while *B. longum* PRL2022 up-regulated 578 genes. However, regarding B. *bifidum* PRL2010 the highest up-regulation was demonstrated to be 400 genes, when co-cultured with *B. breve* PRL2012 (Figure 1a) (Table S2-S10). Therefore, while the tri-association seemed to better stimulate cross-talk between *B. longum* PRL2022 and *B. breve* PRL2012, the presence of the single *B. breve* PRL2012 strain appeared to induce the most relevant impact on the transcriptome of *B. bifidum* PRL2010, with the number of *B. bifidum* PRL2010 up-regulated genes in the tri-association is slightly lower (338) (Figure 1a). This observation was further confirmed by the assessment of the cross-talk index, calculated for each bi-association and for the tri-association as the ratio between the number of over-expressed genes respect to the total gene repertoire for each genome, as previously described (Turroni et al., 2016) (Figure 1b). In detail, the determination of the cross-talk index emphasized that the highest modulation of *B. bifidum* PRL2010 gene expression was achieved when *B. breve* PRL2012, regardless of the bi- or tri-associations, was present in the co-cultivation, suggesting a higher response of *B. bifidum*

PRL2010 towards the interaction with *B. breve* PRL2012 (Figure 1b), reflecting also the frequent co-occurrence of these bacteria in the same ecological niche, i.e., the infant gut (Turroni et al., 2015). In this regard, it has been demonstrated by previous studies that strains of *B. bifidum* and *B. breve* generally establish an intimate trophic dialogue based on metabolic cross-talk/cross-feeding events (Turroni et al., 2015;Turroni et al., 2018b).

Furthermore, a functional categorization of the up-regulated genes was performed using cluster of orthologous genes (COGs). Interestingly, both bi- and tri-associations of the assessed bifidobacterial strains caused a higher production of transcripts belonging to the amino acid and carbohydrate metabolism and transport families (COG-E and COG-G, respectively) (Figure 1c), unveiling a metabolism-biased response to the co-association (when compared to the mono-cultivation). Interestingly, while *B. longum* PRL2022, in all the tested conditions showed a similar number of up-regulated genes belonging to the metabolism-related COG families (Figure 1c), for *B. bifidum* PRL2010 the highest number of up-regulated genes related to the COG-E and COG-G families was recorded when in bi-association with *B. breve* PRL2012 and in the tri-association. Similarly, *B. breve* PRL2012 exhibited a higher number of up-regulated genes belonging to these COG families when co-cultivated with *B. bifidum* PRL2010 or in tri-association. These findings suggest that *B. longum* PRL2022 does not exhibit an affinity

preference for a particular bifidobacterial species, as it interacts similarly from a metabolic point of view with each of the two tested species. In contrast, *B. bifidum* PRL2010 appears to exhibit an enhanced metabolic response when *B. breve* PRL2012 is present, and vice versa. Overall, despite differences in strain-specific gene modulation, these results indicate that these microorganisms, when exposed to particular (bifido)bacterial players, activate a range of metabolic options, ranging from cross-feeding events/cooperation to competitive behavior, to possibly improve their ecological fitness in a human gut-like environment. Furthermore, to assess whether co-associations among bifidobacterial strains led to growth differences compared to single strains, a qPCR assay was conducted using aliquots from the same samples on which RNAseq was performed. Notably, a statistically significant growth increase for *B. longum* PRL2022 was observed in both bi-associations and tri-association when compared to the mono-cultivation under the same conditions (Table S11). Furthermore, even if only a tendency towards significance was observed, higher cell counts for *B. breve* PRL2012 were recorded when in bi-association with *B. longum* PRL2022 as well as for *B. breve* PRL2012 and *B. bifidum* PRL2010 in the case of tri-association and in bi-association with *B. longum* PRL2022 when compared to the mono-culture (Table S11). Thus, suggesting a tendency towards better growth performances of the

bifidobacterial strains when in bi- and tri-association when compared to mono-culture.

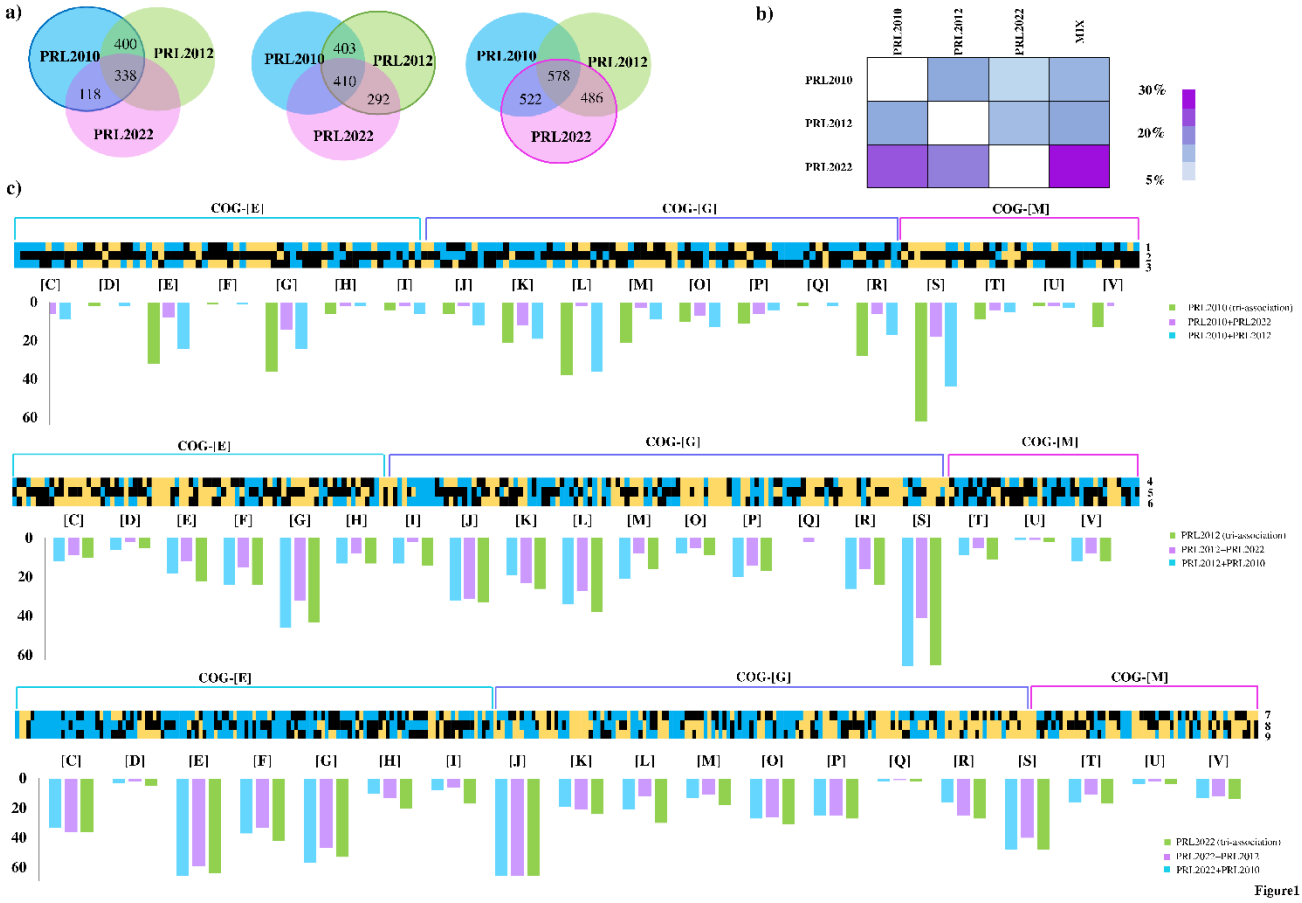

Figure1

Figure 1: Transcriptome analyses of co-cultivated bifidobacterial strains. Panel a) depicts the Venn diagrams representing the up-regulated genes in *B. bifidum* PRL2010, *B. breve* PRL2012, and *B. longum* PRL2022, respectively, in bi- and tri-association. Panel b) reports on the cross-talk index of each bifidobacterial strain for all tested conditions. Panel c) shows the *B. bifidum* PRL2010, *B. breve* PRL2012, and *B. longum*

PRL2022 differentially expressed genes by transcriptome analysis in response to bi- and tri-associations. Each heat map displays fold change in gene expression for *B. bifidum* PRL2010, *B. breve* PRL2012, and *B. longum* PRL2022 according to the condition indicated by the number reported on the right of the heat maps. The numbers next to each individual column of the heat map correspond to the various associations of bifidobacteria: 1, *B. bifidum* PRL2010 + *B. breve* PRL2012; 2, *B. bifidum* PRL2010 + *B. longum* PRL2022; 3, *B. bifidum* PRL2010 (tri-association); 4, *B. breve* PRL2012 + *B. bifidum* PRL2010; 5, *B. breve* PRL2012 + *B. longum* PRL2022; 6, *B. breve* PRL2012 (tri-association); 7, *B. longum* PRL2022 + *B. bifidum* PRL2010; 8, *B. longum* PRL2022 + *B. breve* PRL2012; 9, *B. longum* PRL2022 (tri-association). The heat maps solely report on transcriptional differences of genes belonging to the COG-E, G and M categories. The bar plots under each heat map show the functional annotation of significantly differentially transcribed genes of bi- and tri-association separated according to the cluster of orthologous gene (COG) categories they belong to. Each COG family is identified by one-letter abbreviations: C, energy production and conversion; D, cell cycle control and mitosis; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism; I, lipid transport and metabolism; J, translation; K, transcription; L, replication and repair; M, cell wall/membrane/envelop biogenesis; N, cell motility; O, post-translational modification, protein turnover, chaperone functions; P, inorganic ion transport and metabolism; Q, secondary structure; R, general functional prediction only; S, function unknown; T, signal transduction; U, intracellular trafficking and secretion; V, defense mechanisms; Z, cytoskeleton.

Scrutiny of the co-association effect on the expression of bifidobacterial nutrient-related metabolic pathways. To further evaluate the impact that bifidobacterial co-cultivation may have on the modulation of metabolic pathways

of a *Bifidobacterium* strain, an in-depth functional investigation into the observed up-regulated genes that belong to carbohydrate transport and metabolism (COG-G) was performed. Interestingly, the expression data revealed up-regulation of several genes encoding Glycosyl Hydrolases (GHs) and Glycosyl Transferases (GTs) in all bifidobacterial strains regardless of the co-association. Indeed, the higher abundance of transcripts related to genes involved in carbohydrate metabolism, as a consequence of co-cultivation with other microorganisms, may be considered a competitive response to advantageously access carbon sources, ensuring bifidobacterial growth and/or survival. On the other hand, it can also underline the establishment of cross-feeding events, which may contribute to promoting better metabolic fitness for all strains in co-association. (Turroni et al., 2010a;Milani et al., 2014;Duranti et al., 2019;Luo et al., 2021;Diaz and Garrido, 2024) (Table S2-S10). In detail, *B. breve* PRL2012, both in bi- and tri-association, produced a higher number of transcripts encoding for a predicted extracellular GH13 which acts as α-glucan-active enzyme (Turroni et al., 2016). Substrates of this GH13 include starch and related polysaccharides which are present both in the culture medium used here and are also widely available in the intestinal environment as nutrients internalized through the host diet (Alessandri et al., 2022;Fernandez-Julia et al., 2022) (Table 1). Analysis of the *B. bifidum* PRL2010 transcriptome in bi-association with *B. breve* PRL2012 highlighted up-regulation

of GH42, GH84, and GH33, i.e., enzymes predicted to be involved in mucin-associated O-/N-glycan degradation (Bell and Juge, 2021) (Table 1). Specifically, while GH84 is an N-acetylglucosaminidase predicted to degrade the enteric mucin, the GH33 acts as an exo-sialidase capable of releasing sialic acid into the surrounding environment. In this context, since *B. breve* strains, unlike *B. bifidum* PRL2010, do not possess in their genomes exo-sialidases predicted to cleave mucin to release sialic acid, it is possible that the co-association of *B. bifidum* PRL2010 with *B. breve* PRL2012 specifically induced over-expression of genes involved in carbohydrate degradation/utilization to favor cross-feeding metabolic events, as has previously been described (Egan et al., 2014a;Egan et al., 2014b;Nishiyama et al., 2018;Turroni et al., 2018b;Yokoi et al., 2022). Therefore, this data suggests that the co-association of these bifidobacterial prototypes (when compared to the mono-cultivation) in a human gut-mimicking medium causes enhanced transcription of genes involved in the metabolism of both diet- and host-derived complex carbohydrates, thus enhancing their fitness by inducing cross-feeding interactions (Tannock et al., 2012;Egan et al., 2014a).

Beyond genes related to carbohydrate metabolism, it was also investigated whether the transcription of genes belonging to metabolic pathways involved in amino acid and coenzyme transport and metabolism (COG-E and COG-H families) underwent significant modifications for these bifidobacterial co-

cultures. Interestingly, all three bifidobacterial strains, in every tested condition when compared to the mono-associations, showed transcriptional increase of several genes belonging to these COG families. Amino acids are fundamental for bifidobacterial growth and metabolism (Ventura et al., 2007). Additionally, since amino acid metabolism in bifidobacteria is closely associated with the production of metabolites (D'Aimmo et al., 2024;Dinger et al., 1991), including acetic acid, an in-depth assessment of the transcriptome associated with acetic acid metabolic pathway revealed a significantly higher number of transcripts of several genes in the three bifidobacterial prototypes regardless of the co-association (Table S2-S10). Thus, it can be inferred that co-association of these three bifidobacterial strains, compared to single mono-culture, promotes and stimulates metabolic pathways that may enhance the metabolic flux of the bifidobacterial strains (Arboleya et al., 2016;O'Callaghan and van Sinderen, 2016;Turroni et al., 2018a). In addition, a detailed dissection of the bifidobacterial transcriptomes revealed the up-regulation of genes implicated in the biosynthesis of B vitamins. Specifically, *B. longum* PRL2022 in all the tested conditions, *B. bifidum* PRL2010 in the tri-association, and *B. breve* PRL2012 in the bi-association with *B. longum* PRL2022 unveiled the up-regulation of certain genes for thiamine production (vitamin B1) (Table 1). Interestingly, thiamine serves as a co-factor for pyruvate, an integral component of the metabolic pathway involved in the

production of acetic acid (Soto-Martin et al., 2020). Since the latter may be used as a precursor to produce butyrate, i.e., a major short-chain fatty acid (SCFA) exerting numerous health benefits upon the host, including anti-inflammatory and anticarcinogenic effects (Louis et al., 2014), by butyrogenic members of the gut microbiota such as *Faecalibacterium prausnitzii*, this data suggests a beneficial cross-feeding interaction. Indeed, when co-cultivated with other intestinal players, bifidobacteria can indirectly contribute to the activity of butyrogenic gut microbiota members through this interaction (Gibson et al., 2016;Soto-Martin et al., 2020). Moreover, a gene involved in the biosynthesis of folate (vitamin B9) (Table 1), i.e., a vitamin linked to various physiological functions, including host immunity, gut barrier integrity, and even neurological health (Park et al., 2022), was shown to be up-regulated in *B. bifidum* PRL2010 in the tri-association. Additionally, the bi-association of *B. bifidum* PRL2010 and *B. breve* PRL2012 led to a significantly higher expression, in both strains, of genes involved in the biosynthesis of pyridoxine, along with *B. longum* PRL2022 in bi- and tri-associations (Table 1). Pyridoxine is crucial for the proper functioning of the metabolic pathways of bifidobacteria, participating in different pathways related to amino acid and carbohydrate metabolism, and regulating bifidobacterial growth and energy metabolism (Roberfroid et al., 2010;LeBlanc et al., 2013). Therefore, this data indicates that the co-cultivation of bifidobacterial strains is

able to promote a higher expression of vitamin biosynthesis associated-genes, which may confer benefits to the host, compared to when the same bifidobacterial strains are individually cultivated.

Table 1: List of up-regulated genes when *B. bifidum* PRL2010, *B. breve* PRL2012 and *B. longum* PRL2022 were in bi-association and tri-association in IGSM. The first column specifies the bifidobacterial species used in the experiments. In the second column are listed all the up-regulated genes cited throughout the text in the bi-association for each bifidobacterial species. When not specified, the genes are up-regulated in both the bi-associations.

Species	Bi-association		Tri-association	Gene annotation
	***B. breve* PRL2012**	***B. longum* PRL2022**		
***B. bifidum* PRL2010**	BBPR_RS00830	-	-	beta-galactosidase (GH42)
	BBPR_RS08290	-	-	beta-N-acetylglucosaminidase domain (GH84)
	BBPR_RS09085	-	-	exo-alpha-sialidase (GH33)
	BBPR_RS09090	-	-	exo-alpha-sialidase (GH33)
		-	BBPR_RS06710	1-deoxy-D-xylulose-5-phosphate synthase
		-	BBPR_RS02915	folylpolyglutamate synthase/dihydrofolate
	BBPR_RS01945	-	-	pyridoxal 5'-phosphate synthase glutaminase
	BBPR_RS09240	-	-	class C sortase (pili)
	BBPR_RS09250	-	-	LPXTG cell wall anchor domain-containing (pili)
	BBPR_RS08655	-	-	SpaA isopeptide-forming pilin-related protein (pili)
	BBPR_RS01460	-	-	isopeptide-forming domain-containing fimbrial (pili)
	BBPR_RS08910	BBPR_RS08910	BBPR_RS08910	DUF4244 domain-containing protein (pre-pilin Tad)
	BBPR_RS08920	BBPR_RS08920	BBPR_RS08920	pilus assembly protein (TadB)
	BBPR_RS08925	BBPR_RS08925	BBPR_RS08925	ATPase, T2SS/T4P/T4SS family (TadA)
	BBPR_RS08930	BBPR_RS08930	BBPR_RS08930	septum site-determining protein minD (TadZ)
	BBPR_RS08900	-	-	flp pilus-assembly TadE/G-like family protein
	BBPR_RS08905	-	-	TadE family type IV pilus minor pilin (TadE)
	***B. bifidum* PRL2010**	***B. longum* PRL2022**		
***B. breve* PRL2012**	PRL2012_0103	PRL2012_0103	PRL2012_0103	glycoside hydrolase family 13 protein (GH13_30)
	PRL2012_0115	PRL2012_0115	PRL2012_0115	type I pullulanase (GH13)
	-	PRL2012_0617	-	sulfur carrier protein ThiS
	-	PRL2012_0620	-	rhodanese-like domain-containing protein
	PRL2012_1179	-	-	type I glyceraldehyde-3-phosphate dehydrogenase
	PRL2012_0107	PRL2012_0107	PRL2012_0107	class C sortase (pili)
	-	PRL2012_1610	-	SAF domain-containing protein (pili)
	-	PRL2012_1613	-	FHA domain-containing protein (pili)
	PRL2012_0174	PRL2012_0174	PRL2012_0174	GtrA family protein (EPS)
	PRL2012_0528	PRL2012_0528	PRL2012_0528	GtrA family protein (EPS)
	PRL2012_1735	PRL2012_1735	PRL2012_1735	polysaccharide ABC transporter ATP-binding (TA)
	PRL2012_1736	-	-	ABC transporter permease (TA)
	***B. bifidum* PRL2010**	***B. breve* PRL2012**		
***B. longum* PRL2022**	67B_0509	67B_0509	67B_0509	1-deoxy-D-xylulose-5-phosphate synthase
	67B_0889	67B_0889	67B_0889	SufS family cysteine desulfurase
	67B_1137	67B_1137	67B_1137	transketolase
	67B_1138	67B_1138	67B_1138	transketolase
	67B_0815	67B_0815	67B_0815	pyridoxamine kinase
	67B_1943	67B_1943	67B_1943	ATP-binding cassette domain-containing protein (EPS)
	67B_1944	67B_1944	67B_1944	ABC transporter ATP-binding protein/permease (EPS)
	67B_0488	67B_0488	67B_0488	S-ribosylhomocysteine lyase (LuxS)

The impact of co-culture on bifidobacterial ability to interact with the host and other *Bifidobacterium* members. In order to evaluate whether co-association plays a role in enhancing bifidobacterial fitness in the human gut environment through genes other than those associated with metabolism, we performed an investigation into the genetic sequences that were shown to be up-regulated in the co-associations when compared to the mono-culture, and that were predicted to be involved in the interaction with the host and/or with other microbial players. In this context, a comprehensive analysis of the COG functional characterization revealed up-regulation of several gene clusters putatively involved in bifidobacterial interaction with other intestinal microorganisms or with the host (Table S2-S10). Specifically, all assessed co-associations caused up-regulation of a gene in *B. breve* PRL2012 predicted to belong to a sortase-dependent pilus locus (Table 1). Similarly, two genes of a homologous pilus locus were shown to be up-regulated in *B. bifidum* PRL2010 when in bi-association with *B. breve* PRL2012 as well as in the latter strain when co-cultivated with *B. longum* PRL2022 (Table 1). In this context, since sortase-dependent pili have mainly been depicted as extracellular structures directly involved in host-microbe interactions promoting adhesion to the human intestinal epithelial cells and aggregation between microbial cells, as well as cross-talk with the immune system (Turroni et al., 2013;Turroni et al., 2014a), these findings

suggest that exposure of a bifidobacterial strain to another member of the genus *Bifidobacterium* plays a fundamental role in enhancing bifidobacterial ability to adhere to enterocytes. Therefore, this exposure favors their persistence in the human gut. Moreover, in all tested conditions, transcriptional up-regulation in *B. bifidum* PRL2010 of four genes belonging to the locus encoding for the Tad pilus, was observed (Table 1) (Alessandri et al., 2021). Particularly, enhanced transcription of *tad*Z, *tad*A (the ATPase) and *tad*B, involved in the formation and assembly of the pilus, was detected, along with the *flp* gene, which contributes to the production of the pilus structural proteins (O'Connell Motherway et al., 2011). Additionally, the presence of *B. breve* PRL2012, both in the bi-association with *B. bifidum* PRL2010 and in the tri-association, induced the up-regulation of two genes *tad*E also involved in the synthesis of the structural proteins of the pilus. In this context, the *tad* locus has been poorly characterized in bifidobacteria, except for *B. breve* UCC2003, and thus, the extracellular filament has been postulated to be involved in promoting adhesion of bifidobacteria to epithelial intestinal cells and cellular proliferation (O'Connell Motherway et al., 2011;Alessandri et al., 2021). Therefore, up-regulation of certain genes of the *tad* locus in *B. bifidum* PRL2010 indicates that co-association with other bacteria primes a cascade response aimed to produce extracellular structures to ensure its intestinal establishment and persistence. Similarly, beyond the significantly higher number

of transcripts related to pilus production, in *B. breve* PRL2012 and *B. longum* PRL2022, both in bi- and tri-associations, an up-regulation of two genes implicated in EPS (exopolysaccharide) biosynthesis was observed (Table 1). Furthermore, in all tested conditions, *B. breve* PRL2012 was shown to exhibit an up-regulation of two genes which are predicted to play a role in the production of teichoic acids (Table 1). In this context, since both EPS and teichoic acids, i.e., glycan layers that cover the bacterial cell surface and negatively charged polymers present in/on the cell surface of Gram-positive bacteria, respectively, have been widely depicted as extracellular structures that participate in the interactions of bifidobacteria with the host or other bacterial players of the gut microbiota (Fanning et al., 2012;Alessandri et al., 2023;Argentini et al., 2023), these results strengthen the notion that bifidobacterial co-association stimulates the production of structures directly involved in bifidobacterial colonization of the intestine (Weidenmaier and Peschel, 2008;Colagiorgi et al., 2015;Rizzo et al., 2023).

Furthermore, the co-association of *B. longum* PRL2022 with the other two bifidobacterial strains led to enhanced expression of a gene predicted to encode the S-ribosylhomocysteine lyase (LuxS), i.e., the enzyme accountable for synthesizing autoinducer-2 (AI-2), along with the autoinducer-2 ABC transporter (Table 1). These two proteins contribute to the synthesis and transport of AI-2,

triggering a complicated cell-to-cell communication system known as quorum sensing. Specifically, since AI-2 has been demonstrated to be involved in biofilm formation favoring bifidobacterial colonization of the gut ecosystem (Christiaen et al., 2014;Sun et al., 2014;Alessandri et al., 2023), the higher expression of *luxS* could be interpreted as evidence that the presence of *B. breve* PRL2012 and *B. bifidum* PRL2010 enhances the ability of *B. longum* PRL2022 not only to adhere to human host cells compared to when the strain is in mono-culture, but also to induce inter-microbial communication.

Altogether, these findings highlight that exposure of a single bifidobacterial strain to other members of the genus *Bifidobacterium* not only elicits a metabolism-biased response, but also triggers enhanced transcription of genes involved in the production of extracellular structures. Therefore, since the latter are known to play an important role in the establishment of a molecular dialogue with the host, probably, the presence of other bacterial partner promotes in individual strains the establishment of a host-interacting bifidobacterial consortium. Clearly, more experiments with other bifidobacterial strains are needed to confirm this hypothesis.

The molecular dialogue of bifidobacterial tri-association with human cell monolayers. The transcriptomic data originating from different combinations of bifidobacteria demonstrated that co-association of the three bifidobacterial

prototypes caused transcriptional upregulation of a higher (compared to the mono-/bi-association) number of genes involved in host interaction, i.e., adhesion to the intestinal epithelia. To confirm the effect of increased up-regulation of genes mediating host interaction, *B. bifidum* PRL2010, *B. breve* PRL2012 and *B. longum* PRL2022 were cultured in tri-association on a Caco-2/HT-29-MTX cell monolayer for 4 h. Following this, differences in gene expression were evaluated through RNA sequencing between the bifidobacterial tri-associations in contact with human cell lines and the bifidobacterial mono-association, designated as bacterial control cells. Specifically, RNA sequencing generated a total of 10,110,619 quality-filtered reads with an average of 561,701 reads per sample (Table S12). As described above, only genes showing a fold-change of ≥ 2 in combination with a p-value of ≤ 0.05 calculated through correction for multiple comparisons using the False Discovery Rate (FDR) procedure were considered as significantly differentially expressed between the two conditions. An in-depth investigation of the transcripts revealed a significant up-regulation of 126, 518, and 429 genes in *B. bifidum* PRL20120, *B. breve* PRL2012, and *B. longum* PRL2022, respectively, when in tri-association with respect to the mono-association (Table S13). Particularly, a detailed investigation of the functional characterization of the up-regulated genes revealed that, in all three selected prototypes, the highest number of up-regulated genetic sequences in the tri-

association, when compared to each mono-culture, belonged to carbohydrate and amino acid transport and metabolism COG families (Figure 2a). Interestingly, among the carbohydrate-utilization genes, several of the latter encode enzymes predicted to be secreted outside the bacterial cell. These enzymes, including various pullulanases, sialidases, and amylases, are involved in the cleavage of complex sugars that could increase their bioavailability for other bacteria. These findings confirm that also under conditions of direct contact with human intestinal cells, the tri-association among these three bifidobacterial strains induces a metabolic-biased response promoting the up-regulation of genes useful to promote a higher metabolic fitness of the bifidobacterial prototypes. Furthermore, in addition to genes involved in the metabolism and transport of carbohydrates and amino acids, several genes involved in bifidobacteria-host interaction underwent a significantly higher expression in the three prototypes when they were cultured on Caco-2/HT-29-MTX cell layers as tri-association with respect to mono-associations. Interestingly, in *B. breve* PRL2012 up-regulation of four genes belonging to different loci involved in EPS biosynthesis was observed (Table 2 and Figure 2b). Similarly, three genes belonging to two loci for EPS assembly displayed significantly higher levels of expression in *B. longum* PRL2022 (Table S13). Moreover, genes encoding for transaldolases were detected in all three bifidobacterial strains when placed in contact with human

cell lines as tri-association (Table 2 and Figure 2b). In this context, since both EPS and transaldolases have been identified as extracellular structures that facilitate attachment to the mucosal surface of the host, it can be inferred that exposure to other bacteria may have a role in activating strategies to enhance bifidobacterial binding capabilities to the intestinal epithelial surface (Nishiyama et al., 2020;Shang et al., 2022). Additionally, the tri-association induced in *B. longum* PRL2022 the up-regulation of two genes encoding two fibronectin type III domain containing-proteins as well as a higher number of transcripts encoding a GH136 (Table 2 and Figure 2b). In detail, since proteins harboring a fibronectin type III domain have been shown to promote anchoring to the extracellular matrix protein and the GH136 has been described as a mucin-degrading glycosyl hydrolase also implicated in bifidobacterial gut persistence, this data strengthens the notion that the tri-association promotes the up-regulation of genes responsible for microbe-host interaction to improve strain fitness in the human intestine and favoring their persistence (Yamada et al., 2017;van Leeuwen et al., 2021;Alessandri et al., 2023;Rizzo et al., 2024). Notably, *B. bifidum* PRL2010 did not show a significant increase in transcripts corresponding to genes involved in the production of extracellular structures when the tri-association seeded on Caco-2/HT-29-MTX cell line monolayer was compared to the mono-association. However, upon analyzing the transcriptomic data, it was observed that these

genes were highly expressed in both mono- and tri-association after the exposure to human intestinal epithelial cells, thus suggesting that the production of these extracellular structures is more likely due in response to the contact with the human cell lines regardless of the interaction with other bifidobacteria. Furthermore, beyond those genes aimed at interacting with the host, the tri-association stimulated the up-regulation of genes involved in mucin degradation, i.e., an N-acetylgalactosaminidase in both *B. breve* PRL2012 and *B. longum* PRL2022 (GH129 and GH101, respectively), along with the induction of a mucin glycan degradation enzyme, i.e., GH42, in both bifidobacterial strains (Table 2 and Figure 2b). In this context, since the HT-29-MTX is a mucin-secreting cell line (Bianchi et al., 2019), the bifidobacterial tri-association may have favored a significantly higher production of transcripts corresponding to enzymes that utilize mucin as a carbon source. This is a prerogative of a restricted set of intestinal bacteria, which provides bifidobacteria with a selective advantage in a competitive environment such as the intestine, ensuring their growth, proliferation and persistence (Gotoh et al., 2023) (Alessandri et al., 2021) (Egan et al., 2016;Katoh et al., 2017;Katoh et al., 2020).

In addition, it is worth noting that the tri-association favored transcriptional up-regulation of genes involved in the toxin-antitoxin system in *B. bifidum* PRL2010 and *B. longum* PRL2022 when they were in contact with human cell lines (Table

2 and Figure 2b). In this context, since the toxin-antitoxin system is implicated in bacterial persistence, biofilm formation, and antibiotic tolerance (Klimina et al., 2019;Klimina et al., 2020), the observed up-regulation of the two genes constituting this system may be considered as an additional strategy to improve bifidobacterial survival in the human intestine.

Finally, a notable number of up-regulated genes belonging to the category of genes whose function is unknown (COG-S) were detected in all three bifidobacterial strains when in contact, as a tri-association, with human cell monolayers (Figure 2a). In this context, the significantly higher number of transcripts corresponding to genes encoding proteins with unknown functions in the three bifidobacterial prototypes when they are in contact with human cell lines as a tri-association, not only underlines that a plethora of yet-to-be-characterized proteins may play a role in enhancing bifidobacterial persistence in the human gut by improving their dialogue with host cells as well as by promoting their interaction with the host and other bacterial players. It also emphasizes the need to characterize these bifidobacterial proteins of unknown function to gain in-depth insight into the strategies that bifidobacteria may use and activate to interact with the host and other bacteria, ensuring their persistence in the human gut.

Overall, these data suggest that the association of the three bifidobacterial prototypes induces activation of various strategies that bifidobacteria may exploit

to successfully interact with the human intestinal epithelium, ensuring their persistence within the human intestinal environment.

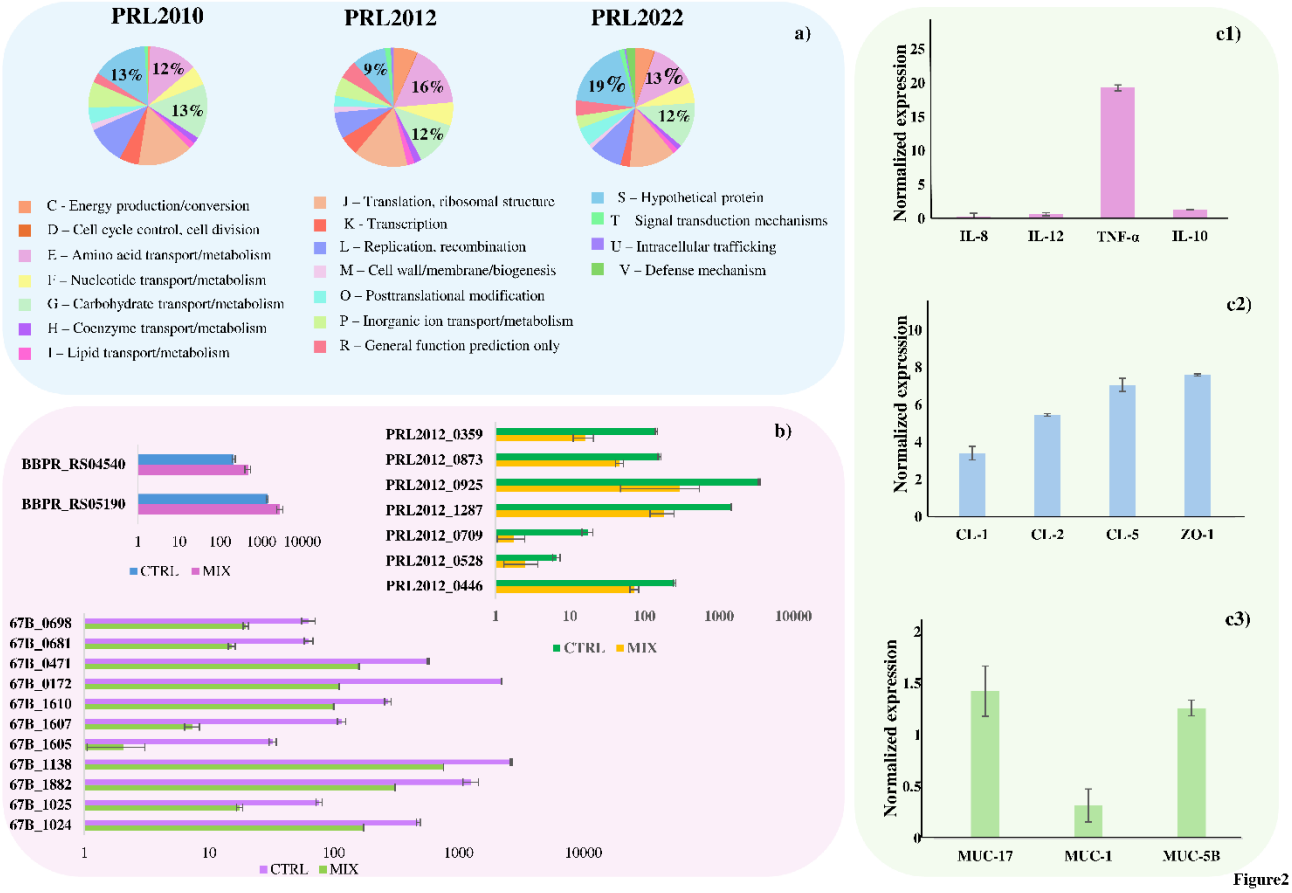

Figure2

Figure 2: Effect of bifidobacterial tri-association exposure to human cell monolayers. Panel a) depicts pie charts representing the percentage, calculated on the total number of overexpressed genes, of the number of up-regulated genes in *B. bifidum* PRL2010, *B. breve* PRL2012, and *B. longum* PRL2022, respectively, after being in contact for 4 h with a Caco-2/HT-29-MTX human cell monolayer of the tri-association when compared to the mono-association. Genes are divided according to the COG category

they belong to. Panel b) shows the transcriptional modulation of genes, cited in the text, of B. *bifidum* PRL2010, *B. breve* PRL2012, and *B. longum* PRL2022 after being in contact for 4 h with a Caco-2/HT-29-MTX human cell monolayer of the tri-association when compared to the mono-association. Transcriptional modulation of genes was reported as average of the normalized count reads obtained from each independent biological triplicate.

Panel c1), c2) and c3) represents the transcriptome levels of different genes of Caco-2/HT-29-MTX monolayer human cells after 4 h of incubation with the bifidobacterial tri-association. The y-axis represents the normalized expression level (ΔCt) according to CFX96 Bio-Rad software relative to the control (Caco-2/HT-29-MTX without bifidobacteria). The vertical bars indicate standard deviations.

Table 2: List of up-regulated genes when *B. bifidum* PRL2010, *B. breve* PRL2012 and *B. longum* PRL2022 were in contact in tri-association with Caco-2/HT-29-MTX. The first column specifies the bifidobacterial species used in the experiments. In the second column are listed all the up-regulated genes cited throughout the text in the tri-association for every bifidobacterial species.

Species	Tri-association	Gene annotation
B. bifidum PRL2010	BBPR_RS05190	transaldolase
	BBPR_RS04540	type II toxin-antitoxin system HicB
B. breve PRL2012	PRL2012_0446	amino acid ABC transporter ATP-binding (EPS)
	PRL2012_0528	GtrA family protein (EPS)
	PRL2012_0709	IS3 family transposase (EPS)
	PRL2012_1287	amino acid ABC transporter ATP-binding (EPS)
	PRL2012_0925	transaldolase
	PRL2012_0873	glycoside hydrolase (GH129)
	PRL2012_0359	beta-galactosidase (GH42)
B. longum PRL2022	67B_1024	glycogen synthase (EPS)
	67B_1025	ATP-binding cassette domain-containing (EPS)
	67B_1882	ATP-binding cassette domain-containing (EPS)
	67B_1138	transaldolase
	67B_1605	glycoside hydrolase family 3 C-terminal (GH3)
	67B_1607	glycoside hydrolase family 3 C-terminal (GH3)
	67B_1610	right-handed parallel beta-helix repeat (GH136)
	67B_0172	endo-alpha-N-acetylgalactosaminidase(GH101)
	67B_0471	beta-galactosidase (GH42)
	67B_0681	type IV toxin-antitoxin system AbiEi
	67B_0698	type II toxin-antitoxin system RelB/DinJ

Evaluation of host response after exposure to bifidobacterial tri-association.

To evaluate whether and how the interaction between the bifidobacterial co-culture and the Caco-2/HT-29-MTX cell monolayer modulates the gene expression not only in the bacterial cells but also in the eukaryotic cells, RNA extracted from the human cell line after exposure to bifidobacterial tri-association was analyzed through the evaluation of the induction of specific sets of genes

through RT-PCR. Caco-2/HT-29-MTX cell monolayers not exposed to any bifidobacterial strains were used as controls. Specifically, genes encoding different cytokines were analyzed to assess if and how these human cells may respond from an inflammatory perspective when placed in contact with bifidobacterial strains when compared to the absence of any microbial cells. Additionally, genes responsible for maintaining the integrity and homeostasis of the intestinal epithelial barrier, including genes coding for tight junction proteins as well as genetic sequences involved in mucous layer production, were also examined. Interestingly, contact between the bifidobacterial tri-association and the human cell monolayer revealed higher levels of tumor necrosis factor (TNF)-α mRNA, a mild induction of the anti-inflammatory interleukin (IL)-10 and a lower induction of the pro-inflammatory IL-12 and IL-8 response compared to the human cells not exposed to bifidobacteria (Figure 2c-1). This finding supports previous reports; indeed, bifidobacteria have been described as strong stimulators of TNF-α but less effective inducers of other pro-inflammatory cytokines, typically associated with systemic immune responses (Fink et al., 2007;Okada et al., 2009;Weiss et al., 2010). In fact, the IL-10/IL-12 ratio (ratio of 1.97) suggests a higher induction of the anti-inflammatory cytokines with respect to the IL-12 specifically involved in a systemic inflammatory response (Hildenbrand et al., 2023). These results suggest that bifidobacterial tri-association plays a role in

initiating communication among immune cells without a system triggering inflammation, instead, alerting the immune system to quickly respond to potential pathogens (Latvala et al., 2011;Fanning et al., 2012;Turroni et al., 2013;Turroni et al., 2014b). Moreover, different genes involved in the regulation of the homeostasis and integrity of the intestinal epithelial barrier were substantially up-regulated when compared to the unexposed control (Pope et al., 2014;Kuo et al., 2021;Ahmad et al., 2023) (Figure 2c-2). This indicates that the bifidobacterial tri-association may induce beneficial effects upon the human host contributing to the reinforcement of the intestinal barrier.

Additionally, the MUC5B and MUC17 genes which encode for a mucin-related epithelial glycoprotein and a major gel-forming mucin protein, respectively, were also slightly over-expressed (gene expression of 1.26 and 1.43, respectively) in the Caco-2/HT-29-MTX cell layer in contact with the bifidobacterial cells, with respect to the human cells without bifidobacterial association (Figure 2c-3). Instead, the MUC1 gene showed an opposite trend (gene expression of 0.32), displaying a down-regulation compared to the control. This gene encodes a transmembrane glycoprotein abnormally over-expressed in colorectal carcinoma also associated with venous thrombosis in cancer patients (Guo et al., 2018;Szlendak et al., 2020;Kawano et al., 2024). The observed down-regulation of MUC1 may open a potential avenue for studying the impact that bifidobacteria

might have on limiting/reducing the likelihood of tumor onset and possible complications. Thus, these findings showed that the bifidobacterial co-association activated the up-regulation of genes involved in mucin degradation, which may indeed be a stimulus for mucin production by eukaryotic cells, potentially aiding in strengthening the integrity of the intestinal barrier along with the down-regulation of a gene involved in the onset of colorectal cancer. Clearly, although these data suggest potential beneficial effects of bifidobacteria towards intestinal health, since the eukaryotic-prokaryotic cell interactions are deeply complicated and depend on multiple factors, other experiments aimed at characterizing the molecular aspects behind these interactions are necessary.

However, these data confirm the hypothesis that the bifidobacterial co-association with the human intestinal cells contributes to the enhancement of the epithelial barrier, thereby potentially promoting health benefits for the host.

Conclusions

Although bifidobacteria are common residents in the human gut and promote health, few studies evaluate the impact of their co-association on both bacteria and host. To explore potential synergistic interactions, we compared the transcriptomes of bi- and tri-associations of *B. bifidum* PRL2010, *B. breve* PRL2012, and *B. longum* PRL2022 to monocultures. Co-association enhanced gene transcription related to carbohydrate, amino acid, and vitamin metabolism and transport, suggesting improved energy harvest and cross-feeding activities. Additionally, the tri-association up-regulated genes for producing extracellular structures like pili, EPS, and teichoic acids, enhancing the bacterial ability to adhere to and interact with the host. Transcriptomic data from tri-associations on Caco-2/HT-29-MTX cells showed higher expression of genes for extracellular structures and mucin degradation, a potential for increased adherence and persistence in the intestine and activation of carbohydrate-degrading activities for growth. Gene expression analyses on human cells in contact with the tri-association revealed changes, particularly in inflammatory response genes. The tri-association did not induce systemic inflammation but stimulated a localized response, pointing out the potential that this interaction may have on educating the immune system for a rapid response against potential pathogens, suggesting that further experiments would be useful to demonstrate this hypothesis. This

study demonstrates the transcriptional response of the tri-association, which may be associated with increased survival, metabolism, growth, and persistence in a simulated healthy intestinal environment.

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Chapter 5

Exploring molecular interactions between human milk hormone insulin and bifidobacteria

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Abstract

Multiple millennia of human evolution have shaped the chemical composition of breast milk towards an optimal human body fluid for nutrition and protection, and for shaping the early gut microbiota of newborns. This biological fluid is composed of water, lipids, simple and complex carbohydrates, proteins, immunoglobulins and hormones. Potential interactions between hormones present in mother's milk and the microbial community of the newborn are a very fascinating yet unexplored topic. In this context, insulin, in addition to being one of the most prevalent hormones in breast milk, is also involved in a metabolic disease that affects many pregnant women, i.e., Gestational Diabetes Mellitus (GDM). Analysis of 3,620 publicly available metagenomic datasets revealed that the bifidobacterial community varies in relation to the different concentrations of this hormone in breast milk of healthy and diabetic mothers. Starting from this assumption, in this study, we explored possible molecular interactions between this hormone and bifidobacterial strains that represent bifidobacterial species commonly occurring in the infant gut using 'omics' approaches. Our findings revealed that insulin modulates the bifidobacterial community by apparently improving the persistence of the *Bifidobacterium bifidum* taxon in the infant gut environment when compared to other typical infant-associated bifidobacterial species.

Importance

Breast milk is a key factor in modulating the infant's intestinal microbiota composition. Even though the interaction between human milk sugars and bifidobacteria has been extensively studied, there are other bioactive compounds in human milk that may influence the gut microbiota, such as hormones. In this manuscript, the molecular interaction of the human milk hormone insulin and the bifidobacterial communities colonizing the human gut in the early stages of life has been explored. This molecular cross-talk was assessed using an in vitro gut microbiota model and then analyzed by various omics approaches, allowing the identification of genes associated with bacterial cell adaptation/colonization in the human intestine. Our findings provide insights into the manner by which assembly of the early gut microbiota may be regulated by host factors such as hormones carried by human milk.

Introduction

The human gastrointestinal tract is inhabited by a myriad of microorganisms that collectively form the so-called gut microbiota [1, 2]. Colonization of this ecological niche occurs immediately after birth, and is influenced by various circumstantial variables such as delivery mode (natural or C-section), type of feeding (breastfeeding or infant formula) [3-5], gestational age, health issues and habits of the mother [6], along with the environment [7]. In this regard, in recent decades, the scientific community has invested considerable research efforts in studying the biology of members of the genus *Bifidobacterium* since they are not only recognized as pioneering microbial colonizers of the human gut, but are also able to exert multiple beneficial effects to the host, i.e., defense against pathogens, immune system modulation and enhancement of the mucus layer that covers the intestinal epithelium [2, 8-14]. Furthermore, certain bifidobacterial taxa have been shown to be transferred to the newborn through vertical transmission from their mother, and this process seems to be affected by the mother's gut microbiota, through the birth canal, along with the microbiota present in breast milk [15, 16]. In this regard, it has been proposed that the infant gut is stratified into compositional patterns based on their bifidobacterial communities, resulting in four so-called infant gut bifidotypes with a predominance of *B. longum* subsp. *infantis* and *B. bifidum*; *B. breve; B. longum* subsp. *longum;* and *B. adolescentis*,

respectively [12]. Maternal milk is considered a key factor in modulating the composition of the gut microbiota during infancy and in shaping the neonatal immune system through various bioactive molecules it contains, prime among these being human milk oligosaccharides (HMOs) that elicit bifidogenic and other beneficial effects [17-20]. Even though the interaction between HMOs and bifidobacteria has been extensively studied in recent years, there are other compounds in human milk, such as hormones, which may influence the neonatal gut microbiota. [21]. In this context, given that it is well recognized that human milk-associated hormones play a crucial role in influencing infant health [22-27], it is somewhat surprising that the interaction between these hormones and bifidobacteria that colonize the neonatal gut is still essentially unexplored. Therefore, to unravel the molecular interactions between human milk hormones and bifidobacteria, we investigated the impact of insulin on members of the genus *Bifidobacterium*. Specifically, our interest focused on insulin since it is not only one of the most abundant human milk hormones, but it is also responsible for a significant disorder in pregnant women known as Gestational Diabetes Mellitus (GDM) [28, 29]. GDM is described as one of the most prevalent metabolic complications during pregnancy since it can cause short- and long-term adverse outcomes in both mothers and newborns [30]. In detail, it has been demonstrated that GDM plays a crucial role in altering the intestinal microbiota of pregnant

women and neonates in terms of both taxonomic composition and functional features [31-33]. At the same time, GDM has been associated with modification of mother milk characteristics, i.e., alteration of the milk microbiota and concentrations of milk-associated hormones that may be causing modifications of the infant gut microbiota [21, 34, 35]. Notably, there is reliable scientific evidence showing that breast milk insulin levels are lower in women with GDM when compared to levels present in milk from healthy mothers [29]. In this context, to evaluate if GDM has an impact on bifidobacterial communities present in the newborn gut, a meta-analysis was performed by comparing the microbiota of fecal samples of infants born from healthy mothers with that of samples of infants delivered by mothers affected by GDM. This comparison revealed that the gut microbiota of infants born from mothers with GDM is depleted of certain bifidobacterial taxa, indicating that specific bifidobacterial species are highly responsive to insulin. Furthermore, the possible molecular impact of insulin on bifidobacterial species typical of the infant gut was investigated through transcriptomic analyses. Finally, the modulatory effects of insulin on infant bifidotypes [12] were evaluated under *in vitro* conditions combining a bioreactors.

Materials and methods

Metagenome dataset. In this project, 3,620 publicly available metagenomic datasets belonging to 35 cohorts from various locations across the globe were obtained through scrutiny of microbiome-based literature (Table S1). In detail, we selected shotgun metagenomic datasets of fecal samples from breastfed infants aged between a few days and six months and delivered by healthy mothers (3557 publicly available metagenomic datasets), and from mothers affected by gestational diabetes mellitus during pregnancy (63 publicly available metagenomic datasets).

Taxonomic classification of short reads at species level. Metagenomic datasets were subjected to a filtering step to remove low-quality reads (minimum mean quality score 20, window size 5, quality threshold 25, and minimum length 100) using the fastq-mcf script (https://github.com/ExpressionAnalysis/ea-utils/blob/wiki/FastqMcf.md). The remaining high-quality sequence data were then taxonomically classified by the METAnnotatorX2 pipeline [36] using an up-to-date RefSeq (genome) database retrieved from NCBI.

Bifidobacterial strain genome selection for identification of novel model strains. To select a model or prototype strain of the *B. bifidum; B. breve, B. longum* subsp. *longum* and *B. longum* subsp. *infantis* species, we used a previously described methodology (Table S2) [37]. In addition, we applied the RefBifSelector

tool in order to identify strains that are genetically most closely related to the model prototype of such species in our local bacterial repository [37].

Bifidobacterial growth conditions. Bifidobacteria used in the current study, i.e., *B. bifidum* PRL2010, *B. bifidum* LMG 11582, *B. bifidum* 156B, *B. breve* 31L, *B. longum* subsp. *longum* 1886B, and *B. longum* subsp. *infantis* 1888B were grown at 37°C under anaerobic conditions (2.99% H_2, 17.01% CO_2 and 80% N_2) (Concept 400; Ruskin) in de Man-Rogosa-Sharpe (MRS) broth (Sharlau Chemie, Barcelona, Spain) supplemented with 0.05% (wt/vol) L-cysteine-HCl.

Bifidobacterial growth assay on insulin. To evaluate insulin susceptibility of *B. bifidum* PRL2010, *B. breve* 31L, *B. longum* 1886B and *B. longum* subsp. *longum* 1886B, these strains were cultivated in the presence of 19 different concentrations of insulin using the broth microdilution method. Specifically, starting from a level of 8 μM/L insulin, a two-fold dilution series was obtained until reaching an amount of 58.59 pM/L insulin and aliquoted in a 96-well microtiter plate. In addition, insulin at a physiologically relevant concentration of 157 pM/L was also included in the assay [38]. Subsequently, an overnight culture of the abovementioned bifidobacterial strains was diluted to obtain an Optical Density at 600 nm (OD_{600nm}) of ~ 1, and 15 μl of the diluted cells were inoculated in 135 μl of MRS supplemented with a specific insulin amount. Microtitreplates were incubated under anaerobic conditions at 37°C for 48h. Optical densities (measured at a

wavelength of 600 nm) were recorded using a plate reader (BioTek, Winooski, VT, USA). The OD_{600nm} values were read in intermittent mode, with absorbance readings performed at 3-min intervals for three times after 48 h of growth, where each reading was preceded by 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.

Exposure of bifidobacterial strains to insulin. Bifidobacterial strains were grown overnight from glycerol stock in de Man-Rogosa-Sharpe (MRS) broth as described above. Subsequently, cells were inoculated in 30 ml of freshly prepared MRS supplemented with 2μM (wt/vol) of insulin [39]. Specifically, cells were inoculated to reach a final Optical Density at 600 nm (OD_{600nm}) of 0.1. After inoculation, growth was monitored and at an OD_{600nm} between 0.6 and 0.8 (exponential growth phase), cells were harvested by centrifugation at 7000 rpm for 5 min. The same procedure was used to obtain the control samples, i.e., the selected bifidobacterial strains inoculated in MRS without the addition of any insulin. Growth assays were carried out in triplicate. The collected cells were subsequently subjected to RNA extraction and sequencing (see next section).

RNA extraction and sequencing. Total RNA from each bifidobacterial culture was isolated as previously described [40]. Briefly, cell pellets were resuspended in 1 ml of QIAZOL (Qiagen, United Kingdom) and placed in a tube containing 0.8

g of glass beads (diameter, 106 μm; Sigma). Cells were lysed by alternating 2 min of stirring the mix on a bead beater with 2 min of static cooling on ice. 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-containing sample was further processed by the use of a RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. The quality of the RNA was verified employing a Tape station 2200 (Agilent Technologies, USA). RNA concentration and purity were evaluated using a spectrophotometer (Eppendorf, Germany). For RNA sequencing (RNA-Seq), from 100 ng to 1 μg of extracted RNA was treated to remove rRNA by employing QIAseq FastSelect – 5S/16S/23S following the manufacturer's instructions (Qiagen, Germany). RNA yield following rRNA depletion was checked by the use of a Tape station 2200 (Agilent Technologies, USA). Subsequently, a whole transcriptome library was constructed using the TruSeq Standard mRNA preparation kit (Illumina, San Diego, USA). Samples were loaded into a NextSeq high output v2.5 kit (150 cycles, single end) (Illumina) according to the technical support guide. The obtained reads were filtered to remove low-quality reads (minimum mean quality 20 and minimum length 150 bp) as well as any remaining ribosomal locus-encompassing reads using the METAnnotatorX2 [36]. Subsequently, the retained reads were aligned to the specific reference genome of each employed bifidobacterial strain through

Bowtie2 software (25621011). Quantification of reads mapped to individual transcripts was achieved through htseq-counts script of HTSeq software in "union" mode [41]. Raw counts were then normalized using CPM (Counts per million mapped reads) for filtering genes with low counts (CPM <1) and TMM (Trimmed Mean of M-Values) for statistically robust differential gene expression analysis through the EdgeR package [42]. Evaluation of expression differences was calculated for each gene as log2 fold change (logFC) of average expression between the control (no contact between human cell lines and strain PRL2022) and "treated" samples (contact between human cell lines and strain PRL2022). Additionally, for each comparison, a Volcano plot was created to simultaneously visualize expression changes (log fold change) and their statistical significance (*p*-value).

Adhesion of *B. bifidum* PRL2010 to Caco-2 cells. Bifidobacterial adhesion to Caco-2 cells was assessed following the protocol described by Serafini *et al.* [43, 44]. Briefly, human colon adenocarcinoma Caco-2 cells (purchased from the ATCC collection) were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 g/mL streptomycin, and 100 U/mL penicillin and maintained in standard culture condition. For the experiments Caco-2 were seeded on microscopy cover glasses previously settled into 10 cm^2 Petri dishes. Confluent cells were carefully

washed twice with phosphate-buffered saline (PBS) before bacterial cells were added. *B. bifidum* PRL2010 was grown as previously described, with and without insulin until a concentration of 2 x 10^8 CFU mL^{-1} was reached. The two conditions were then centrifuged at 3000 rpm for 8 min, resuspended in PBS (pH 7.3), and incubated with monolayers of Caco-2 cells. After 1 h at 37 °C, the cultures were washed twice with 2 mL of PBS to remove unbound bacteria. 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, cover glasses were removed from the petri plate, mounted on a glass slide and examined using a phase contrast microscope Zeiss Axiovert 200 (objective; 100X/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 Caco-2 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 Caco-2 cells [44]. An unpaired Student's T- test was applied for statistically significant differences. All assays were performed at least in triplicate.

Mucin adhesion assay of *B. bifidum* PRL2010. The effect of bifidobacterial adhesion on mucin was performed by adapting the protocol described by Valeriano

et al. [45]. Briefly, 100 µL of a 1 mg mL^{-1} sterile mucin dissolved in a buffer saline solution (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, and filled with 100 µL of a 20 mg mL^{-1} sterile bovine serum albumin solution, and incubated at 4 ˚C for 2 hours. The bifidobacterial strain *B. bifidum* PRL2010 was grown under two different conditions, at 37°C under anaerobic conditions (2.99% H_2, 17.01% CO_2 and 80% N_2) (Concept 400; Ruskin) in de Man-Rogosa-Sharpe (MRS) broth (Sharlau Chemie, Barcelona, Spain) supplemented with 0.05% (wt/vol) L-cysteine-HCl or in MRS supplemented with 2 µM (wt/vol) of insulin. Bifidobacterial growth was monitored until a concentration of 10^8 CFU mL^{-1} was reached. Afterwards, 100 µL of the bacterial suspension, previously washed and resuspended in PBS, was added in each well and incubated under anaerobic condition at 37˚ C for 1 hour. After incubation, each well was washed two times with 200 µL of PBS to remove unbound bacteria. Then, 200 µL of 0.5% (v/v) Triton X-100 was added and incubated at room temperature for 2 hours, 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:

$$\% \text{ relative adhesion} = (\log CFU_{\mathbf{Nadhered}} / \log CFU_{\mathbf{Ninoculum}}) \times 100.$$

Evaluation of growth effects of insulin exposure on the bifidotype I strains. Bifidobacterial strains corresponding to the representative species of the bifidotypes I [12] were routinely grown anaerobically in MRS at 37°C. Subsequently, to evaluate the impact of insulin exposure on these strains, the latter were inoculated in two parallel bioreactor systems (Solaris Biotech Solutions, Italy) in the presence and absence (control sample) of 2 μM of insulin. Strains were inoculated in a final volume of 400 mL of MRS, while cultivations were carried out at 37°C with a mechanical agitation set at 200 rpm. Furthermore, the pH was maintained at the pH of the MRS medium, i.e., 6.2, by the addition of 2.5 NaOH. Furthermore, selected strains were added based on their abundance in the bifidotype I, i.e., *B. longum* subsp. *infantis* 49.73%, *B. bifidum* 34.71%, *B. longum* subsp. *longum* 9.32% and *B. breve* 7.24% [12].

Bifidobacterial ITS sequencing. Partial ITS sequences were amplified from extracted DNA using the primer pair Probio-bif_Uni (5=-CTKTTGGGYYCCCKGRYYG-3=) and Probio-bif_Rev (5=-CGCGTCCACTMTCC AGTTCTC-3=), which targets the spacer region between the 16S rRNA and the 23S rRNA genes within the rRNA locus [46]. Illumina adapter overhang nucleotide sequences were added to the partial ITS amplicons, which were further processed employing the 16S metagenomic sequencing library preparation protocol (part no. 15044223 rev. B; Illumina). PCR amplifications and

library preparation, including the negative control, were performed as described above for the 16S rRNA microbial profiling analyses. Following sequencing, the .fastq files were processed using a custom script based on the QIIME software suite [47]. Paired-end read pairs were assembled to reconstruct the complete Probio-bif_Uni/Probiobif_Rev amplicons. Quality control retained sequences with a length between 100 and 400 bp and mean sequence quality score of 20 were retained, while sequences with homopolymers of 7 bp in length and mismatched primers were removed. To calculate downstream diversity measures, alpha- and beta-diversity (BrayCurtis), ITS operational taxonomic units (OTUs) were defined at 100% sequence homology using uclust [48], generating ESVs. All reads were classified to the lowest possible taxonomic rank using QIIME2 [47, 49] and a reference data set, i.e., an updated version of the bifidobacterial ITS database [46].

Statistical analysis. Student's T-test was performed by means of IBM SPSS Statistics v2. For differential gene expression analysis, EdgeR package was used to estimate the statistical significance of differences between fold changes as the False Discovery Rate (FDR).

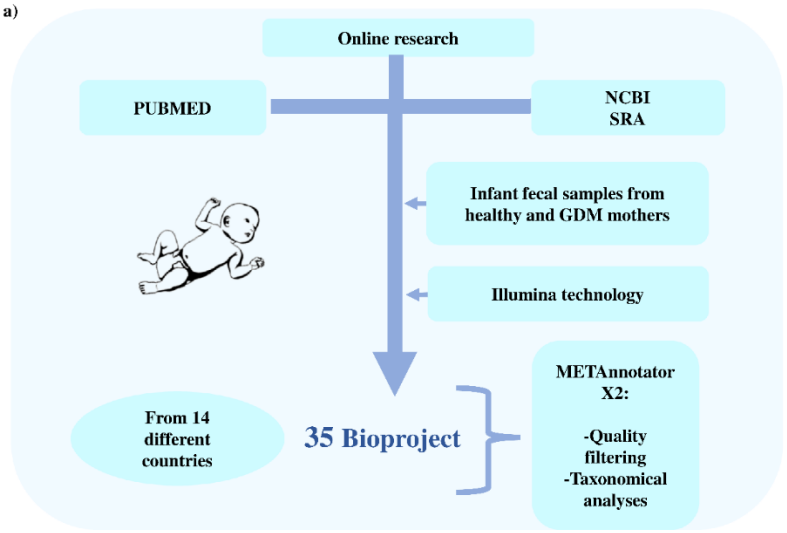
Data availability. Raw sequences of RNA sequencing data are available in the SRA database with accession number PRJNA932965.

Results and Discussion

Gestational Diabetes Mellitus (GDM) affects the infant gut bifidobacterial communities. We analyzed the taxonomic profiles obtained from 63 publicly available metagenomic datasets corresponding to fecal samples of a cohort of infants delivered by mothers affected by GDM. The latter were then compared to the intestinal microbial composition of 3,557 publicly available datasets corresponding to stool microbiota of infants born from healthy mothers (Table 1). To the best of our knowledge, this represents the largest shotgun metagenomic dataset concerning GDM that can be used to evaluate the gut microbiota composition of infants delivered from GDM mothers at a taxonomic resolution down to species level. Thus, collected data were filtered based on several parameters reported in the Materials and Methods section to remove those samples that did not meet DNA quality standards. Then, taxonomic analysis allowed us to identify those bifidobacterial species whose relative abundance significantly differed between the two infant cohorts. Specifically, the infant fecal samples from healthy mothers were shown to contain a statistically significant higher relative abundance of *Bifidobacterium bifidum* (4.04%) when compared to infant fecal samples from GDM mothers (1.42 %). Furthermore, *Bifidobacterium breve* and

Bifidobacterium dentium showed an opposite trend, being present at a statistically significant lower relative abundance in infant fecal samples associated with healthy mothers when compared to infant fecal samples born to GDM mothers (Figure 1). Starting from these findings, in addition to the previously reported finding that the amount of milk insulin in mothers with GDM is lower compared to that for healthy mothers [29], we decided to explore possible molecular interactions between insulin and bifidobacterial species whose levels in fecal samples differed between infants born to healthy or GDM mothers.

a)



b)

Bifidobacterial species	Infant group	Relative abundance	p-value
Bifidobacterium adolescentis	Infant from healthy mothers	0.655%	0.350
	Infant from GDM mothers	1.218%	
Bifidobacterium animalis	Infant from healthy mothers	0.308%	0.507
	Infant from GDM mothers	0.000%	
Bifidobacterium bifidum	Infant from healthy mothers	4.044%	0.001*
	Infant from GDM mothers	1.421%	
Bifidobacterium breve	Infant from healthy mothers	7.536%	0.021*
	Infant from GDM mothers	15.364%	
Bifidobacterium catenulatum	Infant from healthy mothers	0.273%	0.449
	Infant from GDM mothers	0.065%	
Bifidobacterium dentium	Infant from healthy mothers	0.476%	0.033*
	Infant from GDM mothers	4.502%	
Bifidobacterium gallinarum	Infant from healthy mothers	0.005%	0.388
	Infant from GDM mothers	0.035%	
Bifidobacterium longum	Infant from healthy mothers	15.048%	0.707
	Infant from GDM mothers	13.775%	
Bifidobacterium pseudocatenulatum	Infant from healthy mothers	1.450%	0.510
	Infant from GDM mothers	0.903%	
Bifidobacterium scardovii	Infant from healthy mothers	0.139%	0.369
	Infant from GDM mothers	0.384%	
Bifidobacterium unknown_species	Infant from healthy mothers	0.748%	0.206
	Infant from GDM mothers	1.236%	

Figure 1

Figure 1. Bifidobacterial community of infant fecal samples from healthy and GDM mothers. Panel a depicts the flow diagram showing the salient details regarding sample selection and analysis. Panel b shows the average relative abundance of different bifidobacterial species found in infant fecal samples of healthy and GDM mothers. The right column displays the Student's t-test p-value. Asterisks indicate statistically significant p-value.

Table 1: Metadata associated to the fecal samples included in this study.

Study (PMID)	Bioproject	Number of samples	Geographical origin	Technology
30559407	PRJNA497734	159	Finland Russia Estonia	Illumina HiSeq 2500
34335499	PRJNA695570	130	North America	Illumina MiSeq
34083435	PRJNA290380	100	Finland Russia Estonia	Illumina HiSeq 2500
33328245	PRJEB39610	644	United Kingdom	HiSeq X Ten
30374198	PRJNA473126	447	North America	Illumina NextSeq 500
33665175	PRJNA630999	293	North America	Illumina NovaSeq 6000
35685890	PRJNA475246	246	North America	Illumina HiSeq 2500
33479326	PRJNA633576	227	North America	Illumina NovaSeq 6000
30001516	PRJNA352475	99	Italy	Illumina HiSeq 2500
30505830	PRJEB29052	184	Norway	Illumina MiSeq
-	PRJNA300541	4	North and South America	Illumina HiSeq 2500
-	PRJNA557731	191	North America	Illumina HiSeq 2500
24236055	PRJNA215106	31	North America	Illumina Genome Analyzer Iix
34991704	PRJEB42363	30	Malawi	Illumina NextSeq 500
31832638	PRJNA549787	165	South Africa	Illumina NextSeq 500
31279007	PRJEB32135	27	North America	Illumina NextSeq 500
32958861	PRJNA644725	150	Bangladesh	Illumina HiSeq 2500
34253606	PRJNA486782	44	North America	Illumina HiSeq 2500
33732655	PRJNA648487	94	New Zealand	Illumina NovaSeq 6000
30504906	PRJNA379120	38	Luxembourg	Illumina MiSeq
-	PRJNA436562	31	Bangladesh	Illumina HiSeq 4000
28073918	PRJNA327106	45	North America	Illumina HiSeq 2500
34630385	PRJNA730640	16	China	Illumina MiSeq
34278055	PRJNA542703	30	America	Illumina HiSeq 2500
27583441	PRJEB12669	1	China	Illumina HiSeq 4000
34362295	PRJEB24015	27	United Kingdom	Illumina NextSeq 500
31332384	PRJEB24006	26	North America	Illumina HiSeq 4000
28144631	PRJNA339914	5	Italy	Illumina HiSeq 2500
35776122	PRJNA272371	29	Singapore	Illumina MiSeq
31676793	PRJNA555020	20	Netherlands	Illumina NovaSeq 6000
28149696	PRJEB15257	15	United Kingdom	Illumina MiSeq
33258724	PRJEB41463	1	North America	Illumina MiSeq
-	PRJNA489693	6	North America	Illumina MiSeq
24468033	PRJNA221723	2	North America	Illumina HiSeq 2000
35966074	PRJNA845806	63	North America	Illumina NovaSeq 6000

Evaluation of insulin effects on bifidobacterial growth. We first wanted to know if insulin affects bifidobacterial growth in the intestinal environment by enhancing or reducing their loads. For this purpose, a selection of representative strains for each of the relevant bifidobacterial species was made, by identifying

strains that appeared to be more responsive to insulin as based on metagenomic data concerning the gut microbiota of infants born from healthy or GDM mothers. The identification of such representative bifidobacterial strains for each of the abovementioned species was performed by applying a recently developed tool, i.e., RefBifSelector [37]. Based on the scores obtained from the RefBifSelector tool, only those strains with the highest score isolated from infant fecal samples or from human milk, which belong to at least one of the four bifidotypes previously described [12] were considered for subsequent experiments, i.e., *B. bifidum* PRL2010, *B. breve* 31L, *B. longum* subsp. *infantis* 1888B, and *B. longum* subsp. *longum* 1886B (Table S3). Subsequently, to evaluate the possible impact of insulin on bifidobacterial growth, the above described bifidobacterial strains were cultivated in MRS supplemented with different amounts of insulin, i.e., ranging from 8 μM to 58.59 pM. Interestingly, this growth assay did not reveal any statistically significant differences in growth performance of bifidobacterial strains between the various tested insulin amounts nor with respect to the control (strain grown in absence of insulin) (Anova p-value > 0.05) (Figure S1). These findings therefore suggest that insulin neither promotes nor inhibits growth of bifidobacterial strains. Based on these data, taken together with previous studies focused on the interaction between hormones and/or non-antibiotic drugs and the

human intestinal microbiota, led us to select the concentration of 2 μM of insulin for subsequent experiments [39, 50].

Dissecting the molecular impact of insulin on bifidobacteria.

Although insulin does not significantly modify growth, we decided to further evaluate whether or not this hormone exerts a molecular impact on the selected bifidobacterial reference strains. Therefore, to investigate if insulin modulates gene expression in bifidobacteria, the transcriptomes of the reference strains grown in presence or absence of 2 μM insulin were evaluated through RNAseq analyses. Illumina sequencing generated an average of 2,080,016 quality-filtered reads per sample (Table S4). Furthermore, only genes showing a fold-change of ≥ 2 in combination with a *p*-value ≤ 0.05 calculated through correction for multiple comparisons using the False Discovery Rate (FDR) procedure were considered as significantly differentially expressed between the two conditions. Interestingly, insights into the obtained transcriptome profiles of *B. bifidum* PRL2010 revealed that 97 genes were shown to be significantly up-regulated in the presence of insulin when compared to the control (Table S5). Conversely, the transcriptomes of *B. longum* subsp. *longum* 1886B, *B. longum* subsp. *infantis* 1888B, and *B. breve* 31L appeared only mildly affected by the presence of this hormone with only 56, 43, and 35 significantly up-regulated genes, respectively, when exposed to insulin

(Figure 2a). Specifically, in depth functional scrutiny of the up-regulated genes of PRL2010 in the presence of insulin revealed the transcriptional induction of three genes predicted to belong to the locus involved in teichoic acid biosynthesis, i.e., a sugar nucleotide binding protein (BBPR_RS00400), a dTDP-glucose 4,6-dehydratase (BBPR_RS00405), an AAA family ATPase (BBPR_RS00425), coupled with an ABC transporter permease (BBPR_RS03550) that, although not belonging to the above mentioned locus, are predicted to play a role in teichoic acid production (Figure 2b). Interestingly, these extracellular structures are described as negatively charged polymers exposed on the cell surface of Gram-positive bacteria and have been implicated in the interaction between the microorganism and its host, promoting bacterial adhesion to the intestinal epithelial cells and, therefore, favoring bacterial colonization of the intestine [13, 51, 52]. One can therefore speculate that insulin plays a role in stimulating the expression of extracellular structures of *B. bifidum* PRL2010 cells to favor their colonization and persistence in the infant gut, providing a possible explanation for the higher abundance of *B. bifidum* identified in the gut microbiota of infants born from healthy mothers compared to those delivered by mothers with GDM.

In order to support this notion, the adhesive performances of *B. bifidum* PRL2010 to human intestinal mucosa were assessed. For this purpose, the adhesion ability of *B. bifidum* PRL2010 cells to Caco-2 cells in the presence or absence insulin was

calculated, following a previously described protocol [43, 44]. Interestingly, a significant increment in the adhesion index to Caco-2 cell layers was observed for *B. bifidum* PRL2010 cells when grown in presence of insulin (adhesion index of 457,667 ± 26,870) when compared to *B. bifidum* PRL2010 cells cultivated without insulin (adhesion index of 296,000 ± 9,899) (T-test p-value < 0.001). (Figure 3). In addition, an adhesion assay on mucin was performed. Comparison between *B. bifidum* PRL2010 cultures grown in absence and presence of insulin shows that the latter exhibits a relative adhesion to mucin of 85.64% as compared to 81.5% of the control, i.e., *B. bifidum* PRL2010 grown in absence of insulin. These results confirm our previous observations where *B. bifidum* PRL2010 in presence of insulin expresses particular genes predicted to be involved in enhancing the colonization of the human intestinal mucosa.

Conversely, no modification was observed in the transcription of orthologous genes nor other genes involved in promoting microbe-host interactions for the *B. longum* subsp. *longum*1886B, *B. longum subsp. infantis* 1888B, and *B. breve* 31L (Table S5). These findings suggest that insulin does not induce a unique/uniform molecular effect on bifidobacterial gene expression, but rather it seems to modulate bifidobacterial response in a strain/species-dependent manner.

To further explore the molecular impact that insulin may have at intra-species level, we performed transcriptomic experiments on other *B. bifidum* strains.

Specifically, based on data obtained by the application of the RefBifSelector tool [37], the *B. bifidum* strains that are genetically and functionally closest to or furthest apart from the identified model *B. bifidum* PRL2010, i.e., *B. bifidum* 156B and *B. bifidum* LMG11582, respectively, were selected for subsequent RNA sequencing experiments (Table S3). Illumina sequencing generated an average of 4,005,432 quality-filtered reads per sample (Table S4). Only genes showing a fold-change of ≥ 2 in combination with a p-value ≤ 0.05 calculated through correction for multiple comparisons using the False Discovery Rate (FDR) procedure were considered as significantly differentially expressed between the two conditions.

Specifically, insights into the transcriptomic data revealed the up-regulation of 53 and 145 genes for strains LMG11582 and 156B, respectively, when exposed to insulin and when compared to the control. These up-regulated genes were used to perform a comparative analysis to identify possible orthologous genes, among those up-regulated, between the three strains of *B. bifidum* exposed to insulin (Table S6). Interestingly, the up-regulation of a gene involved in the biosynthesis of teichoic acid, i.e., an ABC transporter permease, was observed in both *B. bifidum* PRL2010 and *B. bifidum* 156B (Figure 2b-c). Furthermore, in the latter strain, the up-regulation of another gene belonging to the presumed teichoic acid biosynthesis locus, an ABC transporter-associated ATP-binding protein, was recorded, corroborating our hypothesis that the presence of insulin may promote

the expression of genes implicated in bacterial cell interaction with the host (Figure 2c). At the same time, these two strains were characterized by the up-regulation of a 1,3-beta-galactosyl-N-acetylhexosamine phosphorylase (Figure 2d-e), which is an enzyme involved in the metabolism of N-acetyl-glactosamine, a monosaccharide originated from the degradation of certain oligosaccharides or host-related glycans, including both mucins and HMOs [53-55]. Mucins are highly O-glycosylated proteins formed by monomers such as N-acetylglucosamine, N-acetylgalactosamine, fucose, and galactose that coat the intestinal epithelial cells and are secreted from goblet cells providing necessary nutritional support for the enteric microbial colonization [56, 57]. Therefore, based on the obtained data, we can hypothesize that the presence of insulin in the growth medium can somehow mimic the intestinal niche providing a stimulus to the strain that responds by inducing the expression of genes involved in mucin degradation, favoring its colonization and persistence in the intestinal environment. Moreover, in *B. bifidum* LMG11582 and *B. bifidum* 156B, the over-expression of a pyridoxal phosphate-dependent transferase was observed (Figure 2e-f), which is an enzyme involved in amino acid metabolism [58]. In addition, transcriptomic analyses of these two strains revealed the up-regulation of another enzyme, i.e., argininosuccinate synthase, involved in arginine biosynthesis. Furthermore, beyond orthologous genes, in all tested *B. bifidum* strains, the activation of numerous genes involved

in both the metabolism of amino acids and carbohydrates was observed (Figure 2g).

However, the lack of a considerable number of orthologous up-regulated genes shared by all the three tested *B. bifidum* strains transcriptomes supports the notion mentioned above, according to which the molecular impact exerted by insulin seems to be strain-specific (Table S6).

Figure 2

Figure 2. Transcriptional modulation of bifidobacterial strains when exposed to insulin. Panel a displays the number of statistically significant up-regulated genes of *B. bifidum* PRL2010, *B. breve* 31L, *B. longum* subsp. *longum* 1886B, and *B. longum* susp. *infantis* 1888B in contact with insulin. Panels b and c represent transcriptional modulation of genes of *B. bifidum* PRL2010 and *B. bifidum* 156B, expressed as the

average of normalized count reads obtained from each independent biological triplicate, involved in the synthesis of teichoic acids. Each bar plot shows the average normalized count reads obtained. Panels d, e and f depict the transcriptional modulation of up-regulated orthologous genes of *B. bifidum* PRL2010, *B. bifidum* 156B and B. *bifidum* LMG 11582B, expressed as the average of normalized count reads obtained from each independent biological triplicate, involved in amino acids and carbohydrates metabolism. Each bar plot shows the average of the normalized count reads obtained. Panel g shows the total number of statistically significant up-regulated genes of *B. bifidum* PRL2010, *B. bifidum* 156B and B. *bifidum* LMG 11582B involved in amino acid and carbohydrate metabolism.

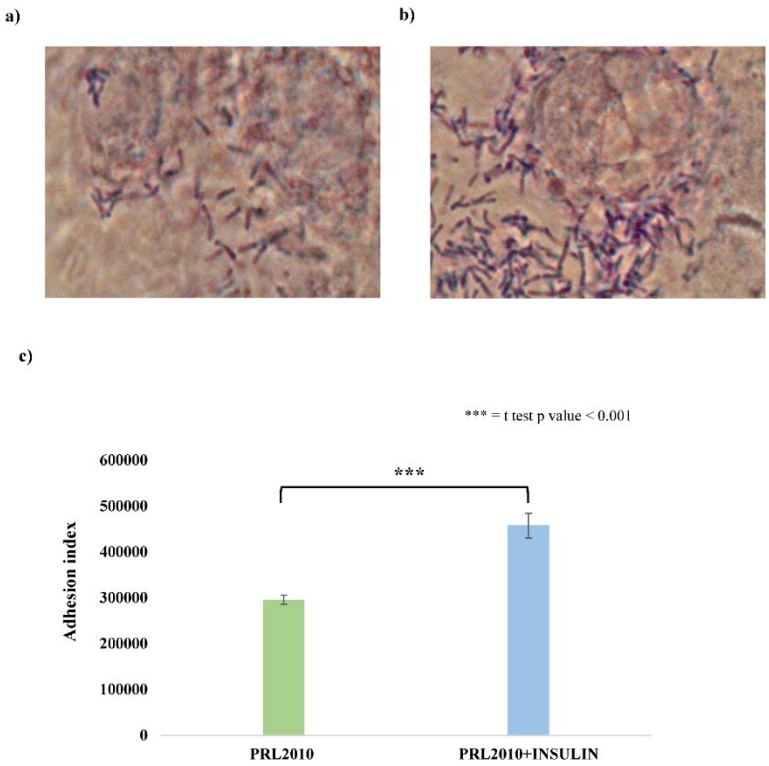


Figure 3. Adhesion of *B. bifidum* PRL2010 cells to Caco-2 cells monolayers. Panel a and b display light microscopic images of Caco-2 monolayer cells as observed with Giemsa staining of *B. bifidum* PRL2010 cells grown under standard conditions and in the presence of insulin, respectively. Panel c depicts the quantification of adhesion ability of *B. bifidum* PRL2010 cells grown in absence and in presence of insulin, respectively. The vertical bars indicate standard deviations, the three asterisks indicate the t- test p value < 0.001.

Effect of insulin on growth performances of the *in vitro* reproduced bifidotype I of the infant gut microbiota. Since insulin plays a role in modulating bifidobacterial gene expression, we decided to assess whether insulin may have a

modulatory effect on growth performances of the infant gut bifidobacterial communities. We decided to focus on bifidotype I since it is characterized by a high relative abundance of *B. bifidum* that was found to be statistically represented in the above performed meta-analysis (Figure 1). In detail, we reproduced the infant gut bifidotype I by inoculating in two parallel bioreactor systems (in presence and absence of 2 μM of insulin), bifidotype I-representative bifidobacterial species according to their observed relative abundances [12], i.e., *B. longum* subsp. *infantis* 1888B at 49.73%, *B. bifidum* PRL2010 at 34.71%, *B. longum* subsp. *longum* at 9.32% and *B. breve* 31L at 7.24%. After 24h of cultivation, the composition of microbial communities was assayed using a bifidobacterial ITS microbial profiling approach [46]. Illumina sequencing generated a total of 40,545 sequenced reads, with an average of 20,275 reads per sample. Quality and chimera filtering then generated a total of 39,993 filtered sequence reads with an average of 19,996 reads per sample (Table S7). Interestingly, after 24h, in the control, the bifidobacterial relative abundances remain essentially stable compared to the inoculum. Instead, in presence of insulin, we noticed that the abundance of *B. bifidum* PRL2010 increased almost two-fold when compared to the inoculum (from 34.71% to 62.25%), with a concomitantly drastic reduction of the predominant species of the bifidotype I in physiological condition with the relative abundance of *B. longum* subsp. *infantis* 1888B which

was considerably reduced from 49.73% to 15.18% (Table S8). Probably, although insulin does not promote nor interfere with growth of bifidobacterial strains, its presence in the bioreactor together with other bifidobacterial strains may have induced a specific cross-talk among strains favoring growth of *B. bifidum* PRL2010. These findings are consistent with the transcriptomic data reported above, according to which *B. bifidum* PRL2010 cells, when placed in contact with insulin, triggers the expression of genes such as those involved in the mucin metabolism and genes encoding for teichoic acid, that are considered to be crucial to enhance the persistence of *B. bifidum* PRL2010 cells in the competitive human gut environment. These findings emphasize the role of insulin as a host-derived compound conferring a possible ecological advantage to *B. bifidum* PRL2010 cells by promoting its ecological fitness within a bifidobacterial community in the human gut.

Conclusions

Gestational Diabetes Mellitus is a common metabolic disorder that affects many pregnant women inducing various physiological alterations that can cause short- and long-term adverse outcomes for both mothers and their infants [30], including an impact on their intestinal microbiota. Interestingly, GDM has been associated with modification of mother milk characteristics, including a reduced insulin concentration in the breast milk [29, 31-33]. Based on these observations, we performed a meta-analysis on 3,620 datasets, comparing the bifidobacterial communities of infants born from healthy and GDM mothers, revealing significant differences in the average abundance of some bifidobacterial species that are well represented in the infant gut microbiota, including *B. bifidum* and *B. breve* species. In this context, to investigate the impact that insulin may have on bifidobacterial species, *B. bifidum* PRL2010, *B. breve* 31L, *B. longum* subsp. *longum* 1886B and *B. longum* subsp. *infantis* 1888B, i.e., representative strains for each of the bifidobacterial species typical of the infant gut microbiota and thus constituting the different infant gut bifidotypes [12], were used for a growth assay on different amounts of insulin. This analysis revealed that this hormone does not favorably or adversely influence bifidobacterial growth performances. However, transcriptomic analysis of the selected bifidobacterial strains when grown in the presence or absence of insulin revealed significant differences in gene expression,

suggesting that, even if insulin does not impact growth performances, it induces a molecular effect on these bifidobacterial strains. Furthermore, transcriptomic results highlighted that insulin seemed to induce a species-specific molecular response, specifically related to *B. bifidum* species whose transcriptome revealed the over-expression of genes involved in the microbial persistence in the infant intestinal epithelium. To understand if this characteristic is strain-specific the transcriptome of two other different strain of *B. bifidum* was performed. Transcriptomic analyses highlighted that each strain differentially responded to insulin exposure, suggesting that the effect of the insulin may be strain-specific rather than species-specific. In addition, when the selected bifidobacterial species were grown together in a bioreactor mode, modulation of their relative abundances was observed in the presence of insulin when compared to the control. All our findings corroborate our initial hypothesis that the lower concentration of milk insulin in GDM women can somehow exert an effect on the bifidobacterial gut composition of newborns. However, these results are representing the starting point of a very interesting topic about the molecular cross-talk between hormones and the human microbiome and about how hormones could elicit species specific bacterial response that could functionally impact on the human health.

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Chapter 6

Investigating drug-gut microbiota interactions: Reductive and hydrolytic metabolism of oral glucocorticosteroids by *in vitro* artificial gut microbiota

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Abstract

Elucidation of the role of gut microbiota in the metabolism of orally administered drugs may improve therapeutic effectiveness and contribute to the development of personalized medicine. In this study, ten different artificial gut microbiota (AGM), obtained by culturing fecal samples in a continuous fermentation system, were challenged for their metabolizing capacity on a panel of six glucocorticoids selected from either prodrugs or drugs. Data from metabolic stability assays highlighted that, while the hydrolysis-mediated conversion of prodrugs to drugs represented only a minor metabolic pathway, significant differences in the stability of parent compounds and in their conversion rates to multiple reductive metabolites were obtained for the selected drugs. In the latter case, a taxonomic composition-dependent ability to convert parent drugs to metabolites was observed. Indeed, the artificial microbial communities dominated by the genus *Bacteroides* showed the maximal conversion of parent glucocorticoids to several metabolites. Furthermore, the effect of drugs on AGM was also evaluated through shallow shotgun sequencing and flow cytometry-based total bacterial cell count highlighting that these drugs can affect both the taxonomic composition and growth performances of the human gut microbiota.

1. Introduction

Emerging scientific evidence suggests that the relationship between orally administered drugs and gut microbiota is complex and bidirectional (Javdan et al., 2020; Spanogiannopoulos et al., 2016). It is well known that the composition of gut microbial communities can be altered as a result of antibiotic (Jernberg et al., 2010; Yassour et al., 2016) and non-antibiotic (Forslund et al., 2015; Jackson et al., 2016; Maier et al., 2018; O'Reilly et al., 2023; Rogers et al., 2016) drug treatments. Conversely, gut microorganisms can directly modify the structure of drugs through transformations catalyzed by their metabolic enzymes, leading to drug deactivation (Guo et al., 2019), prodrug activation (Peppercorn et al., 1972) and/or toxicity issues (Wallace et al., 2010). For instance, it has been recently shown that several gut bacteria are able to metabolize up to 271 orally administered drugs, representative of the drug chemical space, and that each bacterial strain can metabolize a variety from 11 to 95 different drugs (Zimmermann et al., 2019a). Gut microbiota can also act in an indirect way by producing microbial metabolites that can activate or inactivate host gene transcription, ultimately leading, to a modulation of the host metabolic machinery or immune functions (Meinl et al., 2009; Simpson et al., 2023). Microbiome-derived metabolites can also compete with administered drugs for the same host metabolizing enzymes, potentially affecting the drug metabolic fate (Clayton et al., 2009).

There are emerging evidences suggesting that the success of a pharmacological therapy is influenced by various factors, including the diversity and richness of the gut-resident microbial community (Aziz et al., 2018; Zimmermann et al., 2019a,b). To develop personalized, effective, and safer drug therapies, it is essential to investigate the interplay between drugs and gut microbiota, as well as the biological mechanisms correlating microbial associations with clinical

outcomes (Javdan et al., 2020; Spanogiannopoulos et al., 2016; Zhao et al., 2023), which is an ambitious and challenging task.

Human gut microbiota has been categorized into taxonomic enterotypes based on the dominance of three different bacterial genera (*i.e., Bacteroides, Ruminococcus,* or *Prevotella*); still, there are significant inter-individual variations in the taxonomic composition and in the genetic and functional potential (Arumugam et al., 2011; Costea et al., 2018; Healey et al., 2017; Zhu et al., 2015).

From a pharmaceutical perspective, *in vitro* and *ex vivo* workflows are currently being developed and applied to assess the impact of gut microbiota-derived metabolism on various classes of oral drugs; however, this aspect remains underexplored in the drug development field (Javdan et al., 2020; Simson et al., 2023; Spanogiannopoulos et al., 2016). Indeed, assessing the inter-individual variability of microbiota composition in health and disease states and incorporating pharmacomicrobiomics into the drug development pipeline (Doestzada et al., 2018) could enhance research efficacy and support more personalized pharmacological interventions.

In the present work, we set up an experimental workflow to investigate *in vitro*, by a combination of liquid chromatography-mass spectrometry and shotgun metagenomic analyses, the cross-talk between representative gut microbial communities and a panel of oral glucocorticoids. Glucocorticoids (GC) were selected as model drugs as they represent a reference class of orally administered drugs, widely used in clinical practice due to their diverse effects in regulating inflammation, stress, immune response, electrolyte balance, and water retention (Czock et al., 2005). Despite their long-standing use in therapy, dosage regimens in clinical settings have traditionally been determined empirically. This has led to

significant variability in efficacy and severe side effects among patients, particularly during chronic treatments (Czock et al., 2005).

Metabolism plays a relevant role in determining GC bioavailability, which subsequently impacts their therapeutic index. Metabolic pathways derived from host and gut-resident microbiota are known to intersect and influence each other (Ly et al., 2021). Notably, the family of host- and microbial-derived hydroxysteroid dehydrogenases (HSDH) targets various positions of GC steroid ring and side chain (Devendran et al., 2017; Ly et al., 2021). As a relevant example, the fine regulation of local concentrations of the endogenous active glucocorticoid cortisol involves its conversion into the inactive 11-keto form, cortisone, by the 11β-hydroxysteroid dehydrogenase isoforms 1 and 2 (11β-HSDH1/2) within host tissues (Czock et al., 2005; Diederich et al., 2002). It has been reported that 5a-dihydro metabolites of cortisol and corticosterone could act as competitive inhibitors of 11β-HSDH2 in the kidney and that gut microbiota could be involved in their production, with consequent modulation of mineralocorticoid receptor (MR)-mediated side effects (Honour, 1982; Morris et al., 2007).

Based on these premises, six oral glucocorticoids were selected for our investigation (Figure 1). Hydrocortisone (11β,17,21-trihydroxypregn-4-ene-3,20-dione), which is the active pharmaceutical ingredient corresponding to the endogenous GC cortisol (Derendorf et al., 1991); prednisone (17,21-dihydroxypregna-1,4-diene-3,11,20-trione), inactive *per se*, but reversibly interconverted to the active form prednisolone (11β,17,21-trihydroxypregna-1,4-diene-3,20-dione) by 11β-HSDH1, as reported for the cortisone-cortisol redox couple (Garg et al., 1994); budesonide (16,17-butylidenebis(oxy)-11,21-dihydroxypregna-1,4-diene-3,20-dione), an equimolar mixture of 22*R* and 22*S* epimers, in which the introduction of the unsymmetrical 16a, 17a-acetal group produces an extensive CYP3A4-mediated metabolism by the intestinal cell wall and liver (Ellul-Micallef et al., 1980; Jönsson et al., 1995).

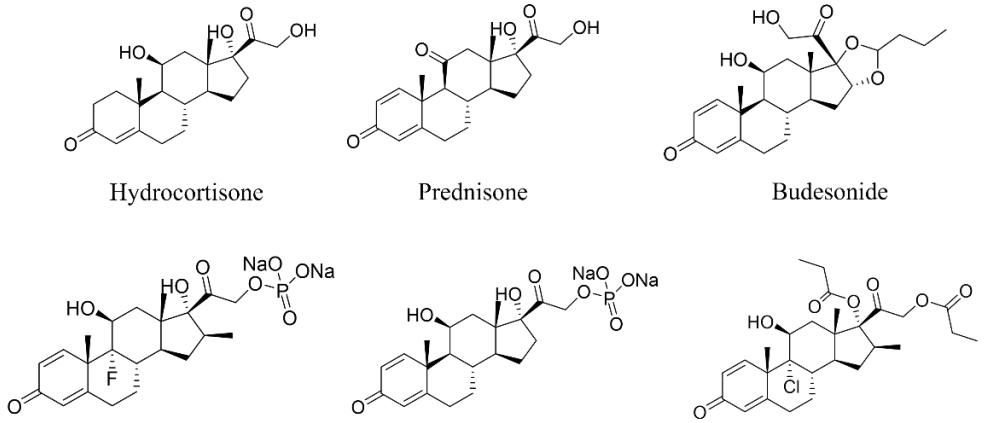

Figure 1. Panel of selected oral glucocorticoids for investigation of metabolic transformation by artificial gut microbiota.

Since the pharmacokinetics of corticosteroids is also strongly influenced by their physicochemical properties (Czock et al., 2005), prodrugs were designed to

ameliorate their hydrophilic-lipophilic balance and water solubility. Therefore, three prodrugs, which are hydrolytically converted *in vivo* to the active GC, were also selected: betamethasone sodium phosphate and prednisolone sodium phosphate, the two phosphate prodrugs of betamethasone and prednisolone, respectively, and the diester beclomethasone dipropionate which is hydrolyzed to the active beclomethasone-17-monopropionate (Würthwein et al., 1990). Their inclusion in this study was aimed to explore the hydrolytic conversion to active drug catalyzed by gut microbiota.

Very few studies have employed human simulated colonic fluids or have set up *in vitro* artificial gut microbiota (AGM), mimicking gut-residing microbial communities, to study drug-microbiota cross-talk on oral glucocorticoids (Berkhout et al., 2022; Isenring et al., 2023; Javdan et al., 2020; Yadav et al., 2013). Indeed, there are few investigations regarding both the effect of gut microbiota on drugs, i.e., how they are modified and whether active, inactive, or toxic metabolites are produced, and, vice versa, the impact that drugs may have on the microbial community colonizing the human intestine.

2. Materials and Methods

2.1. Chemicals and reagents

The European Pharmacopeia standards of hydrocortisone, prednisone, budesonide, prednisolone sodium phosphate, bethametasone sodium phosphate, beclomethasone dipropionate, prednisolone and bethametasone were purchased from the European Directorate for the Quality of Medicine (EDQM), Bruxelles, Belgium. Metabolite standards beclomethasone-17-monopropionate, beclomethasone-21-monopropionate 20a- and 20b-dihydrocortisol, 5a- and 5b-dihydrocortisol were provided by Steraloids (Newport, USA). LC-MS-grade acetonitrile (ACN), water, and methanol were supplied by Scharlab (Valencia, Spain). Waters Sep-pak RP18 SPE columns (Waters, Milan, Italy) were used for sample preparation in metabolite identification workflow.

Tryptone, casein, yeast extract, peptone, meat extract, pectin, guar gum, lactose, L-cysteine hydrochloride, mucin from porcine stomach type III, KCl, NaCl, $NaHCO_3$, sodium chenodeoxycholate, hemin, $FeSO_4$, $MnSO_4$, KH_2PO_4, K_2HPO_4, Tween 80, vitamin K, d-biotin, pantothenate, nicotinamide, vitamin B_{12}, thiamine, para-aminobenzoic acid, $MnSO_4$, gellan gum, xanthan gum, and sodium citrate were purchased from Sigma-Aldrich, USA. Arabinogalactan, inulin, and xylan were supplied by Biosynth, while sodium cholate and starch were purchased from Carlo Erba, Italy.

2.2. Subject recruitment and sample collection

Fresh fecal samples (n=10), here designated from F1 to F10, were collected from different adult individuals. To be enrolled, donors had to be healthy and not having undergone any treatment with prebiotics, probiotics, or drugs during the three months prior sample collection. Approximately five grams of stool were collected immediately after defecation using a dedicated sterile tube provided of

a sampling spoon. After collection, fecal samples were immediately shipped to the laboratory under anaerobic conditions using a jar containing an anaerobic atmosphere generation bag (Thermo Scientific, USA).

2.3. Fecal inoculum and immobilization

Once in the laboratory, tubes were transferred into an anaerobic workstation (2.99% H_2, 17.01% CO_2 and 80% N_2) and peptone water was added to the fecal sample to obtain a final concentration of 20% v/w (Bircher et al., 2018). Subsequently, each fecal sample was immobilized in 1-2 mm gellan-xanthan gel beads (2.5% gellan gum, 0.25% xanthan gum, and 0.2% sodium citrate) as previously described (Cinquin et al., 2004; Cleusix et al., 2008; Zihler Berner et al., 2013).

2.4. In vitro cultivation of gut microbiota based on a bioreactor system

To obtain "artificial gut microbiota" (AGM) from the enrolled donors, a colonic fermentation system (Solaris Biotech Solutions, Italy) was set up and inoculated with immobilized gut microbiota. Specifically, 120 mL of freshly prepared fecal beads were transferred to 380 mL of sterile human colon environment-simulating growth medium (Alessandri et al., 2022; Macfarlane et al., 1998) supplemented with a vitamin and mineral solution previously sterilized through a 0.2 µm filter (Macfarlane et al., 1998). Furthermore, to mimic the intestinal environment, the cultivations were performed under anaerobic conditions through the flushing of an anaerobic mix (2.99% H_2, 17.01% CO_2 and 80% N_2) in the fermentation system, the temperature was kept at 37°C, continuous stirring at 120 rpm, while the pH was maintained at 6.8 by the addition of 2.5 M NaOH (Alessandri et al., 2022, 2024; Macfarlane et al., 1998). To allow the colonization of the beads, the bioreactor was first run as a closed system by replacing the exhausted medium every 12h for a total fermentation time of 60h. Subsequently, the cultivation was

switched to a continuous mode by an inflow and outflow of fresh and exhausted growth medium, respectively, via peristaltic pumps with a mean retention time of 8h (50 mL/h) mimicking the total colonic transit time in healthy adults (Isenring et al., 2022; Poeker et al., 2018). The stabilization of the gut microbiota was operated for 15 days in continuous mode. This growth period was selected as the average time required to stabilize a human fecal bacterial community by using an *in vitro* continuous cultivation system, as previously reported (Asare et al., 2023; Pham et al., 2019). At the end of the cultivation, an aliquot of the obtained AGM was directly collected from the bioreactor through a dedicated sterile sampling system (Solaris Biotech Solutions, Italy) by means of a sterile needle connected to a 50 mL syringe. The effluent was immediately transferred to a sterile pre-reduced 50 mL tube and transported into an anaerobic chamber, where all further steps were performed.

2.5. In vitro drug assay

To evaluate *in vitro* the effect of AGM on glucocorticoids (GCs) and vice-versa, each AGM was individually cultivated in presence of one of six GCs, i.e., beclomethasone dipropionate, betamethasone sodium phosphate, prednisolone sodium phosphate, hydrocortisone, prednisone, and budesonide. Each AGM was inoculated at a final inoculum concentration of 2% (v/v) in the same culture medium used to stabilize fecal samples, while GCs were added to the culture medium to a final concentration of 10 μM (stock solution in dimethylsulfoxide, DMSO; Merck, Germany). In detail, cultivations were carried out in 2 mL of culture medium following the MiPro model (Li et al., 2019), i.e., encompassing 96-deep well plates covered with a silicone gel mat provided with a vent hole on each well created with a sterile syringe needle. During cultivation, plates were shaken at 500 rpm. In addition, each AGM was cultivated without the addition of

any GC as control sample. Cultivation was carried out for a total of 6 h, when the bacteria are in the exponential growth phase and at a peak of their metabolic activities, and three biological replicates for each AGM and for each drug treatment were set up (Zimmermann et al., 2019a). Samples were collected at three different time points (0h, i.e., immediately after the inoculum, 3h, and 6h). All samples were stored at -80°C until they were processed for DNA extraction, flow cytometry-based bacterial cell count, *in vitro* metabolic stability and metabolite identification assays.

2.6. Evaluation of bacterial cell density by flow cytometry

Each replicate was subjected to flow cytometry-based total bacterial count. In detail, replicates were 100,000 diluted in physiological solution (PBS). Subsequently, 1 mL of the obtained bacterial dilution was stained with 1 µL of SYBR®Green I (ThermoFisher Scientific, USA) (1:100 diluted in DMSO; Merck, Germany), vortex-mixed and incubated in the dark for at least 15 minutes before measurement. Count experiments were carried out using an Attune NxT flow cytometer (Thermo Fisher Scientific, USA) 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, i.e., forward and side scatter, while SYBR Green I fluorescence was detected on BL1 530/30 nm channel. Cell debris were excluded from the acquisition analysis by setting a BL1 threshold. In addition, the gated fluorescence events were evaluated on the forward-sideways density plot to exclude remaining background events and to obtain an accurate microbial cell count, as previously described (Vandeputte et al., 2017). Collected data were statistically analyzed with the Attune NxT flow cytometer software.

2.7. DNA extraction and Illumina shotgun sequencing

Artificial gut microbiota as well as one replicate per each AGM exposed to glucocorticoids were subjected to DNA extraction using the QIAmp DNA Stool

Mini Kit, following the manufacturer's instructions (Qiagen, Germany). The extracted DNA was prepared using the Illumina Nextera XT DNA Library Preparation kit and following the Illumina Nextera XT protocol. Specifically, DNA samples were enzymatically fragmented, barcoded, and then purified encompassing magnetic beads. Subsequently, samples were quantified through the Qubit quantification system (Life Technologies, USA), loaded on a 2200 Tape Station Instrument (Agilent Technologies, USA) and normalized to 4 nM. Furthermore, a paired-end sequencing was performed on a MiSeq instrument (Illumina) using a 2x250 bp output sequencing kit together with a deliberate spike-in of 1% PhiX control library for shallow shotgun sequencing.

2.8. Analysis of shallow shotgun metagenomic datasets

Taxonomic profiling of sequenced reads was performed by employing the METAnnotatorX2 bioinformatics platform (Milani et al., 2021). In detail, the obtained fastq files were filtered to remove reads with quality of <25, and to retain reads with a length of > 100 bp. Subsequently, a human host DNA filtering was performed by using the bowtie2 software (Langmead et al., 2012), following the METAnnotatorX2 manual (Milani et al., 2021). Finally, the taxonomic classification was carried out through MegaBLAST (Chen et al., 2015) employing a manually curated and pre-processed database of genomes retrieved from the National Center for Biotechnology Information (NCBI), following the METAnnotatorX2 instructions (Milani et al., 2021). The obtained taxonomic profiles were used to assess α-diversity through the calculation of the Shannon index and species richness. Bray–Curtis dissimilarity matrices based on species abundance calculated through the Rstudios software were used to evaluate similarities between samples (β-diversity). The similarity range was calculated as a value between 0 and 1.

2.9. Metabolic stability studies in the presence of different artificial gut microbiota

In metabolic stability assays, AGM incubated with each glucocorticoid at three different time points (i.e., immediately after the inoculum, 3h, and 6h) were submitted to two centrifugation steps (5000 rpm, 20°C, 10 min and 10000 rpm, 20°C, 10 min) to pellet microbial cells. Then, each sample was further diluted 1:10 with ice-cold acetonitrile containing 500 nM corticosterone as internal standard and centrifuged (13000 rpm, 4°C, 10 min). The supernatant was directly injected into the HPLC-MS/MS system. All AGM containing GCs were analyzed in triplicate per each time point. Means of each time point together with their standard deviations are reported.

2.10. HPLC-MS/MS analytical method for metabolic stability studies

An Accela 1250 HPLC system (Thermo, USA) coupled with a TSQ Quantum Access Max Triple Quadrupole mass spectrometer (Thermo, USA), with a heated electrospray (H-ESI) ion source was employed for HPLC-MS/MS analysis in metabolic stability assays. Column used was a Waters Xselect HSS T3 (100 x 2.1 mm, 3.5 μm; Waters, USA). Mobile phases A and B were ACN and ultra-pure water, both containing 0.1% v/v formic acid. Glucocorticoids were eluted by a linear gradient: 0-1 min: 5%A; 1-6 min: 5-100%A; 6-8 min: 100%A; 8.5 min: 100-5%A; 8.5-10 min: 5%A. Total run time: 10 min. For the elution of beclomethasone dipropionate and its hydrolytic metabolites the following linear gradient was used: 0-1 min: 5%A; 1-10 min: 5-100%A; 10-15 min: 100%A; 15.5 min: 100-5%A; 15.5-18 min: 5%A. Flow rate: 0.22 mL/min; injection volume: 10 μL. Mass spectrometer operated in positive ion mode (ESI^+) and in Multiple Reaction Monitoring (MRM) acquisition. Instrumental parameters were set as follows: ion source voltage: 4000 V (ESI^+); Capillary temperature: 270 °C; sheath gas (N2): 35 psi; auxiliary gas (N2): 15 psi; collision gas (Ar) pressure: 1.5 mtorr.

Xcalibur software version 2.2 (Thermo, USA) was employed for both data acquisition and processing. Tube lens (TL) and collision energies (CE) voltages were optimized for each compound by Flow Injection Analysis (FIA). The optimized MRM transitions for each precursor-product ion are reported in Supplementary Table S1.

Calibration curves for each compound were prepared by spiking bacterial growth medium with stock solutions of each compound in DMSO (final DMSO concentration = 1%). Calibration ranges were between 10 μM – 100 nM for tested GCs and 1 μM – 10 nM for hydrolytic metabolites of GC prodrugs. Coefficients of determinations (r^2) were always >0.995 for all regression curves. The accuracy of calibration standards was considered acceptable within ± 15% (± 20% at the Limit of Quantification (LOQ) of the nominal concentrations, while precision, expressed as percent relative standard deviation (%RSD), had to be <15% (<20% at the LLOQ), according to published guideline on bioanalytical method validation (ICH guideline M10, 2019).

2.11. Sample preparation for metabolite identification (Met ID) studies

For Met ID studies, AGM samples deriving from a 6h incubation in deep-well plates were purified from matrix interferences by Solid Phase Extraction (SPE) employing Sep-Pak RP18 columns (Waters, USA). Briefly, 500 μL of each sample were loaded onto the SPE column, previously activated and conditioned following supplier's instructions; salts and polar compounds were washed with 2 mL of water and GCs and their metabolites were eluted by 1 mL of acetonitrile. The eluate was dried with a gentle nitrogen flux, reconstituted into 150 μL of a 70:30 acetonitrile:water mixture and centrifuged (13000 rpm, 4°C, 10 min) before UPLC-HRMS analysis.

2.12. UPLC-HRMS analytical method for Met ID studies

An UPLC Acquity I-Class coupled to a Vion IMS QToF (Waters, USA) was employed for ion mobility-enabled acquisitions in Met ID studies, as previously described (Ferlenghi et al., 2020). Before analysis, mass spectrometer was calibrated employing Major Mix (Waters, USA) and LC-HRMS system performance was checked for retention time, accurate mass and Collisional Cross Section (CCS) accuracy and/or precision by injecting a system suitability test mixture (Waters, USA). For chromatographic separation, chosen column was an Acquity UPLC HSS T3 (2.1 x 100 mm, 1.7 µm; Waters, USA); eluent A: acetonitrile; eluent B: ultra-pure water, both containing 0.1% v/v formic acid. UPLC gradient was: 0 min: 5%A; 0-2 min: 5-10%A; 2-13.5 min: 10-95%A; 13.5-15 min: 95%A; 15-16 min: 5%A; 16-20 min: 5%A. Total run time: 20 min. Flow rate: 0.40 mL/min; injection volume: 5 µL; column temperature: 40°C. Acquisition range: m/z = 100-800 amu. Instrumental parameters were set as follows: source temperature: 120°C; desolvation temperature: 500°C; source gas flow: 20 L/h; desolvation gas flow: 800 L/h; capillary voltage: 1.0 kV (ESI^-); cone voltage: 40 V; collision energy: low energy: 4 eV; high energy: 10-45 eV; reference mass: leucine enkephalin $[M+H]^+$ *m/z* = 556.2766. The software UNIFI v.1.8.2 (Waters, USA) was employed for data acquisition and processing. The Met ID workflow implemented in the software UNIFI was employed (i) to predict potential phase I metabolites starting from the molecular structure of oral glucocorticoids and (ii) to search for matches in the low-energy full-scan mass spectra. False attributions, which were also detected in control incubations without drugs, were discarded by manual check. For the confirmation of the identities of mono-reduction metabolites of hydrocortisone, 4 pmol of 20b-, 1 pmol of 5a- and 10 pmol of 5b-dihydrocortisol were spiked into microbiota incubates and their LC-HRMS features (retention time, accurate mass, MS/MS spectrum) were compared to those of unknown metabolites.

2.13. Data analysis

Microsoft Excel 365 (Microsoft Corp., USA) was used for data analysis, to plot the graphs and for statistical analysis. In metabolic stability assays, Student's two-tailed unpaired t-test was employed to evaluate the statistically significant differences between the mean concentration values of glucocorticoids in each AGM at t=3h and t=6h timepoints in comparison to t=0 and between them. Statistical significance was set at $p<0.05$.

A correlation analysis between the GC metabolites after incubation with each AGM and the different detected bacterial species of all samples was performed through Spearman's rank correlation coefficient using "rcorr" function (from Hmisc_4.6–0; https://CRAN.R-project.org/package=Hmisc), and only statistically significant results were retained. The false discovery rate (FDR) correction based on Benjamini and Hochberg correction (Benjamini and Hochberg, 1995) and calculated using RStudio through "p.adjust" function (from base package stats) was applied to statistically significant results. SPSS software (version 25; IBM, USA) was used to compute ANOVA statistical analysis for flow cytometry total bacterial cell count.

2.14. Data availability

Raw sequences of shallow shotgun metagenomics data are accessible through SRA under Bioproject number PRJNA1131330.

3. Results

3.1. Taxonomic insights into the composition of the artificial gut microbiota

To obtain artificial gut microbiota representative of the human intestinal communities, ten fecal samples were collected from healthy individuals and subjected to *in vitro* stabilization. The latter was achieved by individually cultivating the fecal samples as bead-immobilized fecal microbial communities in continuous cultivation systems consisting of a single bioreactor simulating the human colon environment, as previously described (Poeker et al., 2018; Pham et al., 2020; Fehlbaum et al., 2015). Upon a cultivation of 15 days, an aliquot of each AGM was subjected to a species-level taxonomic profiling through shallow shotgun metagenomics. The DNA sequencing generated a total of 661,063 reads, resulting in 205,644 reads with an average of 20,564 reads per sample after quality filtering (Table S2). Eight of the obtained AGM (from AGM3 to AGM10) resulted to be dominated by the genus *Bacteroides*, i.e., one of the most abundant and predominant bacterial taxon of the human gut microbiota (Arumugam et al., 2011; Costea et al., 2018) (Table S3). Conversely, the two remaining AGM, i.e., AGM1 and AGM2, were dominated by *Escherichia coli* (Table S3). In addition, in depth insight into the species-level composition of these AGM revealed that, while *Bacteroides uniformis* corresponded to the *Bacteroides* species with the highest abundance in AGM4-9, *Bacteroides cellulosilyticus* and *Bacteroides fragilis* dominated AGM3 and AGM10, respectively (Table S3). Furthermore, beyond the dominant species, other numerous bacterial species typical of the human gut microbiota were stabilized, encompassing *Akkermansia muciniphila*, *Alistipes finegoldii*, *Anaerotignum faecicola*, *Parabacteroides distasonis*, *Flavonifractor plautii*, *Bifidobacterium adolescentis*, and many other members of the genus *Bacteroides* (Alessandri et al., 2022; Mancabelli et al., 2024).

Therefore, the stabilization protocol allowed not only to obtain AGM representative of the human intestinal microbiota, but also provided various taxonomic assemblages useful to evaluate whether different microbial communities differentially act in the biotransformation of orally administered glucocorticoids.

3.2. Evaluation of in vitro hydrolysis of glucocorticoid prodrugs

Evaluation of metabolic stability was conducted to identify which AGM were able to activate glucocorticoid prodrugs and to what extent the transformation was performed. Compounds were tested at the concentration of 10 μM considering two different time points, i.e., 3h and 6h after compound addition to microbial incubates. A drug concentration of 10 μM is in line with estimates of drug concentrations in the gastrointestinal tract (Maier et al., 2018) and it allowed metabolite detection. This concentration falls within the range (2-33 μM) already employed in previous *in vitro* screening of drug-microbiota interactions (Javdan et al., 2020; Maier et al., 2018; Yadav et al., 2013; Zimmermann et al., 2019a).
The metabolic stability of prodrugs prednisolone sodium phosphate, betamethasone sodium phosphate, and beclomethasone dipropionate is reported in **Figure 2**, in which their conversion into prednisolone, betamethasone, beclomethasone-17-monopropionate and beclomethasone-21-monopropionate expressed as percent of conversion of parent prodrug over time is represented.

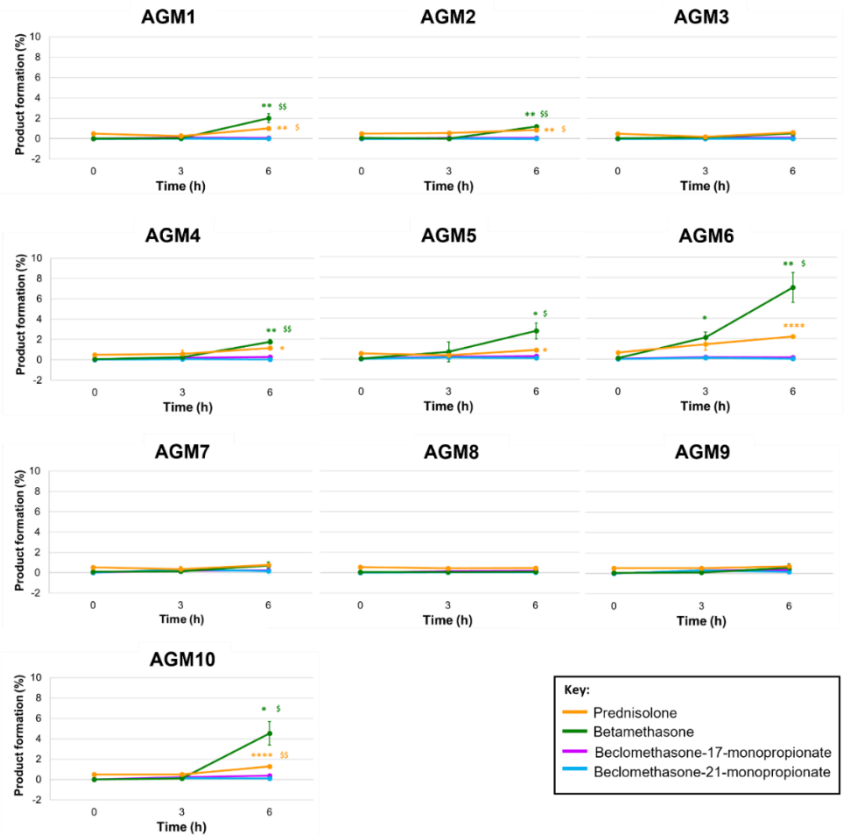

Figure 2. *In vitro* formation of the hydrolysis products prednisolone (orange), betamethasone (green), beclomethasone-17-monopropionate (purple) and beclomethasone-21-monopropionate (light blue), starting from the corresponding prodrugs, in artificial gut microbiota (**AGM1-10**), expressed as percentage of conversion of parent prodrug. Mean values ± SD (n=3) is reported. *: $p \leq 0.05$; **: $p \leq 0.01$; ****: $p \leq 0.001$ (comparison between 0 and 3 or 6h); $: $p \leq 0.05$; $$: $p \leq 0.01$; $$$$: $p \leq 0.001$ (comparison between 3 and 6h).

Prednisolone, the hydrolysis product of prednisolone sodium phosphate, showed a percentage of 0.52 ± 0.04% (T0 mean value ± SD, $n = 10$) at t = 0, which could be due to its presence as an impurity of the prodrug itself or to a chemical

degradation in the gut simulating culture medium. At t=3h, no significant change was observed in almost all the microbial incubates. At the last time point (t = 6h), a small increase in prednisolone concentration was observed in some AGM. In AGM6 and AGM10, the percentages of prednisolone conversion were significantly higher than at t = 0.

As for betamethasone sodium phosphate, the percentage of the hydrolysis product betamethasone showed the most significant increase at 3h, in AGM6, that is 2.12 ± 0.53% (mean ± SD, *n* = 3). Interestingly, after 6h, the concentration of betamethasone arose significantly in several AGM, particularly in AGM1, AGM2, AGM4 and AGM6.

Finally, we evaluated the formation of the two hydrolysis products of beclomethasone dipropionate, namely the active metabolite beclomethasone-17-monopropionate and the inactive beclomethasone-21-monopropionate. We observed very limited biotransformations. After 6h of incubation the percentages of conversion into beclomethasone-17-monopropionate were 0.21 ± 0.12%, while those of beclomethasone-21-monopropionate were 0.06 ± 0.06% (mean values ± SD, *n* = 10).

Overall, the artificial gut microbiota showed very limited ability to hydrolytically activate the glucocorticoid prodrugs.

3.3. Evaluation of in vitro glucocorticoid metabolic stability

We next quantified the levels of three selected corticosteroids, prednisone, hydrocortisone and budesonide, to evaluate their metabolic conversion after 3h and 6h of incubation with the different AGM at 10 µM concentration.

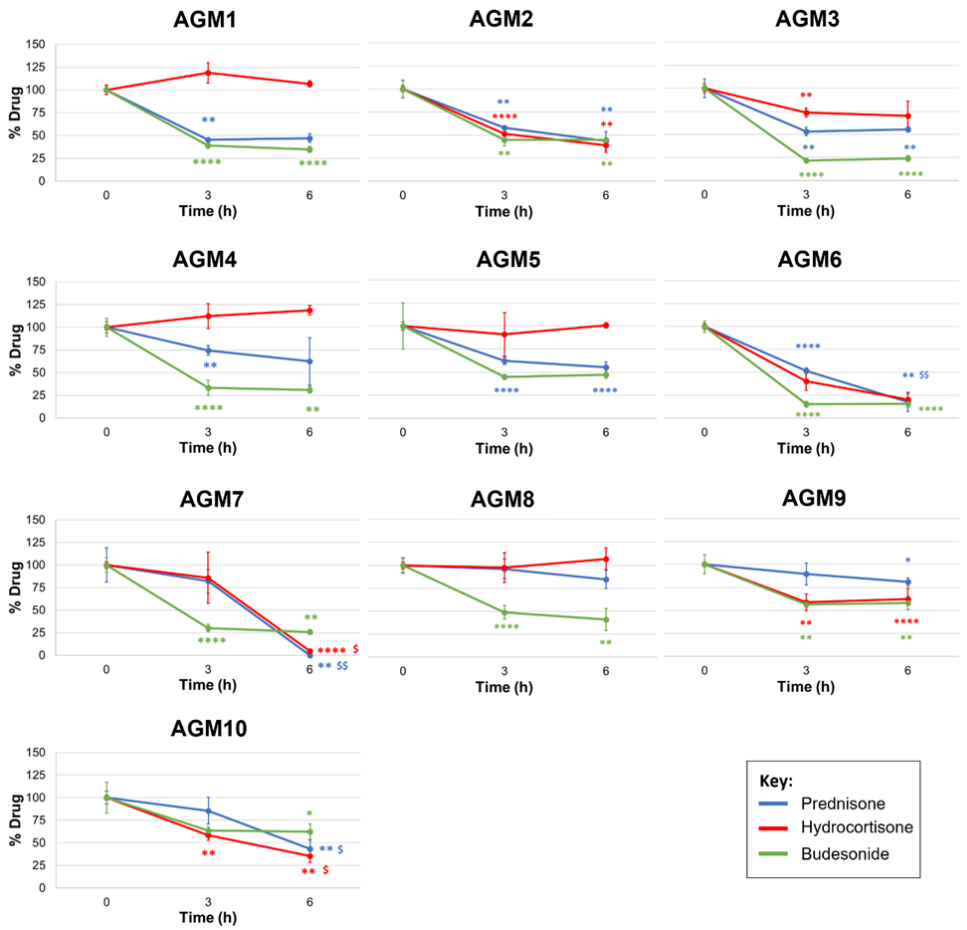

Figure 3. *In vitro* metabolic stability of prednisone (blue), hydrocortisone (red) and budesonide (green) in artificial gut microbiota (**AGM1-10**). Mean values ±

SD (n=3) are reported. *: $p \leq 0.05$; **: $p \leq 0.01$; ****: $p \leq 0.001$ (comparison between 0 and 3 or 6h); $: $p \leq 0.05$; $$: $p \leq 0.01$; $$$$: $p \leq 0.001$ (comparison between 3 and 6h).

Prednisone, as shown in **Figure 3**, presented different stabilities depending on the AGM considered. Notably, after 3h of incubation, the levels of prednisone remained stable in half of the artificial microbiota, except for AGM5 and AGM6 in which there was a significant decrease. On the other hand, after 6h of incubation the concentration of prednisone significantly decreased in almost all the artificial microbiota, other than AGM4, AGM5, and AGM8. In AGM6, 7, and 10 prednisone levels significantly dropped between 3h and 6h of incubation.

After 3h of incubation with the different AGM, hydrocortisone levels were significantly lower than at t = 0 in AGM2, 3, 9, and 10, while they remained stable in the others. Moreover, at 6h, a significant decrease was recorded in AGM7, 9, and 10. Between 3h and 6h in a significant drop of hydrocortisone levels was observed in AGM7 and 10. Opposite to that, in AGM1, 4, 5, and 8 hydrocortisone was not metabolized even after 6h.

Differently from the other two corticosteroids, budesonide levels after 3h of incubation were significantly lower than at t=0 in almost all the AGM, except for AGM5 and AGM10. In addition, we observed a significant reduction of budesonide levels after 6h of incubation in all the artificial gut microbiota, except for AGM5, in which the drop was not significant. Surprisingly, in this case budesonide metabolism did not proceed further after 3h of incubation.

Overall, the observed stability profiles seem to be strongly related to the taxonomic composition of the different AGMs. Despite the three GC sharing a high structural similarity and comparable lipophilicity, with Log P values ranging from 1.46 to 1.91 (Knox et al., 2024), we observed different tendencies to

metabolic transformation and different clearance kinetics across the various AGMs. AGM6 and AGM7 showed the highest metabolic clearances, with less than 25% of starting compound remaining after 6h.

According to the observed gut microbiota profiles (Table S3), AGM6 and AGM7 were characterized by the highest abundance of *Bacteroides uniformis* (47.3% and 61.6%, respectively). The second most abundant bacterial species in both AGM6 and AGM7 was *Bacteroides thetaiotaomicron* (10.9% and 11.8%, respectively).

When single *B. uniformis* and *B. thetaiotaomicron* strains were incubated in a gut simulating medium with different drug classes (Zimmermann et al., 2019a), they showed high metabolic promiscuity (from 50 to 95 metabolized drugs starting from a pool of 271 molecules) and capacity (from 20% to 80% of metabolized drug over 12h). This further reinforces the need to move towards an increasingly personalized and effective medicine that considers the impact of the intestinal microbiota on drugs.

3.4. Profiling of in vitro metabolites generated from corticosteroids by AGM

Once identified which AGM showed the highest metabolic conversion of glucocorticoids, we scrutinized the identities of metabolites by employing an ion mobility-enabled (IM) high resolution mass spectrometry (HRMS)-based workflow (See materials and methods for details). Data were acquired in full scan mode, alternating high and low collision energy states; drift time (dt) and related collision cross section value (CCS, in $Å^2$) were obtained based on the size, shape and charge of each ion as it travelled from the ion source through the ion mobility cell [52]. It was thus possible to align precursors and fragment ions based on both

UPLC retention time (RT) and drift time (dt), allowing an efficient matching of precursor and fragment ions (Giles et al., 2011). The combination of UPLC retention time (RT), accurate masses and CCS values for precursor ions and fragmentation spectra were then employed to identify unknown metabolites across the different AGM.

3.5 Profiling of hydrocortisone metabolites

The UPLC-HRMS traces of parent hydrocortisone and metabolites $M1_{HC}$ -$M3_{HC}$, formed after 6h of incubation in AGM2 and AGM6, are reported in Figure 4. In the ESI negative ion mode (ESI^-), parent hydrocortisone eluted at RT = 6.36 min (See also Supplementary Material, Fig. S.1.1) and did not generate the pseudomolecular ion $[M-H]^-$, but a very stable adduct with formic acid $[M+HCOO]^-$ at m/z = 407.2074 (Figure S.1.2). High energy fragmentation of hydrocortisone gave a very abundant and stable product ion at m/z = 331.1915 corresponding to the loss of formaldehyde CH_2O (Dm = 30 Da) from the side chain (Figure S.1.3).

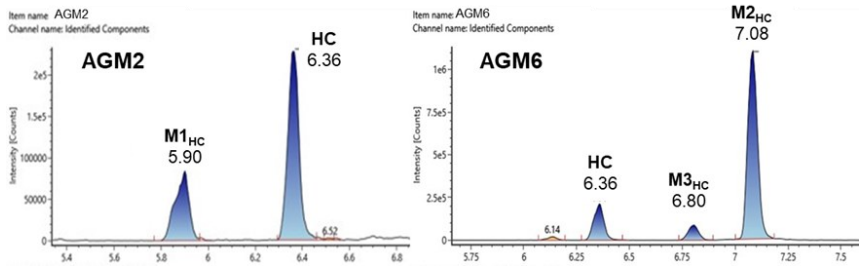

Figure 4. Chromatographic profiles of identified metabolites of hydrocortisone in AGM2 (left panel) and 7 (right panel). Parent hydrocortisone has a RT = 6.36 min. Metabolites derived from a single reduction reaction were identified as 20-beta-

dihydrocortisol (M1HC); RT = 5.90 min; 5-beta-dihydrocortisol (M2HC); RT = 7.08 min and 5-alpha-dihydrocortisol (M3HC); RT = 6.80 min.

In Table 1, the identities of hydrocortisone metabolites are reported. For metabolites **M1$_{HC}$-M3$_{HC}$**, the attribution of the site of reduction and of its chirality was accomplished by comparison of the UPLC-HRMS features of unknown metabolites with those of reference standards, as detailed in the following description. Metabolite **M1$_{HC}$** was identified as the product of reduction of the keto group at position C_{20} on the side chain of the corticosteroid to a hydroxyl group; **M2$_{HC}$** and **M3$_{HC}$** were identified as the two metabolites deriving from the reduction of the double bond D^{4-5} on the steroid nucleus; metabolite **M4$_{HC}$** was a second generation metabolite deriving from a double reduction reaction, with a mass shift of +4 Da (+4H) with respect to parent hydrocortisone.

*Metabolite **M1$_{HC}$***

Metabolite **M1$_{HC}$** was detected in the LC-HRMS traces of AGM1 and AGM2 at RT = 5.90 min (Suppl. Fig. S.1.4-5). The accurate mass values were attributed to the formate adduct ions $[M+HCOO]^-$, at *m/z* = 409.2233 or AGM1 and at *m/z* = 409.2229 for AGM2, of a compound having elemental composition $C_{21}H_{32}O_5$ and a mass shift of +2H (+2 Da) with respect to parent hydrocortisone. It was deduced that **M1$_{HC}$** could be the product of a reduction reaction on hydrocortisone. High energy fragmentation (MS^E) spectrum of **M1$_{HC}$**, detectable in AGM2, showed two ions at 363.2183 and 333.2071 corresponding to the loss of formic acid and also of formaldehyde from the adduct ion at *m/z* = 409.2229. The identity of **M1$_{HC}$** was finally confirmed by comparison of its LC-HRMS features (i.e., RT, accurate masses of precursor and fragment ions, CCS value) with those of a

synthetic reference standard. To achieve this aim, the commercially available reduced metabolites at position C_5, i.e., 5a- and 5b-dihydrocortisol, and C_{20}, i.e., 20a- and 20b-dihydrocortisol, were eluted employing the same LC-MS conditions. The standard 20b-dihydrocortisol shared the same UPLC RT, accurate mass, fragmentation spectrum and CCS value of unknown **M1$_{HC}$** (Suppl. Fig. S.2.2-S.2.4). By spiking 4 pmol of 20b-dihydrocortisol into the incubate of AGM2, a significant increase in **M1$_{HC}$** peak intensity was detected (Suppl. Fig. S.2.4). **M1$_{HC}$** was thus identified as 20b-dihydrocortisol (20b-DHF) (Abel et al., 1993).

*Metabolites **M2$_{HC}$** and **M3$_{HC}$***

As shown in Table 1, metabolites **M2$_{HC}$** and **M3$_{HC}$** were characterized by an observed *m/z* = 409.2233 (mean value in AGM5-11) and *m/z* = 409.2229, which corresponded, with an acceptable mass error, to the formate adduct ion $[M+HCOO]^-$ of other metabolites of mono-reduction of hydrocortisone (see also LC-HRMS features in Supplementary material). MS^E spectrum of **M2$_{HC}$**, detected in AGM10 (Supplementary Figure S.9.2), revealed the ion at *m/z* = 333.2073 that corresponded to the cleavage of the side chain and loss of formaldehyde $[M–CH_2O–H]^-$ and *m/z* = 299.1654 that could derive from the complete cleavage of the side chain. A comparison of the UPLC-HRMS features of **M2$_{HC}$** with those of available chemical standards of hydrocortisone metabolites (Suppl. Fig. S.9.5-S.9.6) allowed to identify **M2$_{HC}$** as 5b-dihydrocortisol (5b-DHF). The same comparisons were also conducted on the other reduced metabolite **M3$_{HC}$**, characterized by a RT = 6.81 min (Suppl. Fig. S.5.5-S.5.6). A spiking experiment (Supplementary Figure S.5.7) in AGM6 allowed to confirm its identity as 5a-dihydrocortisol (5a-DHF).

Metabolite ***M4$_{HC}$***

Searching extensively into the LC-HRMS traces for potential redox transformations occurring to hydrocortisone based on literature references (Abel et al., 1993; Arioli et al., 2022) and on the MetID routine of UNIFI software, a metabolite of double reduction with a mass shift of +4 Da (+4H) with respect to parent hydrocortisone was also retrieved in AGM6, 7, and 10.

Table 1. Metabolites of hydrocortisone observed in different artificial gut microbiota.

Metabolite	ID	Ion type	Retention time (min)	Chemical formula	Observed m/z	Mass error (ppm)	Observed CCS (Å²)	Mass shift	Found in microbiota:
M1	20β-DHC	[M+HCOO]-	5.9	C21H32O5	409.2233	0.2	194.82	+2H	1
		[M+HCOO]-	5.9	C21H32O5	409.2229	-0.7	195.4	+2H	2
M2	5β-DHC	[M+HCOO]-	7.09	C21H32O5	409.2232	0	194.79	+2H	5
		[M+HCOO]-	7.09	C21H32O5	409.2241	2.2	194.85	+2H	6
		[M+HCOO]-	7.08	C21H32O5	409.2229	-0.6	195.13	+2H	7
		[M+HCOO]-	7.1	C21H32O5	409.2236	1	195.71	+2H	8
		[M+HCOO]-	7.08	C21H32O5	409.223	-0.3	194.79	+2H	9
		[M+HCOO]-	7.09	C21H32O5	409.2231	-0.3	194.68	+2H	10
		[M+HCOO]-	7.09	C21H32O5	409.2233	0.2	194.68	+2H	11
M3	5α-DHC	[M+HCOO]-	6.81	C21H32O5	409.2229	-0.6	194.59	+2H	7
M4	-	[M+HCOO]-	6.79	C21H34O5	411.2384	-1.2	193.95	+4H	7
		[M+HCOO]-	6.8	C21H34O5	411.2388	-0.3	194.25	+4H	8
		[M+HCOO]-	6.79	C21H34O5	411.2389	0	193.43	+4H	11

As shown in **Figure 5** (Panel A), AGM6, 7, and 10 were characterized by the highest metabolic activity leading to the generation of reductive metabolite 5b-

DHF in the highest yields. It is plausible that metabolite $M4_{HC}$ could be a second-generation metabolite, deriving from the further reduction of 5b-DHF. Interestingly, two metabolic clusters were also identified leading to a selective reduction at C_{20} (i.e., AGM1 and 2), generating 20b-DHF, and to a reduction at position $C_{4\text{-}5}$ (i.e., AGM5, 7-10) generating 5b-DHF and, in the case of AGM7, also yielding 5a-DHF in lower yields.

3.6. Profiling of prednisone metabolites

Prednisone closely resembles hydrocortisone with two major structural differences: the presence of the keto group at C_{11}, which is *in vivo* reduced to hydroxyl group generating the active metabolite prednisolone (Garg et al., 1994) and of the double bond in C1-2. Table 2 reports the chromatographic (i.e., RT, in min), HRMS (i.e., Experimental Mass and error in ppm related to the corresponding chemical formula) and ion mobility (i.e., CCS in Å²) features for the retrieved prednisone metabolites. Mimicking what had been observed for hydrocortisone, two metabolites deriving from a single reduction reaction (Dm = +2 Da with respect to parent compound) were detected, which were tentatively numbered **M1$_{PRED}$** and **M2$_{PRED}$**. Another metabolite, i.e., **M3$_{PRED}$**, characterized by a mass shift of +4 Da, which likely corresponds to the product of a double reduction on prednisone was detected, together with metabolite **M4$_{PRED}$** which was characterized by a mass shift of +6 Da and which could be considered a third-generation metabolite, deriving from three subsequent reduction reactions. Their LC-HRMS characterization is reported in the following paragraphs.

*Metabolite **M1$_{PRED}$***

Metabolite **M1PRED** was detected in the LC-HRMS traces of AGM1 and AGM2 at RT = 5.82 min. Its experimental mass value corresponded to the pseudomolecular ion $[M-H]^-$ at *m/z* = 359.1861 of a compound having elemental composition $C_{21}H_{28}O_5$ and a mass shift of +2H (+2 Da) *vs.* parent prednisone. (Suppl. Fig. S.10.3-S.11.2). It was concluded that **M1PRED** could be the product of a mono-reduction on prednisone structure. The positions that could be subjected to microbiota-catalyzed reduction were five, either the steroid ring (D^{1-2} and D^{4-5} double bonds; keto groups at C3 and C11) or the side-chain (keto group at C_{20}) and each reduction reaction could give rise to a couple of epimers.

*Metabolite **M2PRED***

In parallel to what had been observed for hydrocortisone, metabolite **M2PRED** was only detected in the cluster of AGM4 and AGM6-10. It was characterized by a RT = 7.02 min and by an experimental mass at *m/z* = 405.1922 which could correspond, within an acceptable mass error (<1.6 ppm) to the formate adduct ion of a compound having chemical formula $C_{21}H_{28}O_5$ (Supplementary Figure S.12.1-S.13.2). Also, **M2PRED** was tentatively defined as a mono-reduction metabolite of prednisone.

Furthermore, after comparing the LC-HRMS features of **M1PRED** and **M2PRED** with those of the reductive and active metabolite of prednisone, i.e., prednisolone, we concluded that neither **M1PRED** nor **M2PRED** corresponded to prednisolone (Supplementary Figure S.17.7-S.17.8).

Table 2. Putative metabolites of prednisone in different artificial gut microbiota.

Metabolite	Ion type	Retention time (min)	Chemical formula	Experimental mass	Mass error (ppm)	Observed CCS (Å²)	Mass shift	Found in microbiota:
M1	$[M-H]^-$	5.82	C21H28O5	359.1861	-0.8	197.67	+2H	1
	$[M-H]^-$	5.83	C21H28O5	359.186	-1	196.87	+2H	2
M2	$[M+HCOO]^-$	7.03	C21H28O5	405.1922	0.9	192.78	+2H	5
	$[M+HCOO]^-$	7.02	C21H28O5	405.1918	-0.2	193.1	+2H	7
	$[M+HCOO]^-$	7.02	C21H28O5	405.1925	1.6	192.73	+2H	8
	$[M+HCOO]^-$	7.03	C21H28O5	405.1924	1.2	192.91	+2H	9
	$[M+HCOO]^-$	7.03	C21H28O5	405.1921	0.6	193.01	+2H	10
	$[M+HCOO]^-$	7.03	C21H28O5	405.1915	-0.9	192.55	+2H	11
M3	$[M+HCOO]^-$	7.22	C21H30O5	407.2077	0.3	192.14	+4H	7
	$[M+HCOO]^-$	7.22	C21H30O5	407.2073	-0.8	192.38	+4H	8
	$[M+HCOO]^-$	7.22	C21H30O5	407.2069	-1.7	191.92	+4H	10
	$[M+HCOO]^-$	7.22	C21H30O5	407.2075	-0.3	191.79	+4H	11
M4	$[M+HCOO]^-$	6.99	C21H32O5	409.2231	-0.5	191.84	+6H	8
	$[M+HCOO]^-$	6.99	C21H32O5	409.2227	-1.5	191.8	+6H	11

Metabolite **M3PRED**

Metabolite **M3PRED** was only detected in AGM6-7 and AGM9-10. It had a RT = 7.22 min and it was characterized by an experimental mass at m/z = 407.2077, which could be attributed to the formate adduct ion of a compound with chemical formula $C_{21}H_{30}O_5$ (Supplementary Figure S.13.3-S.13.4). This could correspond to a metabolite deriving from a double reduction reaction on prednisone structure.

Metabolite **M4PRED**

Finally, only in the case of fast metabolizing AGM7, and, to a minimum extent, AGM10, it was possible to detect metabolite **M4PRED**, which was characterized

by a mass shift of +6 Da if compared to prednisone (Supplementary Figure S.14.5-S.14.6). It had a RT = 6.99 min in the LC-HRMS trace, and its m/z value resembled that of the formate adduct ion of a metabolite of triple reduction on prednisone structure.

As summarized in Figure 5, also in the case of the glucocorticoid prednisone it was possible to detect two metabolic clusters leading to the formation of two different metabolites of single reduction: **M1$_{PRED}$** generated by AGM1 and AGM2 and **M2$_{PRED}$** generated by AGM4 as well as AGM6-10.

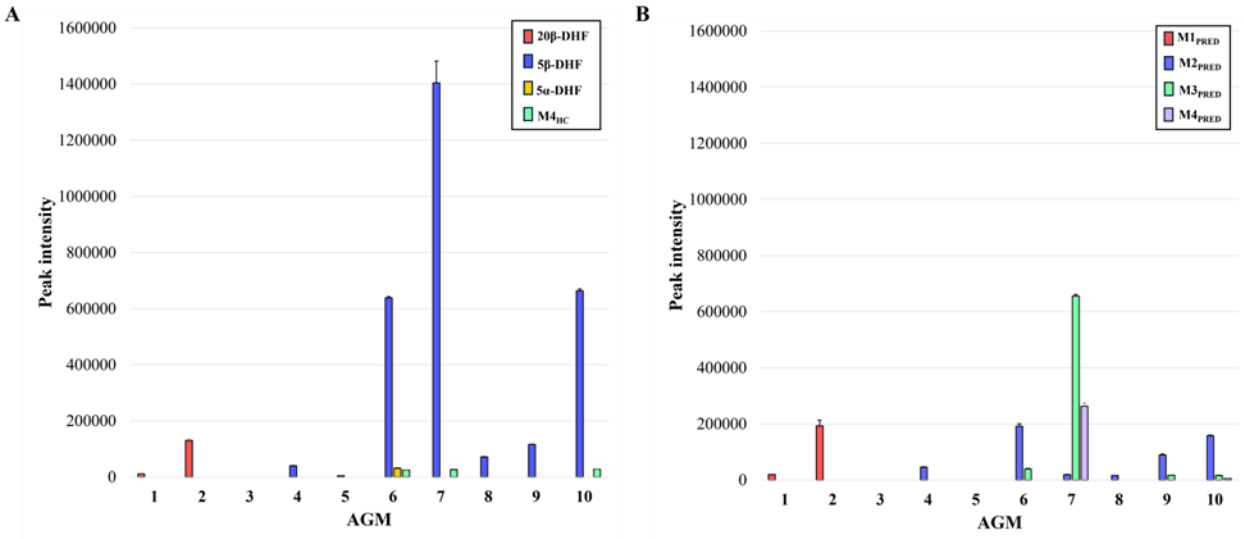

Figure 5. Peak intensities of hydrocortisone (A) and prednisone (B) reductive metabolites found in AGM after 6h of incubation.

3.7. Profiling of budesonide metabolites

Table 3 reports the chromatographic, HRMS and ion mobility features for the budesonide metabolite M1$_{BUD}$, derived from a single reduction (Dm =+2 Da) on

the structure of budesonide. In analogy to the previously analysed corticosteroids, and despite budesonide did not share the same structural features with hydrocortisone and prednisone, the reductive pathway was also active on budesonide, but only in AGM6, 9, and 10, which were already identified as the most efficient metabolizers, a significant amount of $M1_{BUD}$ was detectable after 6h of incubation (Supplementary Figure S.19.1-S.21.2).

Table 3. Putative metabolites of budesonide in different artificial gut microbiota.

Metabolite	Ion type	Retention time (min)	Chemical formula	Observed m/z	Mass error (ppm)	Observed CCS (Å²)	Mass shift	Found in microbiota :
M1	[M+HCOO]-	9.3	C25H36O6	477.2499	1	218.04	+2H	7
	[M+HCOO]-	9.28	C25H36O6	477.2493	-0.3	217.91	+2H	8
	[M+HCOO]-	9.3	C25H36O6	477.2495	0.1	218.04	+2H	11

The main findings related to the microbiota-derived reductive metabolism of prednisone, hydrocortisone and budesonide, with the identification of two microbial metabolic clusters leading to two divergent pathways of reductive metabolism are summarized in Table 4.

Table 4. Reductive metabolites of prednisone, hydrocortisone and budesonide found in AGM after 6h of incubation. The coloured cells represent the presence of the reductive metabolite in the respective AGM.

				Microbiota									
Drug	**Metabolite**	**Mass shift**	**RT**	**1**	**2**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
hydrocortisone	20-beta-dihydrocortisol	+2H	5.9										
	5-beta-dihydrocortisol	+2H	7.09										
	5-alpha-dihydrocortisol	+2H	6.81										
	Metabolite 4	+4H	6.79										
prednisone	Metabolite 1	+2H	5.83										
	Metabolite 2	+2H	7.03										
	Metabolite 3	+4H	7.22										
	Metabolite 4	+6H	6.99										
budesonide	Metabolite 1	+2H	9.29										

3.8. Effects of orally administered corticosteroids on artificial gut microbiota

Alongside with the analysis of metabolites produced after the incubation of oral glucocorticoids with artificial gut microbiota, the impact of the different drugs on both taxonomic composition and growth performances of AGM was assessed through shallow shotgun metagenomics and flow cytometry-based total bacterial cell count, respectively. Since metabolic transformation was observed only in case of drugs, i.e., hydrocortisone, prednisone and budesonide, while the conversion of prodrugs into drugs was almost completely absent, microbiological assays were only conducted on the three drug incubates. To evaluate if the

taxonomic composition of each AGM effectively changed due to the presence of glucocorticoids, a shallow shotgun sequencing was performed on all 10 AGM exposed to the three drugs after 6h of incubation, as well as on the AGM not exposed to any drugs, i.e., control samples. The DNA sequencing generated a total of 4,379,229 reads reduced to a total of 1,662,863 with an average of 41,571 reads per sample after chimera and quality filtering (Table S4).

The generated taxonomic profiles at species-level were used to explore whether the exposure to drugs has a role in influencing α-diversity (Fig. 6a). In detail, the calculation of the Shannon index highlighted that while for some samples, i.e., AGM1 and AGM2, the bacterial complexity remained almost unchanged, for the other samples, an alteration of the α-diversity was observed in response to the drugs when compared to the control sample, as also confirmed by the evaluation of species richness (Fig. 6b). This result suggests that a same drug differentially influences the bacterial complexity of the human gut microbiota, probably depending on the specific composition of this complex microbial ecosystem. Moreover, the taxonomic profiles were also exploited to calculate a Bray-Curtis dissimilarity matrix, to evaluate the impact that drugs may have on bacterial biodiversity. Interestingly, while for certain samples no significant differences in biodiversity were observed when the AGMs were exposed to drugs, in other cases remarkable changes were recorded (Fig. 6c), suggesting that drugs can exert a significant impact on both bacterial complexity and biodiversity of the human gut microbiota.

An in-depth insight into the taxonomic profiles revealed that for some AGM, i.e., AGM1 and AGM2, the treatment with the above-mentioned drugs did not induce

any appreciable modification in the abundance of bacterial species. Conversely, for other AGM a drug-induced decrease of the abundance of certain microbial taxa was observed when compared to the control. Particularly, AGM4, AGM5, and AGM6 showed a 2- to 5-fold decrease in the abundance of various species belonging to the genus Bacteroides when the AGM were exposed to drugs (Table S5), except for Bacteroides finegoldii in AGM4 and AGM8 (in presence of hydrocortisone and prednisone, respectively), as well as for Bacteroides ovatus in AGM5, and Bacteroides caccae in AGM7 whose abundances underwent a 2-fold increase when in presence of hydrocortisone. Similarly, other bacterial species typical of the human gut microbiota were reduced, such as Akkermansia muciniphila which experienced a 2- to a 5-fold decrease in AGM4 and AGM5 in both budesonide and prednisone, as well as in AGM9 when exposed to budesonide and hydrocortisone (Table S5), while Anaerotignum faecicola underwent an abundance reduction from a 2- to a 5-fold in both AGM5 and AGM6 in presence of budesonide or prednisone (Table S5). However, in addition to some species that decreased, other bacterial taxa showed an opposite trend. Alistipes finegoldii in AGM4 and AGM8 underwent a 2-fold increase in presence of hydrocortisone and prednisone, respectively (Table S5). Finally, while Bifidobacterium breve showed an almost 5-fold decrease in AGM3 when exposed to budesonide and prednisone, in AGM5 in prednisone, the same species demonstrated a 2-fold increase (Table S5).

Moreover, to further investigate the impact that glucocorticoids have on the growth performances of AGM bacteria after 6h of incubation with drugs, a flow cytometry-based bacterial total count was conducted. Interestingly, these data

highlighted not only that each drug had a different impact on the various AGM, but also that each AGM generally maintained a consistent trend when exposed to the three drugs (Fig. 6). Indeed, AGM3, AGM5, and AGM6 exhibited a statistically significant reduction in cell count, while AGM1, AGM8, and AGM9 showed no substantial changes in terms of microbial growth.

Finally, a correlation analysis between the microbial taxa characterizing each AGM after being in contact with the drugs, and the metabolites detected for each AGM was performed. Interestingly, the correlation data revealed that the presence of Bacteroides nordii was consistently correlated with the presence of metabolite $M3_{HC}$ produced by hydrocortisone ($p < 0.001$) (Table S6). Similarly, Clostridium spp. positively correlated with the presence of metabolite $M1_{BUD}$ from budesonide and metabolite $M3_{HC}$ from hydrocortisone ($p < 0.05$ and $p < 0.001$, respectively) (Table S6). Finally, Enterococcus casseliflavus was found to be positively correlated with the production of $M1_{BUD}$ from budesonide and $M4_{HC}$ from hydrocortisone ($p < 0.05$) (Table S6). Furthermore, a correlation analysis between taxa revealed that the presence of Clostridium spp. positively correlated with the presence of E. casseliflavus ($p < 0.05$), suggesting that the presence of these two bacterial taxa might be involved in the formation of $M1_{BUD}$ of budesonide (Table S6).

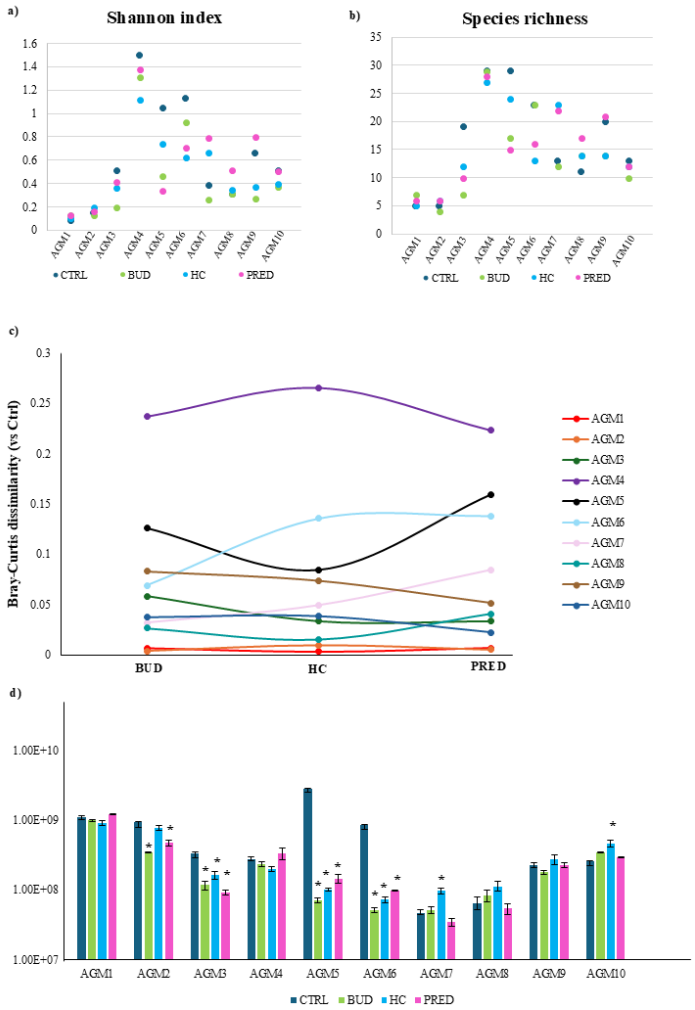

Figure 6. Evaluation of the impact of glucocorticoids on AGMs. Panels a) and b) depict the Shannon index and the species richness, respectively, of each AGM in presence of budesonide (BUD), hydrocortisone (HC), prednisone (PRED) and the control (CTRL). Panel c) represents the Bray-Curtis dissimilarity matrix calculated for every condition tested vs. controls. Panel d) indicates AGM total bacterial count after 6h of incubation with budesonide, hydrocortisone and prednisone at initial 10 µM concentration. Each bar plot depicts the flow cytometry-based bacterial counts obtained by cultivating the AGM with the three glucocorticoids as well as without the addition of any drugs (control samples). The vertical bars indicate standard deviations (n= 3), and the asterisks indicate a p-value < 0.001.

Discussion

One main finding of the present study is that different AGM communities, stabilized through a continuous fermentation system from fresh fecal samples, and representative of the human gut microbiota, were able to metabolize GCs showing significant differences in drug metabolic stability as well as in the number, structure and amount of generated metabolites.

In vitro metabolic stability assays, conducted by incubating a 10 mM solution of GCs with different AGM, allowed to identify those artificial microbiota communities characterized by the highest metabolic clearances. Then, by means of a HRMS-based metabolite identification (Met ID) workflow, the activated metabolic pathways were highlighted. AGM showed a very limited or null ability to hydrolyze GC ester prodrugs, with a maximum 7% betamethasone production after 6h of contact. It has been reported that hydrocortisone is released from the ester hydrocortisone acetate by either *ex vivo* microbial cultures or by single strains of *Parabacteroides distasonis* or *Clostridium bolteae*. Nevertheless, its hydrolytic conversion, which was followed in the case of microbial cultures by a reductive step to 20b-dihydrocortisone, was not quantitative even at longer incubation times (i.e. 24h) with microbial cultures or single isolates (Javdan et al., 2020).

In the case of GC prednisone, hydrocortisone and budesonide, we only detected reductive metabolites, and not products of oxidative metabolism, nor of conjugation reactions.

Interestingly, AGM1 and AGM2 were the only artificial gut communities able to generate the metabolite 20β-DHF (metabolite $M1_{HC}$) from hydrocortisone and

metabolite $M1_{PRED}$ from prednisone. Although the position of reduction for $M1_{PRED}$ was not identified, in both cases the microbial modification was obtained via a single reduction reaction. Considering that AGM1 and AGM2 were the only intestinal bacterial communities to be dominated by *Escherichia coli,* it can be argued that this bacterial taxon may have a role in generating these two metabolites from hydrocortisone and prednisone.

In humans, 20β-DHF production is catalyzed by host 20β-hydroxysteroid dehydrogenases (20β-HSDH) (Eisenschmid et al., 1987) as well as by carbonyl reductase-1 (CBR1) (Morgan et al., 2017), but a role for gut microbiota has also been clearly established. For instance, *Bifidobacterium adolescentis* (Doden et al., 2019)*, Butyricicoccus desmolans* and *Clostridium cadavaris* (Bokkenheuser et al., 1986; Devendran et al., 2017; Winter et al., 1982), belonging to human fecal microbial ecosystem, were found to catalyze the conversion of cortisol to 20β-DHF by means of their 20β-HSDH enzymatic activity. 20β-DHF has been described as a minor urinary metabolite of the glucocorticoid hormone cortisol, accounting for approximately 3% of total urinary cortisol metabolite profile, nevertheless showing plasma and tissue levels comparable to the other endogenous GC corticosterone (Morgan et al., 2017). An increase in urinary concentrations of 20β-DHF has been reported in patients with various disease states including Cushing's syndrome (Schöneshöfer et al., 1983), obesity (Morgan et al., 2017) and hypertension (Kornel et al., 1975). Recently, it has been shown that 20β-DHF can act as an endogenous agonist of the human glucocorticoid receptor (GR), able to suppress inflammatory gene transcription

by induction of the nuclear translocation of the receptor within the same time frame as cortisol (Morgan et al., 2017).

All other detected metabolites were produced exclusively by AGM3 to AGM10 which, unlike AGM1 and AGM2, were characterized by a predominance of various species belonging to the genus *Bacteroides*. It can thus be assumed that microbial taxa of this genus may be involved in the production of metabolites 5b-dihydrocortisol (5b-DHF/$M2_{HC}$), 5a-dihydrocortisol (5a-DHF/$M3_{HC}$) and $M4_{HC}$ from hydrocortisone, as well as metabolites $M2_{PRED}$, $M3_{PRED}$, and $M4_{PRED}$ from prednisone, and metabolite $M1_{BUD}$ from budesonide. Considering the relative peak intensities in the extracted ion chromatograms (XIC) of GC metabolites (Fig. 5), AGM6, 7 and 10 showed the highest conversion rates to 5b-DHF, while 5a-DHF was only detected in AGM6. However, only isolation and individual cultivation of *Bacteroides* species in the presence of corticosteroids could clarify their involvement in drug metabolism or, on the contrary, highlight that it is most likely a concerted activity of various species the responsible for the formation of these metabolites. This also applies to the role of *E. coli* in the generation of metabolites found in AGM1 and 2.

Reduction of the $D^{4,5}$ double bond in the A-ring of the steroid structure is the rate-limiting step in cortisol metabolism in humans. This reaction is catalyzed by either 5a- or 5b-reductases (Gold et al., 1959). The resulting dihydro-metabolites are then further reduced to their tetrahydro-metabolites tetrahydrocortisol (THF) and allo-tetrahydrocortisol (ATHF) by 3a-hydroxysteroid dehydrogenases (Gold et al., 1959). Initially considered inactive, metabolite 5β-DHF has been found to sensitize ocular tissue to GCs by triggering GR nuclear translocation and it is

abnormally accumulated in patients with glaucoma (Weinstein et al., 1983; Southren et al., 1985). 5a-DHF was found to have a weak mineralocorticoid activity and was able to potentiate the activity of low concentrations of aldosterone (Adam et al., 1978).

The differential generation of microbiota-derived GC metabolites could have a significant impact on the *in vivo* regulation of GC action. In fact, in the case of the endogenous GCs cortisol and corticosterone, this delicate tuning is modulated, among other factors, by their metabolism by 11b-hydroxysteroid dehydrogenases (11b-HSDH). In the kidney, the isoform 11b-HSDH2 catalyzes the conversion into the inactive 11-keto forms cortisone and 11-dehydrocorticosterone. 11b-HSDH2, thus, controls the magnitude of mineralocorticoid receptor (MR)-mediated Na^+ retention, playing a chief role in volume regulation and hypertension (Rusvai et al., 1993). When this enzyme is inhibited, cortisol can bind to MR in the distal nephron with an affinity equal to that of aldosterone (Krozowski et al., 1983). As stated before, it has been reported that microbiota-derived 5α-dihydro metabolites of cortisol and corticosterone could act as competitive inhibitors of 11b-HSDH2 leading to potential MR-mediated side effects (Honour, 1982; Morris et al., 2007).

Beyond the investigation of the impact that human gut microbiota may have on corticosteroid metabolism, the evaluation of the possible effects that these drugs may play on AGM was also assessed, confirming, as also previously described, that the interplay between drugs and gut microbiota is complex, bidirectional, and dependent on the specific composition of the gut microbiota (Aziz et al., 2018). Indeed, total bacterial cell count data highlighted that different bacterial

communities showed different susceptibility to the presence of the drugs with some AGM displaying a significant reduction and other AGM a significant increase in the number of bacterial cells when in presence of a drug. In parallel, while for some AGM the relative abundances of the various bacterial species were not drastically altered, for other AGM specific bacterial taxa reported an up to 5-fold increase or decrease in their relative abundances in presence of the drugs when compared to the control. In addition, although eight AGM were characterized by a dominance of the genus *Bacteroides*, they not only showed different growth performances in the presence of drugs, but also induced the formation of different metabolites from the same GC.

5. Conclusions

Precision medicine requires novel approaches to predict drug metabolism phenotypes in order to tailor drug treatments for each individual. We successfully set up a high-resolution mass spectrometry- and shotgun metagenomics-based experimental workflow that was applied to investigate the metabolic transformations performed by a set of artificial gut microbiota, representative of the human intestinal microbiota, on oral corticosteroids, looking for both drug metabolism and prodrug activation. Additionally, the effect of drugs and their metabolites on bacterial populations was also investigated, given the potential impact of these xenobiotics on microbiota growth and composition, which further affect drug fate. These combined approaches shed light on the role that gut microbiota plays in the biotransformation of GCs and can be applied to the characterization of several other pharmacological agents. These studies will help clarifying the relative contribution of intestinal microbial ecosystem and gut epithelium in drug metabolism, and the impact of drug treatments on the metabolic transformations of co-administered drugs in the context of multiple drug therapies.

Additionally, isolation and characterization of drug metabolites could allow to gain further information on new xenobiotics which could sustain and prolong the activity of parent drugs, or, at the worst, could impact on *in vivo* drug bioavailability and toxicity outcome.

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Chapter 7

General Conclusions

The intestine is colonized by a diverse and highly complex community of microorganisms, collectively referred to as the gut microbiota [138]. From birth through old age, this ecosystem evolves in response to numerous factors, both intrinsic (e.g., genetics) and extrinsic (e.g., drugs, diet, and lifestyle), and is intimately linked to the overall health of the host [1]. In recent years, the scientific community has placed large efforts in understanding the mechanisms behind gut microbiota colonization and its dynamic changes, aiming to address dysbiosis and develop strategies to restore a state of eubiosis. Significant knowledge gaps persist despite this focus due to the many interacting variables involved in these processes.

In this context, this Ph.D. thesis sought to address some of these knowledge gaps by focusing on specific factors that influence the composition and function of the gut microbiota, aiming to disentangle their impact and uncover their underlying molecular mechanisms.

The first objective of this Ph.D. project was to investigate the intrinsic factors related to the genetics of microorganisms that constitute the gut microbiota, which play a role in its colonization and modulation. The ability of bifidobacteria to persist in the host throughout life can be attributed to specific genomic characteristics contributing to their persistence in the human gut by promoting their interaction with the host and other commensal microorganisms, such as their ability to produce structures extracellular structures, i.e., exopolysaccharides (EPS), teichoic acids, and pili. Furthermore, bifidobacteria possesses a set of genes encoding a large arsenal of enzymes required for diet-related carbohydrate

breakdown and host-associated glycan degradation. Another genetic adaptation is represented by genes shared among persistent bifidobacterial strains in the human intestine, one of which is *per*B, involved in mucin metabolism [139]. Notably, bifidobacterial persistence is more pronounced in women than men, particularly up until reproductive age. This phenomenon is primarily observed with strains belonging to *Bifidobacterium bifidum* and *Bifidobacterium longum* species, which are also the most frequent taxa that are vertically transmitted from mother to child, providing an evolutionary advantage in maintaining their presence within the female gut. The vertical transmission of these strains is likely a contributing factor to their higher persistence in females, underlying the occurrence of a strict microbe-host co-evolutionary scenario. Notably, mucin is thus sustaining the persistence of those bifidobacteria that can metabolize it through the production of glycosyl hydrolases. Notably, *per*B is predicted to the glycosyl hydrolase 136 (i.e, a lacto N biosidases) that has been shown to exploit a key role in the metabolism of mucin [140]. The women's gut, particularly during reproductive years, is enriched with mucin, especially in its sialidate form, creating a more favorable environment for the colonization of bifidobacteria. Moreover, estradiol, a hormone predominant in women, has been shown to up-regulate mucin production, further enhancing the persistence of bifidobacteria [141, 142]. The genetic support for this persistence, exemplified by the *per*B gene, thus highlights a finely tuned interaction between host biology and microbial adaptation, underscoring the co-evolutionary dynamics between the human host and its gut microbiota. In this context, we demonstrated the predicted role of this gene by generating a mutant in the *per*B gene. *In vitro* experiments using human mucin-

producing cells demonstrated that the presence of the *per*B gene, as seen in *B. bifidum* PRL2010, enhances growth and adhesion to mucin, clarifying the role of the *per*B gene in gut persistence. In addition, *in vivo* experiments with mice further confirmed that the inactivation of *per*B reduces the colonization ability of *B. bifidum* PRL2010 compared to its wild-type counterpart (Chapter 3). These findings are crucial to clarify the genetic determinants of microbes involved in their engraftment and colonization of the human gut. However, since the persistence process involves the involvement of many actors, further biochemical and ecological studies are needed to fully understand the intricate process behind it. In this regard, since microorganisms do not function independently within their ecological niche, it is essential to highlight the importance of microbe-microbe and microbe-host interactions. In this context, bifidobacteria exhibit a form of social intelligence that explains their ability to co-evolve and establish co-occurrence interactions, which are reflected in trophic interactions where there is co-participation in the utilization of complex substrates, such as glycoproteins. Cross-feeding processes are central to these interactions, with a notable example of the metabolic cooperation between *Bifidobacterium bifidum* and *Bifidobacterium breve*. In this relationship, *B. bifidum* metabolizes the enteric mucin, generating sialic acid, a metabolic product that is subsequently utilized by *B. breve*, facilitating its growth [143]. In this context, the impact of co-association among *B. bifidum* PRL2010, *B. breve* PRL2012, and *B. longum* PRL2022 on bacterial and host responses was analyzed. Transcriptomic analyses revealed that co-association enhanced gene expression related to carbohydrate, amino acid, and vitamin metabolism, suggesting improved energy harvest and cross-feeding. This

further confirms the characteristic cross-talk interaction between *B. breve* and *B. bifidum*, underscoring the altruistic behavior of the latter in providing metabolic byproducts that benefit other species [144]. Additionally, tri-association up-regulated genes for extracellular structures, improving bacterial adhesion and interaction with the host. The production of sortase-dependent pili and other extracellular structures further facilitates their engraftment and persistence in the gut as well as their cross talk with the host. Additionally, tests on intestinal cells indicated that the co-association of these strains enhanced mucin degradation and adherence, triggering localized immune responses without inducing systemic inflammation (Chapter 4). These findings demonstrate that co-association among bifidobacterial species significantly enhances their metabolic and structural capabilities, improving energy acquisition, cross-feeding, and adhesion to the host.

In addition to intrinsic genetic factors influencing bifidobacterial colonization, extrinsic factors such as maternal health and drugs intake also play a crucial role in shaping the gut microbiota. It is crucial to emphasize that eukaryotic hormones may serve as important signaling molecules that not only modulate the composition of the microbiota but also impact its functionality. This could represent a significant example of microbiota regulation directly mediated by the host. While much of the current understanding has focused on how host genotype regulates the microbiota, little is known about how host-derived products, such as hormones, can similarly influence these microbial communities. In this circumstance, we were able to correlate the lower insulin concentration in the breast milk of mothers with GDM to a reduced presence of *B. bifidum* in the

intestines of their infants. This allowed us to demonstrate that insulin promotes gene over-expression in host colonization through transcriptomic analysis and experiments using a human gut model (Chapter 5). These findings support the hypothesis that lower insulin levels in the breast milk of GDM mothers may influence infant gut bifidobacterial composition, highlighting the need for further research into the molecular interactions between hormones and the human microbiome and their potential implications for human health.

Moreover, due to hormonal influences, the impact of pharmacological agents on gut microbiota composition and function further underscores the complexity of factors affecting microbial colonization and health. In this framework, the bidirectional relationship between drugs and the gut microbiota was explored in a relatively new field known as pharmacomicrobiomics. Here, for a personalized medicine aimed at finding a specific treatment tailored to the genetics and physiology of each individual, it is crucial to consider how the microbiota can influence the therapeutic efficacy of various drugs by altering their metabolism and, consequently, their bioavailability; and at the same time how drugs can impact the microbiota, shifting it from a state of eubiosis to dysbiosis. To better understand this relationship, we developed an experimental workflow integrating high-resolution mass spectrometry and shotgun metagenomics to investigate how artificial gut microbiota metabolizes oral corticosteroids, representative of the human intestinal microbiota. This approach allowed us to explore both drug metabolism and prodrug activation while also assessing the effects of these drugs and their metabolites on microbial populations. Our findings highlight the crucial role of gut microbiota in corticosteroid biotransformation and offer a framework

for studying other pharmacological agents. This research contributes to understanding the interplay between the gut microbiota and drug metabolism, with implications for multi-drug therapies. The isolation and characterization of drug metabolites also provide insights into new xenobiotics that may prolong drug activity or influence drug bioavailability and toxicity, impacting the individual health status (Chapter 6).

In conclusion, this Ph.D. thesis underlines the intricate interaction between intrinsic and extrinsic factors in shaping the intestinal microbiota and its functional capacity, clarifying some molecular aspects related to the microorganism-microorganism and microorganism-environment interaction. The findings highlight the importance of both microbial relations and genetic determinants in the colonization process and the significant influence of external molecules, such as hormones and pharmacological agents, on microbial dynamics, ultimately contributing to our understanding of gut health and its implications for overall human well-being.

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