MICROBALANCE

White Paper

GUT AND VAGINAL MICROBIOME DNA BARCODING

INTRODUCTION

Human organism is not a sterile environment: both skin and internal tissues house a large variety of microorganisms peacefully living with their host, contributing to its health status. This set of microbes (bacteria, fungi, protozoan, viruses and phages) is called microbiota and comprises 100 trillions (billions of billions) of bacteria.

The collection of the genetic material of all these microorganisms is called microbiome.

The majority of this microbes lives in the gut, where it forms the so called gut microbiota, a community 10 fold more populated than the number of human cells in the organism whose genomes – the gut microbiome – encode a number of genes 100 fold greater than human gene number. In total, gut microbiota comprises more than 50 bacteria phyla – in particular anaerobes such as *Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria* – and more than 15 thousand kinds of bacteria, and weights 1 kg. Colon (the terminal part of the bowel) is the most densely populated region; here, each gram of the intestinal content comprises 100 billions of bacteria.

THE ROLE OF MICROBIOTA IN HUMAN HEALTH PROMOTION

The idea of microbiota playing a role in human health promotion arises from studies on the function of lactobacilli as vaginal ecosystem gatekeepers and the discovery of the association between fermented milk products consumption and prolonged life. Today, it is known that health is shaped by microbiota robustness, that is its capability to resist and recover from change, that acts as a shield preventing pathogen invasion of skin, mouth and gut. Moreover, gut microbiota is critical for immune system development and for the establishment of immunotolerance.

In fact, gut microbiota plays a fundamental role in the induction, education and function of the immune system, establishing an alliance with its host defense weapons to interweave innate and adaptive immunity to select, calibrate and terminate responses. The training of this system during infancy and childhood leads to the establishment of a durable relationship. Immune system continuously works to control this relationship, and the microbiota constantly reinforces the so-called "barrier immunity", that is the mechanisms that allow its containment.

These mechanisms consist in strategies to minimize the contact between microorganisms and bowel epithelial cells. First, intestinal cells produce a mucus layer that limits contact between the microbiota and host tissues and prevents microbial translocation in the bloodstream. Moreover, intestinal cells produces antimicrobial molecules too. Third, immune cells in the intestines produce antibodies (IgA) specific for microbiota antigens, and white blood cells in the intestinal wall (namely, macrophages) rapidly eliminate microbes translocating across the epithelial cell barrier.

Learning to recognize commensal microbiota is a critical step to be taken early in life, during preweaning period. A certain degree of microbiota recognition is a common occurrence, in most cases it is not associated with a pathogenic response. However, gut dysbiosis – that is, alterations of gut microbiota – is associated with a number of health problems. Overall, allergic sensitization, eczema and asthma are associated with a lower relative abundance of *Bifidobacteriaceae* and *Lactobacillaceae* and a greater relative abundance of *Bacteroidaceae, Clostridiaceae*, and Enterobacteriaceae, and a greater microbiota diversity promotes the development of a healthy immune system, reducing the risk of asthma and allergic diseases.

However, gut microbiota role in human health goes far beyond immune system training: it is fundamental also for immune system functioning under steady state and inflammatory conditions, and for tuning the inflammation generated by cells involved in the control of pathogens in the bowel, protecting host tissues from damages.

The mechanisms by which the microbiota shapes immunity are not yet completely understood. Commensal bacteria-derived signals could influence the expression of genes involved in innate responses, enabling a basal level of host-defense factors and a rapid response upon pathogen responses. What is known is that the microbiota directly and dynamically interacts with pathogens. They compete for the same ecological niche; moreover, microbiota metabolites that modulate the activity of the immune system (such as short chain fatty acids, SCFAs) can also down regulate the expression of pathogens virulence genes. What is more, the microbiota promote the establishment of an environment hostile for pathogens, for example by lowering local pH or by producing antimicrobial molecules.

Alterations in gut microbiota can affect both local immune responses and immunity and inflammation in other organs, even if they are distant from the intestine. For example, reduction of gut "good" bacteria via antibiotic treatment reduces immune response against influenza intranasal infection. This control on systemic

immunity has profound consequences also in the context of therapy. For example, gut damage and microbial translocation subsequent to total body irradiation used in some settings of immunotherapy provide an adjuvant effect to bone marrow transplantation. Also dysbiosis, intestinal damage and bacterial translocation associated with cyclophosphamide-based chemotherapy contribute to treatment response. Conversely, disruption of gut microbiota via antibiotic treatment could affect the response to immunotherapy.

Gut microbiota plays a pivotal role in digestion and absorption of dietary components too. In fact, gut microbiome encodes physiological traits that the human organism has not had to evolve, for example the ability to extract from food components, such as fibers, nutrients and energy that would otherwise be lost because humans lack enzymes required to harvest them.

Metabolites produced by commensal bacteria from food components shape and modulate host immune system and metabolic responses. In turn, gut microbiota composition and functional capacity are modulated by the overall balance between proteins, carbohydrates and fats, by the presence of dietary fibers, and by polyphenols.

Among other gut microbiota functions are detoxification of potentially dangerous substances (including carcinogens) and vitamins and amino acids synthesis. Moreover, gut microbiota affects the cardiovascular system.

MICROBIOTA AND HUMAN DISEASES

Several studies suggest the involvement of microbiota in human diseases. Playing an important role in energy homeostasis, intestinal microbial communities may modulate weight loss or gain and obesity-associated disorders. Gut microbiota and its metabolites have been associated with blood pressure regulation, chronic kidney disease, allergy, asthma and critical factors associated with cardiovascular diseases, and dysbiosis is an hallmark of acute infections of the gastrointestinal tract.

Gut microbiota and inflammation

Gut microbiota seems to play an important role in the development of inflammation-associated diseases and conditions. The outgrowth of opportunistic bacteria can drive an increase in inflammation, and the loss of benign bacteria reduces immunoregulation. Dominance of bacteria characterized by enhanced invasiveness and inflammatory properties (in particular γ-proteobacteria) can directly exacerbate inflammation and tissue damage, and mechanisms such as their capability to thrive on metabolites derived from inflammatory settings could contribute to their proliferation.

Moreover, external factors such as the overuse of antibiotics, changes in the diet and the elimination of chronic parasitic infections may select for microbiota lacking the resilience required for the establishment of a balanced microbiota-host interaction in which stimulatory and regulatory signals allow immunity development without compromising the tolerance to innocuous antigens. Such a selection is now believed to contribute to the increase in chronic inflammatory and autoimmune disorders seen in westernized countries.

In fact, many inflammatory diseases are associated with significant alterations in the resident microbiota, shifting from a healthy to a diseased state. Susceptibility to some diseases could be at least in part due to the presence of specific bacteria particularly adept at surviving in, and contributing to, inflammation. Evidences of this phenomenon comes both from mice and human studies.

In particular, the development of Inflammatory Bowel Diseases (IBDs, namely Crohn's disease – CD – and ulcerative colitis – UC) is believed to be the result of a combination of genetic factors, the immune system activity and environmental factors – including the microbiota. Both CD and UC are associated with a reduced complexity of the microbiota and with a dysbiosis characterized by the outgrowth of proteobacteria (in particular, the *Enterobacteriaceae* family). Moreover, in CD patients commensals that are intrinsically inflammatory (such as *Escherichia coli*, *Yersinia* and *Clostridium difficile*) are much more common than in healthy individuals, and there is an increase in serum antibodies against the microbiota. Nowadays, it is thought that IBDs are caused or exacerbated by a loop where host mutations leading to dysregulated immune responses in the gut drive the outgrowth of bacteria that promote more inflammation. In parallel, the loss of bacteria producing SCFAs (that have been shown to limit gastrointestinal inflammatory processes) may contribute to gut inflammation. In particular, *Clostridia* – a class of *Firmicutes* that directly induce immune cells opposing colitis induction and ferment dietary fiber to SCFAs – are reduced in IBDs patients.

A number of studies suggests the involvement of gut microbiota in other inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis and type 1 diabetes. For example, the analysis of the microbiome of rheumatoid arthritis patients suggests the association with microbes belonging to the *Prevotellaceae* family.

Gut microbiota and obesity

Gut microbiota is linked to type II diabetes (T2D), obesity and metabolic syndrome too.

In T2D, the disease can be exacerbated by chronic inflammation driven by complex interactions between the immune system and gut microbiota; SCFAs producing *Clostridia* strains are reduced whereas *E. coli* increases, and blood translocation of bacterial products may rise – as suggested by increased serum lipopolysaccharide (LPS) levels.

Both obesity and its comorbidities are associated with changes in gut microbiota. In general, obesity is associated with a lesser diversity and richness of gut microbiota, and in obese people the ratio of *Firmicutes* to *Bacteroidetes* seems to be higher. Moreover, some studies highlighted a decrease of *Methanobrevibacter smithii* in obese individual and a strong association between obesity and *Blautia hydrogenotorophica, Coprococcus catus, Eubacterium ventriosum, Ruminococcus bromii, Ruminococcus obeum,* and *Lactobacillus reuteri, opposed to a larger proportion of Bacteroides faecichinchillae, Bacteroides thetaiotaomicron, Blautia wexlerae, Clostridium boltae, Flavonifractor plautii, and Bifidobacterium and Lactobacillus* species in lean people.

Low bacterial richness is also associated with obesity-related conditions, in particular insulin resistance and dyslipidemia. In particular, insulin resistance has been associated *with Lactobacillus* increase and *Clostridium* reduction.

In-depth study of the link between obesity and gut microbiota has been conduced mainly in animal model, but several data suggest that similar relationships are present in humans too.

Among proposed mechanisms is the ability of gut microbes to regulate energy intake by fermenting dietary fibers. Thanks to the higher presence of enzymes for complex carbohydrate degradation and fermentation, obesity-associated gut microbiota is characterized by increased capability to harvest energy from the diet; fermentation-derived SCFAs can induce lipogenesis, increase triglycerides stores, inhibit lipolysis and encourages adipocyte differentiation, and obese people feces are characterized by higher SCFAs levels and reduced residual food calories in feces than lean people ones.

Moreover, obesity-associated gut microbiota impairs triglycerides metabolism and promotes fat storage by stimulating gene reprogramming in the colon; it inhibits the enzyme adenosine monophosphate kinase (AMPk), resulting in decreased fatty acid oxidation and, as a consequence, increased fat accumulation.

Gut microbiota LPS can trigger systemic inflammation too, contributing to metabolic disturbances associated with obesity. Dysbiosis increases gut permeability to bacterial products such as LPS and ethanol.

Finally, obesity-associated gut microbiota changes hormones and other bioactive molecules involved in regulating food intake released by intestinal endocrine cells, alters gut-brain communications via molecules such as LPS, gut peptides and hormones, SCFAs and lactate, and seems to contribute to metabolic disorders through an axis of communication with adipose tissue; for example, LPS triggers insulin resistance in adipose tissue, lactate contributes to postprandial satiety, and gut bacteria-derived serotonin and γ-aminobutyric acid affect the central control of appetite.

Microbiota and urogenital infections

Traditionally, urinary tract has been considered a sterile, germ-free environment. However, new tools demonstrate that standard bacteriuria represents a fraction of a diverse microbiota hosted by urinary tract. That is why some experts think it would be better talking about dysbiosis in the case of urinary tract infections (UTIs) too.

Based on actual knowledge, in order to develop such a dysbiosis a complex interplay between host and behavioral risk factor, the immune system and pathogenic bacteria is needed. In terms of pathogenic bacteria, UTIs are characterized by a significantly higher number of different *Escherichia coli* strains, often bearing multidrug resistance. This resistance to antimicrobial seems to be linked to pathogen virulence; moreover, antibiotic treatment increases the risk of UTIs.

Gut microbiota acts as a reservoir for the infecting *E. coli.* Women are more prone to infections, probably because of anatomical reasons; in their case, the initial step in the pathogenesis of UTIs is colonization of the vaginal introitus and periurethra, followed by ascension of the pathogens via the urethra to the bladder and, sometimes, the kidneys. Moreover, in some cases vaginal microbiota seems to serve as a pathogen reservoir on its own.

Looking at gut microbiota, UTIs are associated with more unique *E. coli* clones, often multiresistant. Behavioral factor interacting with pathogen presence to give rise to an infections include sexual intercourse, history of recurrent UTIs, diaphragm-spermicide use, spermicide-coated condom use, and recent antimicrobial treatment. Hormonal contraceptive have been shown to increase the risk of UTIs

too, whereas some data suggest that the presence of an intrauterine device could protect against such an infection.

In the case of vaginal microbiota, risk of UTIs increases with the loss of normally protective lactobacilli species. Infections are more common in the twenties and in postmenopausal women.

Certain bacteria species or characteristics are associated with disease-free conditions. In general, a lactobacilli-dominated vaginal microbiota correlates with a healthy vaginal micro-environment; in particular, *Lactobacillus crispatus* is the predominant species in healthy premenopausal women. Conversely, the absences of vaginal lactobacilli is associated with several disease states, not only UTIs but also, for example, bacterial vaginosis and *Neisseria gonorrhoeae* infection.

Bacterial vaginosis is a dysbiosis associated with subclinical vaginal inflammation and several anaerobic pathogens; it represents the most common dysbiosis of the vaginal microbiome. Other possible dysbiotic states are characterized with a high abundance of streptococci, staphylococci, *Enterobacteriaceae, Candida or Trichomonas*. These vaginal microbiome alterations have been associated with increased susceptibility to sexually transmitted infections, increased risk of pelvic inflammatory disease, preterm birth and maternal and neonatal infections.

Lactobacilli may prevent vaginal colonization via several mechanisms. First, they compete with pathogens for cells adherence. Moreover, they lower vaginal pH by producing lactic acid. Finally, they produce hydrogen peroxide (a microbicidal for many bacterial species), bacteriocines, surfactants and other antimicrobial. In particular, hydrogen peroxide microbicidal activity can be 10- to 100-fold greater in the vagina, where it is combined with chloride anion and myeloperoxidase present in the vaginal environment.

Women with recurrent UTIs are often characterized by increased *E. coli* colonization and depletion of hydrogen peroxide-producing lactobacilli, suggesting that vaginal colonization with such lactobacilli may prevent *E. coli* proliferation. Among factors altering vaginal microbiota are exposure to antimicrobials and the use of some spermicidal products.

Interventions to reduce vaginal microbiota alterations may reduce the risk of UTIs and other vaginal microbiota-associated diseases. The use of oral or intravaginal probiotics is an attractive option to attempt to restore the protective lactobacilli.

Gut microbiota and cancer

Cancer is a disease associated with gut microbiota too. The first example of this association is stomach cancer, whose development depends on the presence of the bacterium *Helicobacter pylori*. Instead, liver cancer has been linked to the microbiota via induction of specific bile acids that damage liver cells DNA. Or, again, the microbiome of colorectal cancer patients is characterized by an increase in *Prevotella*.

Gut microbiome exerts both local and systemic effects on cancer. Tumors other than stomach and liver cancer it has been implicated in include colorectal carcinoma, bladder carcinoma, lymphoma, prostate cancer, breast carcinoma, sarcoma, pancreatic cancer, and ovarian cancer. Organs others than the intestines with no known microbiota, such as liver, can be affected because of anatomical links with the gut allowing exposure to microorganism-associated molecular patterns (MAMPs) and bacterial metabolites.

Among factors influencing cancer development is the relationship between bacteria, inflammatory cytokines and immune system; notably, intestinal cancer is inextricably linked to gut inflammation, and CD is associated with colorectal cancer development. Several mechanisms are involved in this relationship, from innate and adaptive immune cell migration to endocrine and neural pathways, translocation of bacteria or bacterial products and toxins, and systemic inflammation and oxidative stress modulation.

In some cases, as it is in the relationship between *Helicobacter pylori* and stomach cancer, single bacteria strains are responsible for driving the development of some types of tumors, such as *Escherichia coli* in colorectal carcinoma, and *Salmonella enterica typhi* in bladder carcinoma. However, carcinogenesis can be locally modulated also by bacteria groups, which contribute to cancer development interacting with an altered environment.

Examples of gut microbiota alterations associated with cancer are shifts in composition and changes in bacterial density near mucous membranes after dysregulation of tight junctions. Moreover the correlation between obesity, a known risk factor for cancer, and gut microbiota is well documented.

Some bacteria strains can affect cancer development by deregulating signals or pathways. Moreover, recent studies suggest gut microbiota can directly affect carcinogenesis by producing virulence factors, such as toxins, that modulate inflammation in the cancer microenvironment, influence genomic stability of

host cells or epigenetically regulate host gene expression. Colibactin-synthesizing *Escherichia coli*, for example, is associated with DNA damage in colorectal cancer, whereas *Helicobacter hepaticus* promotes hepatic cancer by activation of NF-kB regulated networks.

Finally, gut microbiota can interact with food and inflammation-related host metabolites that increase the growth of potential pathogens and the risk of DNA damage. It can, for example, enhance the production of secondary bile acids hypothesized to modify mucosal barrier allowing antigen penetration and increasing inflammation and the production of factors needed for cancer development, such as vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Other bacteria metabolize products of red meat and high processed food giving rise to molecules believed to damage DNA, such as hydrogen sulfide.

Conversely, some gut bacteria can exert protective effects by producing other metabolites, by inducing immune tolerance, or by outcompeting pathogenic strains. Some prebiotics (substances that enhance the growth or activity of gut microbiota), such as resveratrol, can contribute to cancer prevention working as antioxidants and inflammation downregulators. Butyrate produced by gut microbes through dietary fiber fermentation has tumor suppressing properties, and loss of butyrate-producing bacteria could increase tumorigenesis.

Many other herbal supplements can be converted by gut microbiota in potentially anticancer substances. A possible example is american ginseng.

Finally, microbiome could be important for modulating anticancer therapy efficacy and side effects too. Several studies have been focusing on enhancing the former and reducing the latter via microbiota targeting. Cyclophosphamide effect, for example, is modulated by commensal bacteria, and also oxaliplatin, cisplatin and CpG oligonucleotides exert an antitumor response based on gut microbiota. Melanoma patients responding to anti-PD1 immunotherapy microbiota showed a high diversity and abundance of *Ruminococcaceae* and *Faecalibacterium*, and *Bacteroides* species such as *B. thetaiotaomicron* and *B. fragilis* improve anticancer immunity translocating after mucosal damage induced by anti-CTLA-4 antibodies. In other cases, commensal bacteria can induce side effects, such as a dose-limiting diarrhea following CPT-11 administration.

Microbiota and other diseases

Among other diseases for which an association with gut microbiota alterations has been highlighted are constipation (associated with an increase in *Enterobacteriaceae* and methanogens), irritable bowel syndrome (associated with a lower abundance of *Ruminococcaceae*), cholelithiasis, urinary incontinence, acne and osteoarthritis (negatively associated with, respectively, *Mollicutes, Odoribacteraceae, Deltaproteobacteria and Lentisphaeria*).

In general, diseases are associated not only to an increase or a decrease of a type of bacteria but also to a reduction of microbiota diversity.

In the case of food allergies, gut microbiota composition in the infancy seems to be fundamental. In fact, fecal microbiota of children with cow's milk allergy is markedly different from that of healthy infants. Fecal transplantation studies indicate that healthy infants' microbiota protects from allergic reaction to cow's milk thanks to the presence of a bacterium belonging to *Clostridia* class, namely *Anaerostipes caccae*, a butyrate-producing microbe whose presence is associated with changes in the expression of intestinal epithelial cells genes that correlate with the protection from cow's milk allergic reaction.

In adults, food allergies have been associated also to an increase in the *Comamonadaceae, Enterococcaceae* and *Bacteroidaceae* families and a decrease in the *Oxalobacteraceae, Bifidobacteriaceae, Ruminococcaceae, Peptococcaceae, Christensenellaceae, Anaeroplasmataceae, Paraprevotellaceae, Prevotellaceae, Syntrophomonadaceae, Porphyromonadaceae*, and *Cerasicocccaceae* families, and in the *Mollicutes, Alphaproteobacteria, Deltaproteobacteria, Barnesiellaceae* and *Lentisphaeria* classes.

Gut microbiota and mood

Gut and brain are two-way linked: mood can influence intestinal wellbeing, and gut can influence mood. This interplay is regulated not only by the so-called "brain-gut axis", but also by gut microbiota.

Intestinal bacteria directly respond to stress-related signals; some stress-related molecules, for example, influence bacterial growth, motility and virulence. The mechanisms underlying gut microbiota influence on mood, stress and anxiety are less known. Gut microbiota may activate immune signals directed to the brain. Brain effect of gut microbiota may depend on the contact between microbes and intestinal cells or nerve terminals in the gut too, and chronic inflammation induced by gut dysbiosis can be associated with mood and behavior changes, stress reactivity increase and stress-associated diseases. Moreover, the presence

of *Faecalibacterium* and *Coprococcus* that produce butyrate is associated with higher quality of life indicators, and microbial γ-aminobutyric acid production seems to be involved in depression, a condition linked to *Dialister* and *Coprococcus* depletion.

Dietary behavior can be influenced too. Constant neural and chemical inputs coming from the gut are integrated by the central nervous system to generate appropriate food-reward signaling to maintain homeostasis by regulating appetite, food intake and energy balance. Bacteria and their metabolites might directly target the brain via vagal stimulation or indirectly influence it through immune-neuroendocrine mechanisms.

MICROBIOTA IN PREGNANCY AND BREASTFEEDING

Endocrine, metabolic and immune alterations taking place during pregnancy are associated with significative variations in gut microbiota. Its composition changes dramatically form the first to the third trimester. In the former, it is similar to that of non-pregnant women, but the progression of the pregnancy is characterized by individual richness reduction and increased presence of members of Actinobacteria and Proteobacteria. Conversely, in the third trimester levels of butyrate-producing *Faecalibacterium* decrease significantly, coupling with between-subject diversity, weight gain, insulin insensitivity, and increased levels of fecal cytokines.

Several changes observed in the third trimester are similar to metabolic syndrome ones, but in the context of pregnancy they have positive and essential effects, contributing to healthy fetal development by fat accumulation and fetus nutrition. Moreover, as pregnancy advances, nearly 70% of women goes through an increase of the abundance of bacteria associated with inflammatory states. The greatest change is the increase in the ratio of *Firmicutes* to *Bacteroidetes*, mimicking that observed in obesity. In the context of a normal pregnancy, also these changes appear to be required.

Also vaginal microbiome undergoes significant changes during pregnancy. In general, vaginal microbiota confers protection against infection; during pregnancy its overall diversity decreases, whereas its stability increases. *Lactobacillus* species increase in association with vaginal pH. However, vaginal microbiota composition varies according to gestational age, resembling those of non-pregnant women at the later stages, and to ethnic group.

Differences in post-partum vaginal microbiota composition have been identified too, with vaginal community more similar to gut one, a gradual depletion of *Lactobacillus* species, increased diversity and enrichment of bacteria associated with vaginosis.

Finally, oral microbiota changes too. Levels of the pathogenic bacteria *Prophyromonas gingivalis* in the subgingival plaque are significantly higher in early and middle pregnancy, along with A*ggregatibacter actinomycetemcomitans* ones, whose elevation seems to persist also in the third trimester. Also *Candida* levels are significantly higher during middle and late pregnancy. This demonstrate a higher prevalence of periodontitis-associated pathogens in pregnancy.

It is always important to distinguish healthy microbial changes during pregnancy from undesired ones, which may be linked to complications such as preterm birth, associated with higher abundance of *Gardnerella* and *Ureaplasma*, lower abundance of *Lactobacillus, Candida albicans* colonization, and higher diversity in vaginal microbiota at early pregnancy stages.

The presence of gut microbes in amniotic fluid of women who experienced preterm premature rupture of membrane suggests that gut microbiome could play a role in intrauterine infections; proposed mechanisms include inflammation induction by LPS, ascending inoculation through the vagina, and leaky gut-associated spread of microbes through blood. However, maintaining an healthy microbiota during pregnancy is important also because the gut microbiota of vaginally delivered infants is first colonized by bacteria from maternal vagina and gut. It has been suggested that disruption of such transmission via C-section can have long-term medical consequences, such as obesity, asthma and celiac disease development. Moreover, *Bacteroides*, more abundant after vaginal delivery, influence the maturity of the immune system.

Along with mode of delivery, the other pivotal factor shaping gut microbiota is feeding (breast milk or formula). In fact, microbiome matures during the first year of life; at the same time, microbial human milk composition changes. It contains bacteria too; in the colostrum they include *Weisella, Leuconostoc, Staphylococcus, Streptococcus,* and *Lactococcus*, whereas 1-6 months post-partum milk contains oral cavity related microbes such as *Veillonella, Leptotrichia*, and *Prevotella*. *Bifidobacteria* are the most abundant microbes in breast-fed infant gut, whereas *Enterococci* and *Clostridia* predominate in formula-fed infants gut; moreover, breast-fed infant gut microbiota is more diverse.

The source of milk microbes is considered to be from the gut; in fact, progesterone may increase gut permeability, allowing microbes translocation to mammary

gland through blood.

Factors potentially modifying gut microbiota during pregnancy include diet; fiber fermentation, for example, increases SCFAs production, positively influencing maternal weight gain, glucose metabolism and metabolic hormones levels. Moreover, dramatic hormonal shifts associated with pregnancy result in inflammatory and immune changes that alter gut function and gut microbiota composition. In particular, estrogen and progesterone affect bacterial metabolism and growth and virulence of pathogenic bacteria, such as *Listeria monocytogenes*. Finally, also stress can affect dysbiosis.

Prebiotics, probiotics, dietary fiber and fermented food rich in probiotics (such as yogurt) can help improving metabolic parameters in pregnant and lactating women.

AGING AND GUT MICROBIOTA

Aging is a process leading to a generalized physiological functions decline. This process depends on individual characteristics such as ethnicity, is genetically determined, and is modulated by environmental factors, including lifestyle, diet, medications use and changes in gut microbiota.

With regard to microbiota variations, they are associated with enteric nervous system degeneration, intestinal motility alteration and mucosal barrier disfunction-mediated defense function reduction. There is an overall decrease in microbiota capability to ferment carbohydrates, whereas its capability to ferment proteins increases. Changes can involve both the composition and the stability of gut microbiota.

Normally, *Firmicutes* and *Bacteriodetes* are the most represented bacterial phyla in human gut microbiota, with *Firmicutes* as the predominant microorganisms in adults. In the elderly the ratio is inverted; *Bacteriodetes* predominate, and the relative proportion of *Firmicutes* subgroups changes. Moreover, microbiota diversity decreases along with the abundance of species producing butyrate, that modulates the immune response by regulating inflammation mediators such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, nitric oxide (NO) and IL-10; among butyrate-producing reduced species is *Faecalibacterium prausnitzii,* a microbe protective against gut inflammation. Bifidobacteria decrease too, whereas levels of *Akkermansia muciniphila*, a mucin-degrading bacterium, increase. Finally, in centenarians it is possible to observe the enrichment in potential pathogens, particularly in *Proteobacteria*. All these changes are also associated with proinflammatory IL-6 and IL-8 blood concentrations.

Some gut microbiota changes, including the decrease of some butyrate-producing microbes and the increase of inflammation-associated species such as *Escherichia coli*, are influenced by external factors less than others; such changes may represent the core features of elderly gut microbiota and may be at least in part linked to the chronic activation of the immune system due to immunosenescence. Nevertheless, gut microbiota composition is also influenced by lifestyle factors such as diet.

First, a less diverse diet is linked to reduced gut microbiota diversity, and reduced diversity correlates with increased frailty, inflammatory markers and impaired health parameters. Second, diet drastic changes often occur with aging resulting in the increase of sugars and fat rich foods and in the reduction of the intake of plant origin foods, and decreased consumption of healthy foods is associated with gut microbiota composition. Altered immune response to foods that promote inflammation may exacerbate microbial changes, and dietary factors altering microbiome may exacerbate inflammation and altered immunity, leading to a two-way link between immunosenescence and dysbiosis.

Other factors that modulate gut microbiota in the elderly are medications, particularly antibiotics. This drugs influence gut microbiota along with residence location; beneficial microbes such as bifidobacteria and lactobacilli are more abundant, respectively, in community-dwelling and short staying-hospital individuals.

Finally, intestinal permeability can increase with age. Epithelial barrier physical alterations are associated with increased IL-6 concentrations and may play a role in dysbiosis and inflammaging, the low-grade proinflammatory state characteristic of aging associated with the expression of proinflammatory cytokines such as IL-6 and TNF-α.

During advanced age the ability to resolve inflammation becomes impaired; this leads to the sustained presence of immunity cells (namely, leukocytes) in tissues, and to the chronic release of such proinflammatory molecules even in the absence of acute infection.

Gut microbiota is thought to have a role both in the induction and the maintenance of inflammaging, that has been hypothesized to predispose to the development of different age-related diseases. In fact, increasing levels of proinflammatory

cytokines, together with a marked reduction of immunological function induced by the low-grade inflammation state, negatively impact metabolism, bone density, strength, exercise tolerance, vascular system, cognitive function, and mood. Among others, pathologies associated with chronic low-grade inflammation include diabetes, cardiovascular diseases, osteoporosis, cancer and dementia.

Gut microbiota alterations can participate in this phenomenon in several ways. For example, the lack of *Akkermansia* and the increase of *Proteobacteria* is associated with the local and systemic inflammatory response, promoting small intestinal inflammation and systemic T cells activation. Some evidences suggest that gut microbiota-derived compounds in the bloodstream can activate specific white blood cells (macrophages) to an atherosclerosis-promoting proinflammatory state associated with cardiovascular diseases and vascular dementia development. Alteration of inflammatory cytokines and SCFAs production associated with gut microbiota changes can modulate central nervous system activity too (in particular microglia function and integrity), contributing to cognitive decline. Gut microbiota seems to also play a role in Alzheimer's disease; in fact, proinflammatory responses stimulated by LPS promote the production of beta-amyloid, the Alzheimer-associated peptide.

Conversely, preliminary studies conduced on mice demonstrate that probiotic oral administration may strengthen the intestinal barrier, reduce circulating LPS and proinflammatory cytokines, increase *Bacteriodetes* and reduce senescence markers both in the colon and the hippocampus, the brain area critical for learning and memory especially vulnerable to damage at Alzheimer's diseases early stages. Antimicrobial activity, enhancement of intestinal barrier function, and immunomodulation are among well-known actions of probiotics, and it has been demonstrated that probiotics exert beneficial effects on gut microflora composition and systemic immunity in the elderly.

Other microbiome-targeted interventions potentially leading to beneficial effects on age-related inflammation include caloric restriction, a mediterranean-style diet implementations, and the use of nutritional supplements containing polyphenol such as resveratrol.

Besides diet, physical activity may be helpful too. Few human studies have been published to date, but preliminary results on animal models suggest that aerobic exercise could enhance epithelial membrane integrity, increase microbial diversity, and attenuate intestinal inflammation.

GUT MICROBIOTA MODIFYING FACTORS

Factors able to modify gut microbiota include medications. Antibiotics are expected to have a large effect, but also other common medication can have an impact on gut microbiota composition.

Protonic pump inhibitors (PPIs) are for example associated with an higher *Streptococcaceae* and *Micrococcaceae* abundance and a lower microbiota diversity. Also paracetamol and opioids are associated with an increase of *Streptococcaceae*, whereas serotonin reuptake inhibitors (SSRIs) are negatively associated with *Turicibacteraceae*. Or, again, inhaled anticholinergic are negatively associated with *Ruminococcaceae* and *Peptococcaceae* abundance and microbiota diversity.

Diet is another major regulator of gut microbiota structure and function. Its contribution to microbiota modulation and host-microbiota crosstalk is evident from the beginning of life, with human milk oligosaccharides participating in microbiota development and solid food introduction increasing bacterial richness, whereas in the elderly, where food diversity is reduced, microbiota richness decreases too.

Some nutrients (such as glycans, quinones, and flavonoids) directly interact with bacteria, promoting or inhibiting their growth. Moreover, diet-derived compounds can indirectly shape gut microbiota by affecting host metabolism and immune system; vitamin D, for example, is associated with a decrease in circulatory levels of LPS, decreased abundance of *Coprococcus* and *Bifidobacterium* and increased abundance of *Prevotella*, and dietary constituents (such as selected emulsifiers) might disrupt the intestinal barrier.

Also herbs like ginseng can modulate gut microbiota; its administration has been associated with the decrease in bacteria possibly promoting tumorigenesis (such as *Bacteroidales* and *Verrucomicrobia*) and the increase in bacteria possibly exerting antiinflammatory and anticancer activity (such as *Firmicutes*).

Carbohydrate restriction and diets rich in fiber and vegetables are associated with health benefits due at least in part to gut microbiota changes. High amounts of plant polysaccharides in the diet are associated with a low abundance of *Firmicutes* and a high abundance of *Bacteroidetes* (in particular *Prevotella*), whereas a paucity of dietary fiber is associated with the increase of *Enterobacteriaceae* (in particular *Shigella* and *Escherichia*). The absence of dietary fiber is instead associated with an

increase in mucus-degarding bacteria (*Akkermansia muciniphila* and *Bacteroides caccae*) at the expense of fiber-degrading species (*Bacteroides ovatus* and *Eubacterium rectale*).

That is why microbiota is responsive to some dietary interventions; for example, in overweight and obese people the consumption of fruits, vegetables and fish is associated with microbiome richness. And that is why the intake of prebiotics, probiotics, and synbiotics (the latter combining prebiotics with probiotics) has long been proposed as a way of modifying metabolic disorders largely depended upon altered microbiota composition. Applying personally tailored diets is associated with shifts in gut microbiota composition after only 1 week of intervention.

Prebiotics are substances enhancing gut bacteria growth or activity. Among them are non-digestible dietary fiber (present in many fruits and vegetables) that are fermented by gut bacteria into SCFAs, and phytoestrogens (present, for example, in some berries). SCFAs, especially butyrate, help maintaining intestinal immune homeostasis and protecting from inflammation and carcinogenesis. Moreover, fiber seems to promote intestinal barrier function and improve glucose tolerance.

Prebiotics intake can significantly reduce body weight, body fat percentage, and desire for high-calorie foods; moreover it can improve insulin sensitivity, low-grade chronic inflammation and lipid metabolism. They can be administered in form of fermetable dietary fiber such as inulin, oligofructose, fructooligosaccharides or galactooligosaccharides, that can increase the abundance of bifidobacteria and lactobacilli, but not only; among others, also conjugated linoleic acid and milk sphingomyelin exert prebiotic activities.

Probiotics are instead live bacteria providing health benefits when consumed. They include lactobacilli and bifidobacteria, and can be easily found both in fermented foods (such as yogurt and kefir) and in food supplements.

Dietary supplements with bacterial strains aim at replenishing the gut with healthy commensal bacteria granting favorable metabolic properties. They exert several and sometimes very different beneficial effect. In some cases they can help reducing intestinal pain, blowing or tensions, or attenuating the immune response associated with acute colitis symptoms. Moreover it seems that probiotics can help improving the intestinal barrier and inhibiting pathogens growth. Moreover, given the central role played by gut microbiota in the brain-gut axis, probiotics can help also in case of psychological diseases; in particular, they can reduce stress-associated visceral hypersensitivity, and lactobacilli and bifidobacteria can help fight anxiety and depression.

Strain mixtures might be more effective than some single-strain preparations.

ANALYZING GUT AND VAGINAL MICROBIOTA: WHY, WHEN AND HOW

Microbiota is thus a key player in maintaining health. Understanding its role has been allowing better disease prevention, diagnosis, and treatment. For example, most healthy gut microbiota are resistant to invasion; conversely, gut microbiota vulnerability (that is the inverse of robustness) makes pathogens invasion simpler. Thus, having a healthy, robust gut microbiota could help preventing pathogens thrive. Or, again, having a healthy, robust gut microbiota is desirable when antibiotics treatments are needed.

Specific microbiota composition are associated with better health conditions. Unravelling individual profiles, microbiota analysis helps promoting a good health status and preventing intestinal or systemic diseases acting on microbiota composition.

Microbiota analysis can be useful:

. in case of intestinal diseases (colitis, recurrent diarrhea, constipation, flatulence, bowel irregularity), to verify gut microbiota involvement and select the best treatment;

. in case of urinary diseases or genital infections (cystitis, urethritis, vaginitis, candidiasis), to verify the involvement of gut or vaginal microbiota and select a treatment effective in preventing recurrence;

. during pregnancy and breastfeeding, to provide the mother and the baby with a good microbiota;

. at menopause, to face metabolism and physiology changes with a healthy microbiota profile;

. in the event of risk factors for intestinal or systemic diseases, to allow prevention;

. to monitor the effectiveness of nutritional or pharmacological treatment aimed at correcting imbalances or improving microbiota composition;

. to protect health and wellbeing throughout life, from infancy to old age, and to develop individualized food plans aimed at correcting imbalances or improving microbiota composition.

First studies on microbes – including those living in the gut – predominantly focused on single species cultured in the laboratory. The first microbial genome sequenced (that of *Haemophilus influenzae*) was published in 1995. However, the vast majority

of microbes – including bacteria living in the gut – cannot be cultured in the laboratory, and therefore cannot be studied with microbiology classical methods. Moreover, culturing favors the selection of microbes best able to thrive under laboratory conditions, and not necessarily the dominant or the most influential one in the gut, and in nature many microbes functions as multicellular – and, often, multispecies – entities, interacting and communicating in complex ways.

Nowadays we are in a new era, called metagenomics, in which the power of genomics (the study of the entire genetic material of an organism), bioinformatics and system biology are combined to analyze the entire community of gut microbiota, bypassing individual microbes isolation and culture. Metagenomics transcends the individual microbe, focusing on genes and their reciprocal influence in the gut microbiota community. New tools enable to study gut microbes in the complex community where they actually live, analyzing the genome of many microorganisms simultaneously. This allows to understand what they are capable of, how they work, and the alterations of gut microbiota that could lead to health problems.

The first truly metagenomics survey of human gut microbiota appeared in 2006, and the first catalog of human microbiota bacterial genome, comprising 178 references, was published in 2010 by the Human Microbiome Project; until 2017, 437 gut microbiota genomes were sequenced. However, more than half of the sequences obtained from the analysis of a human gut microbioma cannot be mapped to existing bacterial reference genomes.

In 2019 a reference catalog of 1,520 nonredundant, high-quality draft bacterial genomes of human gut bacteria isolated using different culturing conditions (the Culturable Genome Reference) deposited in the China National GeneBank (CNGB) improved the mapping rate of selected metagenomics datasets to over 70%.

The first step of a gut microbiota metagenomics study is DNA extraction from stool samples. The following DNA sequencing can capture a massive amount of information on gut microbioma; however, to study microbiota composition and diversity it is possible to focus on so-called ribosomal (rRNA) phylotyping, a culture-independent method based on a database of more than 200,000 rRNA gene sequences.

rRNAs are essential components of cellular protein-making engines, ribosomes. All organisms, including bacteria, have rRNAs different enough to be distinguished one from another. That's why the analysis of rRNA sequences in a stool sample allows to identify gut microbiota composition.

Focusing only on one gene, rRNA phylotyping represents a useful preliminary step providing assessment of gut microbiota diversity.

MICROBALANCE BY BIOSCIENCE INSTITUTE

In this scenario Bioscience Institute presents MicroBalance, a test based on the analysis of 16S rRNA gene, the DNA sequence encoding the rRNA of the smaller ribosome subunit of bacteria.

Individuals are provided with a kit for stool or vaginal fluid sampling and transport, that after sampling has to be sent to Bioscience's laboratories for bacterial DNA extraction and 16S rRNA gene library preparation.

16S rRNA gene is sequenced in Bioscience's laboratories by Next Generation Sequencing with Ion S5™ System (Thermo Fisher Scientific). Obtained sequences are subjected to bioinformatics analysis.

Sequencing output does not represent the diagnosis of a pathology, but a microbiota profile allowing medical doctors and qualified nutritionists to identify food plans and lifestyle adjustments aimed at correcting imbalances or improving microbiota composition.

REFERENCES

Al-Assal K et al. Gut microbiota and obesity. Clin Nutr Exp. 2018 Aug;20:60-64. doi: 10.1016/j.yclnex.2018.03.001

Belkaid Y and Hand T. Role of the Microbiota in Immunity and inflammation. Cell. 2014 March 27; 157(1): 121–141. doi:10.1016/j.cell.2014.03.011

Braak H et al. Staging of Alzheimer-related cortical destruction. Eur Neurol. 1993;33:403–408. doi: 10.1159/000116984

Buford TW. (Dis)Trust your gut: the gut microbiome in age-related inflammation, health, and disease. Microbiome (2017) 5:80. doi: 10.1186/s40168-017-0296-0

Castaner O et al. The Gut Microbiome Profile in Obesity: A Systematic Review. Int J Endocrinol. 2018 Mar 22;2018:4095789. doi: 10.1155/2018/4095789

Committee on Metagenomics: Challenges and Functional Applications – Board on Life Sciences Division on Earth and Life Studies. The new science of metagenomics – Revealing the Secrets of Our Microbial Planet. The National Academies Press, Washington, DC. 2007

Cresci GA and Bawden E. The Gut Microbiome: What we do and don't know. Nutr Clin Pract. 2015 Dec;30(6):734-46. doi: 10.1177/0884533615609899

Danneskiold-Samsøe NB et al. Interplay between food and gut microbiota in health and disease. Food Res Int. 2019 Jan;115:23-31. doi: 10.1016/j.foodres.2018.07.043

De Palma G et al. The microbiota-gut-brain axis in functional gastrointestinal disorders. Gut Microbes. 2014 May-Jun;5(3):419-29. doi: 10.4161/gmic.29417

Edwards SM et al. The Maternal Gut Microbiome During Pregnancy. MCN Am J Matern Child Nurs. 2017 Nov/Dec;42(6):310-317. doi: 10.1097/NMC.0000000000000372

Feehley T et al. Healthy infants harbor intestinal bacteria that protect against food allergy. Nat Med. 2019 Jan 14. doi: 10.1038/s41591-018-0324-z

Finucane TE. 'Urinary Tract Infection' and the Microbiome. Am J Med. 2017 Mar;130(3):e97-e98. doi: 10.1016/j.amjmed.2016.08.018

Foster JA et al. Stress & the gut-brain axis: Regulation by the microbiome. Neurobiol Stress. 2017 Mar 19;7:124-136. doi: 10.1016/j.ynstr.2017.03.001

Jackson MA et al. Gut microbiota associations with common diseases and prescription medications in a population-based cohort. Nat Commun. 2018 Jul 9;9(1):2655. doi: 10.1038/s41467-018-05184-7

Konturek PC et al. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. J Physiol Pharmacol. 2011 Dec;62(6):591-9
Mangiola Fet al. Gut microbiota and aging. Eur Rev Med Pharmacol Sci 2018; 22 (21): 7404-7413. doi: 10.26355/eurrev_201811_16280

Nielsen KL et al. Faecal Escherichia coli from patients with E. coli urinary tract infection and healthy controls who have never had a urinary tract infection. J Med Microbiol. 2014 Apr;63(Pt 4):582-9. doi: 10.1099/jmm.0.068783-0

Nuriel-Ohayon M et al. Microbial Changes during Pregnancy, Birth, and Infancy. Front Microbiol. 2016 Jul 14;7:1031. doi: 10.3389/fmicb.2016.01031

Pacelli S et al. Il microbiota umano: funzioni biologiche e interrelazioni con lo stile di vita
e alimentare. La rivista di scienza dell'alimentazione, numero 1, gennaio-aprile 2016, anno 45

Rea D et al. Microbiota effects on cancer: from risks to therapies. Oncotarget. 2018 Apr 3;9(25):17915-17927. doi: 10.18632/oncotarget.24681

Squire LR. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. Psychol Rev. 1992;99:195–231

Stapleton AE. The Vaginal Microbiota and Urinary Tract Infection. Microbiol Spectr. 2016 Dec;4(6). doi: 10.1128/microbiolspec.UTI-0025-2016

Sun L et al. Insights into the role of gut microbiota in obesity: pathogenesis, mechanisms, and therapeutic perspectives. Protein Cell. 2018 May;9(5):397-403. doi: 10.1007/s13238-018-0546-3

Valles-Colomer M et al. The neuroactive potential of the human gut microbiota in quality of life and depression. Nat Microbiol. 2019 Feb 4. doi: 10.1038/s41564-018-0337-x

Van de Wijgert JHHM and Jespers V. The global health impact of vaginal dysbiosis. Res Microbiol. 2017 Nov - Dec;168(9-10):859-864. doi: 10.1016/j.resmic.2017.02.003

Zhang YJ et al. Impacts of Gut Bacteria on Human Health and Diseases. Int J Mol Sci. 2015 Apr 2;16(4):7493-519. doi: 10.3390/ijms16047493

Zimmermann P et al. Association between the intestinal microbiota and allergic sensitization, eczema, and asthma: A systematic review. J Allergy Clin Immunol. 2019 Feb;143(2):467-485. doi: 10.1016/j.jaci.2018.09.025

Zmora N et al. You are what you eat: diet, health and the gut microbiota. Nat Rev Gastroenterol Hepatol. 2019 Jan;16(1):35-56. doi: 10.1038/s41575-018-0061-2

Zou Y et al. 1,520 reference genomes from cultivated human gut bacteria enable functional microbiome analyses. Nat Biotechnol. 2019 Feb;37(2):179-185. doi: 10.1038/s41587-018-0008-8

AZIENDA CERTIFICATA
UNI EN ISO 9001:2015

BIOSCIENCE
GENOMICS

Numero Verde
800 690 914

www.bioinst.com - info@bioinst.com

SAN MARINO
Strada Rovereta, 42
47891 Falciano RSM

MILANO
Ospedale San Raffaele DIBIT 1
Via Olgettina, 58 Milano - Italy

ROMA
Università di Roma Tor Vergata
Via Ricerca Scientifica, 1 Roma - Italy

DUBAI
Al Razi Building n.64 - Block B
Dubai HealthCare City - UAE

HONG KONG
Unit 802 8/F, No 15 - Science Park
West Avenue - Hong Kong