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## CONNECTIONS BETWEEN GUT MICROBIOTA AND BONE HEALTH

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***CONNECTIONS BETWEEN GUT MICROBIOTA AND BONE HEALTH.***

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## **"CONNECTIONS BETWEEN GUT MICROBIOTA AND BONE HEALTH"**

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**RUNNING TITLE:** bugs and bone

## **ABSTRACT**

The gut microbiota (GM)-host interactions contribute to the maturation of the host immune system, and modulates its systemic response. It is well documented that GM can interact with non-enteral cells such as immune cells, dendritic cells, and hepatocytes, producing molecules such as short-chain fatty acids, indole derivatives, polyamines, and secondary bile acid. The receptors for some of these molecules are expressed on immune cells, and modulate the differentiation of T effector and regulatory cells: this is the reason why dysbiosis is correlated with several autoimmune, metabolic, and neurodegenerative diseases. Due to the close interplay between immune and bone cells, GM has a central role in maintaining bone health and influences bone turnover and density. GM can improve bone health also increasing calcium absorption and modulating the production of gut serotonin, a molecule that interacts with bone cells and has been suggested to act as a bone mass regulator. Thus, GM manipulation by consumption of antibiotics, changes in dietary habits, and the use of pre- and probiotics may affect bone health.

## **KEY WORDS**

Gut microbiota, Bone, Osteoporosis, Immune system, Probiotics, Inflammation.

## **CONTEXT**

In physiological condition, GM relationship with host is complex and comprehends various forms of symbiotic relationship such as parasitic, commensal, and mutualistic. GM helps in food digestion, in fighting pathogens and, during the first years of post-natal life, contributes to the maturation of the host immune system. During the whole life, GM interacts with the host and contributes to the modulation of gut and systemic immunity. Immune homeostasis disruption is the causal mechanism of several chronic non-communicable human diseases (NCDs) such as allergy, asthma, some autoimmune, cardiovascular and metabolic diseases, and neurodegenerative disorders. These disorders are characterized by a low grade of inflammation. Although inflammation and the pathways to disease are multifactorial, the altered gut colonization patterns, associated with decreasing microbial diversity, are a central theme and are increasingly implicated

in the physiologic, immunologic, and metabolic deregulation seen in many NCDs. Altered GM-host interaction has been indicated as a possible cause of immune deregulation and increased inflammation associated with several NCDs (1,2).

## **MOLECULES AND PATHWAYS**

Products of microbial metabolism signal to the host and influence his/her metabolism, GM metabolize some products that are introduced with diet and are not absorbable by the host, these substrates (most commonly complex carbohydrates) are able to influence GM and GM-host interaction and are known as prebiotics. Prebiotics cannot be digested by human enzymes and are fermented in the colon by GM to yield energy for microbial growth and end products such as short-chain fatty acids (SCFAs), mainly acetate, propionate and butyrate.

Butyrate is particularly important as an energy substrate for cellular metabolism in the colonic epithelium, whereas acetate and propionate are taken up by the liver and used as substrates for lipogenesis and gluconeogenesis. SCFAs also affect proliferation, differentiation and modulation of gene expression in mammalian colonic epithelial cells (3,4). However, these effects have been attributed to butyrate acting as a potent histone deacetylase inhibitor and, as such, it may regulate the mammalian transcriptome.

It has been demonstrated that SCFAs have anti-inflammatory effects on intestinal mucosa, thus protecting the bowel from the development of inflammatory bowel disease (5-7). SCFAs signal to several non-enteral cell types through G-protein-coupled receptors and the signal between GM and immune system is fundamental to regulate the homeostasis and to maintain the balance between immune tolerance to commensals bacteria and immunity to pathogens.

It has also been suggested that, depending on the cytokines milieu, interaction between SCFAs and their receptors influences T cells differentiation not only toward T regulatory cells (Tregs), but also toward effector T cells. Furthermore, butyrate and propionate also modulate antigens presentation inhibiting the development of dendritic cells by gene expression regulation (8-11) and by interaction with SCFAs receptors (12,13)

Beyond SCFAs, GM produces other metabolites, such as indole derivatives and polyamines, from digested food that have important immunomodulatory function. These metabolites derive from dietary tryptophan and arginine, respectively, and have an indirect immune function. Indole derivatives favor the integrity of the enteral mucosa and the barrier defense toward pathogens by stimulating the production of anti-microbial peptides, mucins, and proliferation of intestinal goblet cells. Polyamines such as putrescine, spermidine, and spermine fulfill important roles in gene expression and proliferation. They enhance the development and maintenance of the intestinal mucosa and resident immune cells (2).

The primary bile acids are synthesized in the human liver from cholesterol, and are important for ensuring that cholesterol, dietary fats and fat-soluble vitamins from the small intestine are soluble and absorbable. Primary bile acids are conjugated to glycine in humans, and are taken up in the distal ileum for transport to the liver. However, bacteria in this part of the ileum deconjugate these bile acids, which then escape intestinal uptake and can be further metabolized by the GM into secondary bile acids. Bile acids are taken up from the gut and circulate throughout the body, acting as signaling molecules and binding to cellular receptors in peripheral organs, where they may contribute to overall host metabolism. An immunomodulating role has also been postulated for metabolized bile acids; however, physiological role of these metabolites in health and disease is still an open question (14).

Polysaccharide A is a bacterial product that influences T cells fate through its interaction with the toll-like receptor 2. Interacting with T cells, it favors immune tolerance by inhibiting T helper 17 differentiation and favoring Tregs activity (15).

In summary, GM influences T cells differentiation through the production of bacterial metabolites at least at the intestinal mucosa level and T cells differentiation through cognate bacterial antigens (16). The molecular pathways are summarized in **Figure 1**.

## **IN VITRO AND IN VIVO MODELS**

Use of model systems with different levels of microbial complexity reveals how host genes impact the microbiome and how the microbiome regulates host genetic programs. Model organisms provide opportunities to study host–microbiome interplay with a level of experimental control that is not achievable in human studies (17). Model systems are also revealing roles for the microbiome in host physiology ranging from mate selection (18) to skeletal biology (19,20) and lipid metabolism (21,22).

With an ever-increasing number of human microbiome studies completed and under way, experimental systems employing model organisms will prove essential tools to interrogate and validate the associations identified between the human microbiome and disease (23,24).

Germ-free (GF) animals are reared in sterile isolators to control their exposure to microorganisms, including viruses, bacteria and eukaryotic parasites (24). If these animals are colonized with microbial communities of specific donors (human or other animal species) they become gnotobiotic and therefore allow analysis of the systemic impact of specific microorganisms on the xenograft recipient (25).

Being able to associate a specific function to a particular bacterial strain or species is of great importance considering the possibility that specific organisms could be used as a treatment for a given disease.

Therefore, the beneficial impacts of a single bacterial species/strain on the host should be validated by taking into account the full microbial community context. Nevertheless, GF animals show a number of important physiological differences when compared to conventionally raised animals, for this reason data extrapolated from experiments carried out with GF animal models must be considered with caution.

Although different animal models (zebrafish, mice, rats and pigs) have been developed, mice represent the most widely used and best characterized model organisms: in fact, 99%

of mouse genes are shared with humans at the host genetic level, and they share key similarities with the human gut microbiome, making them a powerful model system for evaluating host–microbiota interactions applicable to human biology (17,26).

Mouse genetics, the availability of strains and collections of knockout, knock-in and transgenic mutants, make mice a useful model system for studying the role of host genetics in host–microbiota interactions. However, in some cases, confounding factors have made drawing conclusions from the impact of mouse genetics on the microbiome far from clear: the point is to highlight that studying host–microbiota interactions in mice requires careful experimental design (17).

Furthermore, they often do not yield reliable preclinical results that readily translate into effective human treatments. Two important factors contribute to this failure: bacterial species that colonize the gastrointestinal tract appear to be host-dependent, so a specific microbiota is critical for a given host (27); and the immune responses in non-human mammalian species is often distinct from those seen in human (28,29)

### **GM, IMMUNE SYSTEM AND BONE LOSS**

The interaction between immune system and GM has a central role in the maturation of immune system during the early post-natal period (2,30) and a role in the modulation of immune system and response to self-antigens during the whole life (30,31); thus it has been suggested that dysbiosis may play a role in the development of diseases characterized by immune deregulation such as allergies, autoimmune, and inflammatory disorders.

The role of GM in the development and maturation of host immune system in the early post-natal life has been demonstrated in GF mice: the use of this experimental model has shown that the absence of GM negatively influences the formation of lymphoid organs; in particular, GF mice have defective formation of the spleen and mesenteric lymph nodes, the intestinal Peyer's patches are smaller, and display a reduced number of CD4+ T cells and reduced production of IgA (23,32-36). Also isolated lymphoid follicle and cryptopatches are reduced in GF mice (37,38).

As regards, immune cells of different GM phyla were associated with the development of different T helper (Th) phenotypes: in fact, GF mice have imbalance in T helper cells, reduced Treg, absence of Th17 cells, and altered ratio between Th1 and Th2 with increased Th2 response (39).

Arthritic phenotypes are restored when GF animals are colonized with segmented filamentous bacteria, which enhance the differentiation and function of Th17 cells; also colonization of GF animals with *Bacteroides fragilis* restores a correct balance between Th1 and Th2 cells and redirects lymphoid organogenesis (35). Resident bacteria, such as segmented filamentous bacteria and in particular some Clostridia-related species, have been associated to Th cells development and to Tregs cells induction (40,41).

The majority of the evidences thus suggest that GM metabolites and antigens may influence immune regulation and hence dysbiosis may be the environmental factor responsible for some immune and inflammatory disorders, both at gut level such as inflammatory bowel disease (42) and outside the gut such as Rheumatoid Arthritis (43), type 1 diabetes (44), and asthma (45). However organs distant from gut, skin, and lung are not in direct contact with GM. This implies that GM has the ability to communicate with the host immune system in distant organs as well as in the gut. These signals have been identified in GM-derived products and also in circulating antibodies or immune cells (46).

Osteoporosis increases dramatically the risk of fractures: major osteoporotic fractures are a social and economic burden. In developed countries, the lifetime risk for osteoporotic fractures at the wrist, hip, or spine is 30–40%, very close to that for coronary heart disease. The number of new fractures in 2010 in the EU was estimated at 3,5 million, comprising approximately 620,000 hip fractures; 520,000 vertebral fractures; 560,000 forearm fractures; and 1,800,000 other fractures (47). Osteoporotic fractures impair patients' quality of life and increase mortality: 20% of elderly patients suffering from femoral fractures will die within a year, and 50% of the survivors will lose independence. The most frequent cause of bone loss is postmenopausal osteoporosis (PMO) that is driven by estrogen deficiency at menopause. In PMO, there is an imbalance in bone turnover with

increased bone resorption and reduced bone formation. It has been demonstrated both in experimental models and in humans that estrogen deficiency affects bone cells number and activation and bone turnover partially through its effect on immune system (48). During estrogen deficiency, T cells increase their production of pro-inflammatory and pro-osteoclastogenic cytokines, such as TNF alpha and RANKL (49); however, the reasons of this increased activity in osteoporotic women and not in non-osteoporotic subject are unknown.

GM may be involved in the mechanism of PMO. It has been suggested that the absence of GM influences bone mass: the majority of the findings demonstrate that GF mice have increased bone mass and report an acute effect of GF colonization with GM obtained from conventionally raised mice on reduction of bone mass due to increased bone resorption, whereas the long-term colonization resulted in a net skeletal growth in young animals (50). Studies on mice treated with broad spectrum antibiotics to alter GM bring to different conclusions regarding the effect on bone density. These discrepancies are possibly due to differences in animal age, sex, and protocols applied for antibiotic treatment (19,50-53). However, the majority of the reports suggest that antibiotic-treated mice have increased bone density (19,53,54) and also best bone mechanical properties (54) than conventionally raised mice.

It is possible to induce a pharmacologically bone loss condition in mice with the GnRH agonists leuprolide and to investigate the role of GM in bone loss induced by sex steroid deficiency (55). These studies demonstrated that GM plays an important role in sex steroid deficiency-induced osteoporosis: GF mice are protected against osteoporosis and the increase in bone turnover induced by sex steroid deprivation thanks to the lack of increase in TNF, RANKL, and IL-17. Furthermore, sex steroid depletion augments inflammation in the intestine by increasing gut permeability to bacterial antigens, namely, by decreasing the expression of modulators of intestinal barrier integrity (56,57,58).

In humans, scarce data support results obtained in mice.

Relationship between GM, immune system and bone in PMO are summarized in **Figure 2**.

## **GM AND BONE HEALTH BEYOND IMMUNE SYSTEM**

It has been suggested that GM composition and manipulation may affect bone health beyond immune system by influencing calcium absorption and the production of gut derived serotonin.

A post hoc analyses on the use of *Lactobacillus reuteri* demonstrated that the use of this probiotic in healthy subject increases the level of serum 25OH vitamin D that influences calcium absorption and benefits bone health. The mechanism through which this probiotic influences vitamin D level is not clear; however, this may be due to a modification in the gut environment that specifically favors vitamin D absorption or to indirect effect on increased hepatic 25-hydroxylase activity or 7-dehydrocholesterol concentration due to reduced absorption of dietary and biliary cholesterol (2,59). On the other hand, the relation between GM and vitamin D may also be inverse as it has been proposed that decreased vitamin D intake is associated with different GM profiles (60,61). Another possible mechanism through which GM benefits bone health is the increase in calcium absorption. It is well known that maintaining a positive calcium balance is important in achieving a good peak of bone mass that protects from the development of osteoporosis in older age (62,63). Dietary intake of fibers influences calcium absorption: after being fermented by GM, fibers improve calcium absorption by reduction of gut pH, thus reducing the formation of calcium phosphates and increasing the calcium absorption and by increasing the production of SCFAs such as butyrate (64). The effect of SCFAs may be more complex than the effect on gut pH, and in fact it has been demonstrated that SCFAs increase calcium transport through signaling pathway modulation (65). As previously said SCFAs influence bone health also through immune system modulation; hence dietary fiber intake may be responsible for a healthier immune system and reduced inflammation. In fact, there is a general consensus recognizing that an adequate dietary fiber intake is associated with lower risk of chronic diseases such as cardiovascular disease (66). Another possible mechanism through which GM influences bone health is mediated by its effect on the production of gut serotonin (5HT). In recent past, a dual effect of serotonin in the regulation of bone mass has been described depending on the site of production of this molecule (67): a role as a bone mass regulator

was proposed to gut-derived 5HT (g5HT), which is influenced by GM. Enterochromaffin cells of the duodenum are responsible for the synthesis of g5HT that is partially modulated by GM as SCFAs increase the synthesis of g5HT (68,69). It has been shown that 5HT interacts with bone cells and, in particular, decreases osteoblast proliferation; these observations suggest that regulation of g5HT by GM may be a potential therapeutic strategy to improve bone health.

Indeed, in animal models of ovariectomy-induced bone loss, pharmacological inhibition of g5HT synthesis results in the prevention of osteoporosis mediated by increased bone formation (70). However, data on the effect of 5HT on bone health are quite controversial.

Relationship between GM, and bone beyond immune system are summarized in **Figure 3**.

### **GM MANIPULATION AND BONE HEALTH**

GM composition may be manipulated in several ways such as the use of broad spectrum antibiotics, change in dietary habits and, more easily, by the use of prebiotics and probiotics, change in GM composition may affect bone health.

**Prebiotics** have been defined as “non-digestible food ingredients that benefit the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon” (71). Prebiotics are complex carbohydrates and fibers that influence composition and/or activity of GM in a way that favors host health.

To be classified as a prebiotic, a substance should meet these criteria: be resistant to low gastric pH, hydrolyzed by mammalian digestive enzymes, and not be absorbable by humans, be fermented by GM, and stimulate the growth and activity of gastro intestinal tract (72).

Dietary carbohydrates including resistant starches, non-starch polysaccharides and oligosaccharides can reach the large intestine directly by escaping the digestion of host enzymes and act as the major substrates for the growth of gut bacteria with specific carbohydrate enzymes (73,74). Fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are two well-known dietary prebiotics, which are widely used for improving metabolic disorders (75,76). Moreover, improvement of gut barrier function also contributed to the beneficial effects of FOS (77).

Prebiotic supplementation in animal models favors the proliferation of Bifidobacteria and increases SCFAs production. As regards the effect of prebiotics on bone health, some experimental studies showed that they improved calcium absorption and bone density in animal models (78,79). In humans, the supplementation with different prebiotics in adolescent girls improved calcium absorption and bone density (80,81). Recently, the corn derived non-digestible carbohydrate, soluble corn fiber (SCF), has been evaluated for its ability to increase calcium absorption and improve bone health in humans. In particular, SCF administration enhances calcium absorption and its consumption is associated with a favorable change in GM, namely, increased presence of Bacteroidetes and Firmicutes known to ferment starch and fiber (82,83).

Firmicutes are positively correlated with calcium absorption, suggesting that the role of GM in calcium absorption is complex due to different species (82).

Prebiotic fiber may influence bone metabolism both by the change in the composition of GM-favoring microbes with higher anti-inflammatory potential and by increasing SCFAs production thus increasing calcium absorption. It has also been suggested that prebiotics could have direct effect on immune system modulation and an anti-pathogen effect regardless to their effect on GM (84). However, until now, in human studies on prebiotics only calcium absorption, markers of bone metabolism, and bone density have been investigated, whereas immune phenotype and inflammation have not been investigated, **Table 1** summarizes the results obtained in humans with different prebiotics.

As more and more beneficial intestinal bacteria are being found with the application of advanced sequencing techniques, the definition of prebiotics should be more specific, for instance, which phyla are promoted by the probiotics and possible clinical application, in the near future new prebiotics are expected to be developed (73).

GM may be manipulated also by the administration of **probiotics**.

According to the definition from the Food and Agriculture Organization of the United Nations and the World Health Organization, probiotics are “*live microorganisms which provide a health benefit on the host when administered in adequate amounts*” (73,85)

The majority of experimental data produced in mice demonstrated that modulation of GM by the use of probiotics is able to increase bone mass and reduce sex steroid associated bone loss (55,86-88). Probiotics used were different in different studies, both a single strain or a mixture of strains. The most used were Lactobacilli spp. that were demonstrated to have the higher anti-inflammatory and bone protective effect. Also some data on the use of yogurt that contains different probiotics, but is also a source of calcium and proteins that are fundamental for bone health, have been produced (89). All these studies showed a protective effect of probiotic yogurt on bone health. Moreover, it has been demonstrated that dairy products consumption in early life led to a higher peak bone mass (90). Also in adults older than 60 years, consumption of dairy products was associated to increased bone density and lower risk of osteoporosis (91-94). The use of probiotics has been proposed also as an adjuvant treatment in focal bone loss such as alveolar erosion in periodontitis. The ability of different Lactobacilli strains in reducing osteoclast number, alveolar erosions, and tooth movement in rat and mice has been demonstrated (95-97). In humans, a recent meta-analysis concludes that current evidences suggest a possible use of probiotics as an adjuvant therapy in gingivitis and periodontitis (98). In a geriatric population, the administration of Lactobacillus helveticus increases serum calcium (99). Also in osteopenic women, the administration of a multispecies probiotic (6 different species) increases markers of bone formation, decreases TNF alpha level, but has no effect on bone density during a 6-month period (100).

With the development of high-throughput sequencing, more and more bacteria with probiotic activity will be found, but how to obtain specific conditions to culture them is still a big challenge. However, it is still not clear whether these novel probiotics are absolutely safe to human health. Thus, future works focusing on the understanding of the microbe-host relationships are warranted.

Gnotobiotic animals are valuable tools used to evaluate the physiological functions of each probiotic strain (73).

Also the use of **antibiotics** is known to have significant effects on the intestinal microbiota.

The use of high-throughput metabolomics has shed light on the interactions between the intestinal microbiota, the host and the use of drugs, showing that antibiotic treatment disrupts intestinal homeostasis and has a profound impact on the intestinal metabolome.

A single, high dose of the antibiotic streptomycin can have a profound impact on many crucial host metabolic functions: among some of the pathways affected are those involved in sugar, amino acid, fatty acid, bile acid, steroid, and eicosanoid metabolism. (101).

Thus, it is clear that antibiotics can have profound, previously unappreciated effects on human health, and that the effects of the indiscriminate use of antibiotics extend beyond the development of microbial drug resistance (101).

In turn, GM may be an important environmental factor that influences the inter-individual variations in drug efficiency.

## **DIAGNOSTIC IMPLICATIONS**

A new frontier in medicine is to achieve personalized approaches to monitor, diagnose, and treat the patients according to their genetic and phenotype traits.

Related to this, it is of great interest to individuate samples for a "personalized proteome": the advanced proteome technologies have led to novel opportunities to identify biomarkers based on human microbiome studies and to use them for new clinical diagnostic tests based on these methods. However, it has still to overcome some challenges to reach routine clinical analysis: missing of a worldwide standardization in sample collection, bio-banking, processing, and clinical validation and the high intra- and inter-subject variabilities between samples (102).

To establish causality in the deterioration of the host–microbiota relationship is of great importance; however, discovery of candidate molecules and pathways that underline mechanisms remains a major challenge. Several approaches have been used to achieve an insight into host

responses to the microbiota, such as transcriptional assays, cytokine panels and imaging analyses, applied to animals.

Furthermore, measuring individual's responses requires tissue samples obtained through invasive procedures, thus leading many difficulties to obtain samples, particularly for the purpose of monitoring patient's response to therapeutic interventions (103).

For this reason, stool is the eligible sample for measuring host responses, as it is acquired non-invasively and contains molecules of both host and microbial origin; these molecules can directly describe the gut ecosystem without confounding with non-gastrointestinal contributions, as is the case in other samples such as blood. Host proteins have a key role in the dynamics of host-microbial interactions and are conveniently measured from feces (103,104).

Moreover, stool can provide additional data from previously conducted experiments, as proteins in frozen fecal specimens are available to be further analyzed (103).

Currently, PCR-based assays offer a commercial available test that provides immediate clinical information of gut health. They routinely measure the commensal bacteria in stool, which reveal the composition, diversity, and relative abundance of a key set of clinically relevant genera/species (102).

Also metabolome changes could predict disease, as shown by the data from a pilot study comparing patients bearing colonic diverticula with controls (105). Metabolomics offers a comprehensive qualitative and quantitative overview of the metabolites present in a biological system and could help identifying clinically relevant biomarkers associated with diseases (106).

In conclusion, the combination of microbial community, metabolite, host protein analyses from stool and the use of gnotobiotic animal models, will allow to individuate the proteins that help maintaining balance within this complex ecosystem and those that perturb it.

A multi-dimensional definition of GM state will allow to isolate patients, set up individualized treatment and monitor the disease progression and recovery.

These approaches also provide a first step to the discovery of diseases-related biomarkers: one or a few proteins that individuate "signatures" in patients and could provide a simple tool for diagnosing a spectrum of gastrointestinal diseases. Such markers may be helpful to understand the disease mechanism and to direct pharmaceutical development (103).

### **FUTURE PERSPECTIVES**

Due to the great increase of studies raised in the past few years, we have accessed to a deeper knowledge on GM and its relationship to the host, during health and diseases as well as during whole life course.

The great amount of information will allow to detect potential targets for therapeutical strategies and to manipulate the GM through diet (use of functional food), pre- and probiotics intake and administration of antibiotics directed to specific microbial species.

As we know the mechanisms by which the GM contributes to pathologies we could set up an "intelligent modulation" of the GM and to precisely determine its effects on the intestinal community and the host, thus having the possibility to be extremely beneficial for human health (84,107).

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## **LIST OF ACRONYMS AND ABBREVIATIONS**

GM: gut microbiota

NCDs: non-communicable human diseases

SCFAs: short-chain fatty acids

Tregs: T regulatory cells

Th: T helper cells

GF: germ free

PMO: postmenopausal osteoporosis

5HT/g5HT: serotonin/gut-derived serotonin

FOS: fructooligosaccharides

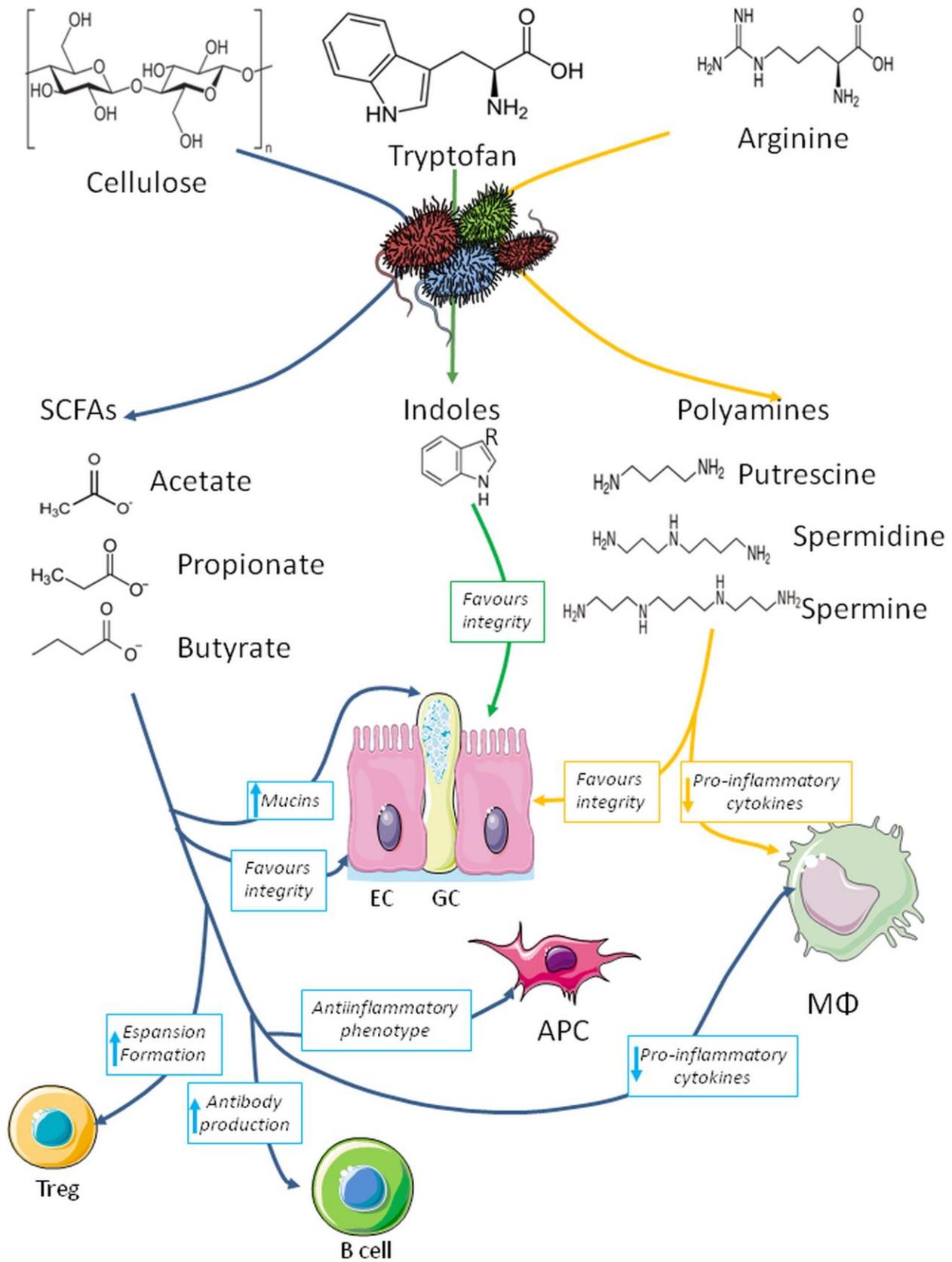
GOS: galactooligosaccharides

SCF: soluble corn fiber

## **FIGURE LEGENDS**

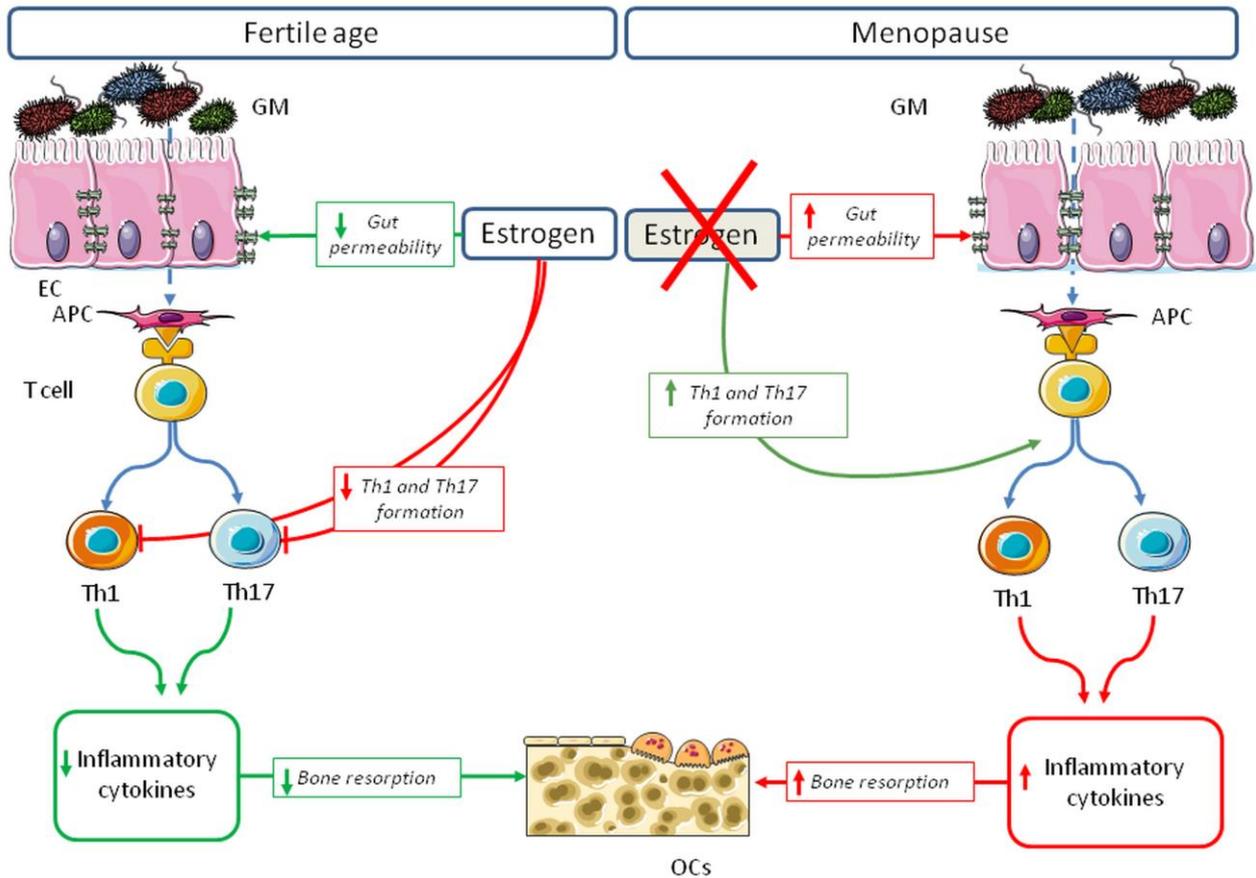
**Figure 1.** The cartoon shows gut microbiota metabolites and their effects on bone cells.

Abbreviations used: short-chain fatty acids (SCFAs); enteral cells (EC); goblet cells (GC); antigen presenting cells (APC); macrophages (MØ); T regulatory cells (Treg).



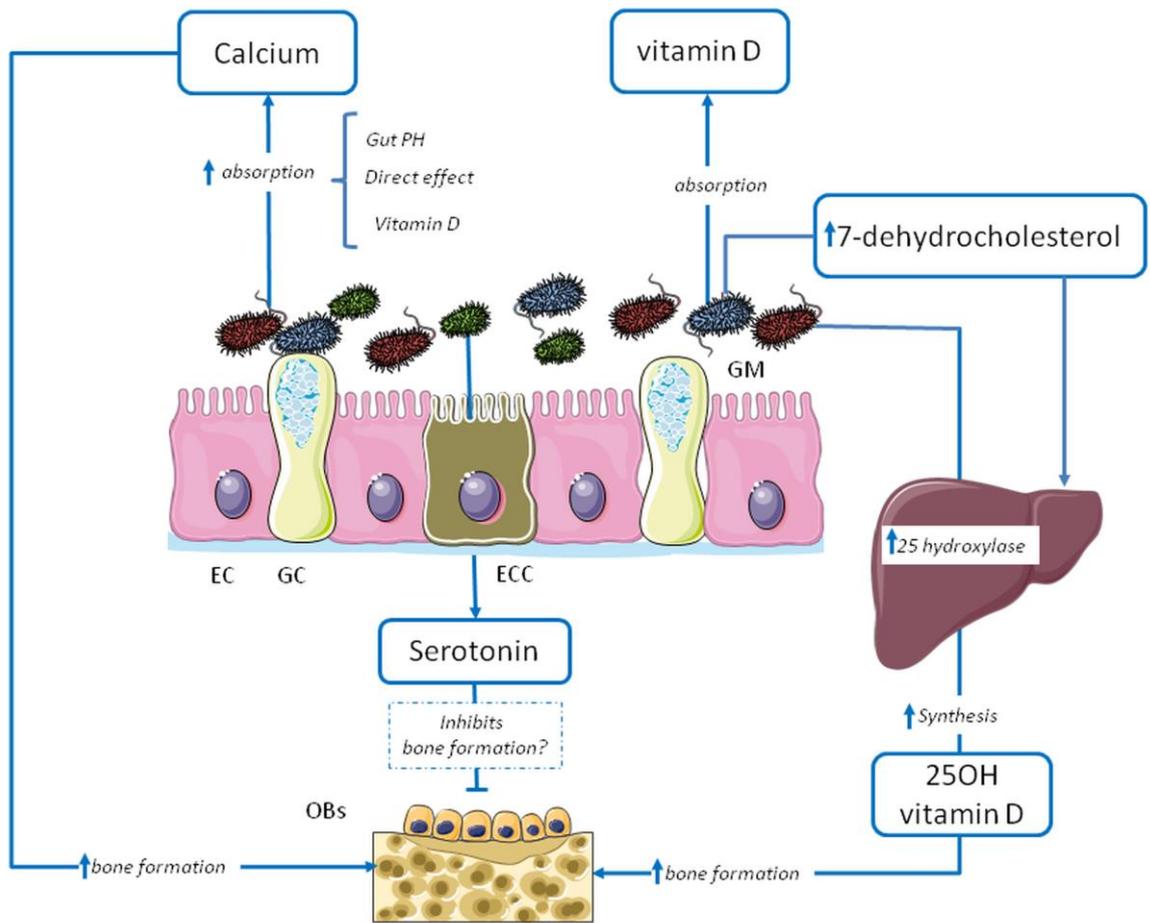
**Figure 2.** The cartoon shows the relationships between immune system, estrogen deficiency-bone loss and gut microbiota: enteral barrier integrity, cytokine production, immune and bone cells are involved.

Abbreviations used: gut microbiota (GM); enteral cells (EC); antigen presenting cells (APC); T regulatory cells (Treg); T helper-1 cells (Th1); T helper-17 cells (Th17); osteoclasts (OCs).



**Figure 3.** The cartoon shows the link between gut microbiota and bone turnover beyond immune system.

Abbreviations used: gut microbiota (GM), enteral cells (EC), enterochromaffin cells (ECC), goblet cells (GC); osteoblasts (OBs).



**Table 1.**

<b>Subjects recruited</b>	<b>Number of subjects</b>	<b>Pre-biotic used</b>	<b>Dose and time</b>	<b>Out-comes</b>	<b>Reference</b>
Adolescents	50 males 50 females	Oligofructanes, long-chain inuline	8 g/die 12 months	↑ Ca absorption ↑ Bone mineral density	81
Preadolescent	31 females	Galattoligosaccarides	5-10 g/die 3 weeks	↑ Ca absorption	80
Adolescents	15 males 9 females	Soluble corn fiber	12 g/die 3 weeks	↑ Ca absorption ↑ Bacteroides	83
Adolescent	28 females	Soluble corn fiber	10-20 g/die 4 weeks	↑ Ca absorption and bone markers ↑ Bacteroidetes, Firmicutes	82