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**MICROBIOME SHAPING AND PERSONALIZED  
PREVENTION IN IBD: THE ROLE OF S100B IN  
INFLAMMATION AND EUBIOSIS AS A PERSPECTIVE  
FOR HEALTH PROMOTION**

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## 1. Abbreviations

|       |                                 |
|-------|---------------------------------|
| AIEC  | adherent-invasive <i>E.Coli</i> |
| AMP   | adenosine monophosphate         |
| AMPK  | AMP-activated protein kinase    |
| APCs  | antigen presenting cells        |
| ASA   | aminosalicylates                |
| ASVs  | Amplicon sequence variants      |
| AZA   | azathioprine                    |
| BAT   | brown adipose tissue            |
| BMD   | bone mineral density            |
| BMI   | body mass index                 |
| CagA  | cytotoxin-associated gene A     |
| cAMP  | cyclic adenosine monophosphate  |
| CARD3 | caspase recruitment domain 3    |
| CD    | Crohn's disease                 |
| CI    | confidence interval             |
| CLR   | centered log-ratio              |
| COX-2 | cyclooxygenase-2                |
| CRC   | colorectal cancer               |
| CS    | caesarean section               |

|                  |                                       |
|------------------|---------------------------------------|
| DNA              | deoxyribonucleic acid                 |
| DSS              | dextran sulfate sodium                |
| EDTA             | ethylenediaminetetraacetic acid       |
| EEN              | exclusive enteral nutrition           |
| ELISA            | enzyme-linked immunosorbent assay     |
| ENS              | enteric nervous system                |
| EtOH             | ethanol                               |
| FDR              | false discovery rate                  |
| FFQ              | food frequency questionnaire          |
| GFA              | glial fibrillary acidic               |
| H <sub>2</sub> S | hydrogen sulfide                      |
| HADS             | hospital anxiety and depression scale |
| HFD              | high fat diet                         |
| HIV              | human immunodeficiency virus          |
| HR               | hazard ratio                          |
| IBD              | inflammatory bowel disease            |
| IFN              | interferon                            |
| IgA              | immunoglobulin A                      |
| IRR              | incidence rate ratio                  |
| IL-1             | interleukin-1                         |
| IL-10            | interleukin-10                        |

|          |                                                               |
|----------|---------------------------------------------------------------|
| IL-12    | interleukin-12                                                |
| IL-17    | interleukin-17                                                |
| IL-23    | interleukin-23                                                |
| IL-6     | interleukin-6                                                 |
| iNOS     | inducible nitric oxide synthase                               |
| IPAQ     | international physical activity questionnaire                 |
| IQR      | interquartile                                                 |
| JAK      | janus kinase                                                  |
| JAK-STAT | janus kinase/signal transducer and activator of transcription |
| KEAP1    | kelch-like ECH-associated protein 1                           |
| LPS      | lipopolysaccharides                                           |
| MD       | Mediterranean diet                                            |
| MDS      | Mediterranean diet score                                      |
| MDSS     | Mediterranean diet serving score                              |
| MeSH     | medical subject headings                                      |
| MET-min  | metabolic equivalent per minute                               |
| mTOR     | mammalian target of rapamycin                                 |
| MTX      | methotrexate                                                  |
| NCDs     | noncommunicable diseases                                      |
| NF-kB    | nuclear factor-kB                                             |

|           |                                                                |
|-----------|----------------------------------------------------------------|
| NGAL      | neutrophil gelatinase-associated lipocalin                     |
| NGS       | next-generation sequencing                                     |
| NLRX1     | nod-like receptor X1                                           |
| NO        | nitric oxide                                                   |
| NOD2      | nucleotide-binding oligomerization domain-containing protein 2 |
| NOS       | newcastle–ottawa quality assessment scale                      |
| NRF2      | nuclear factor erythroid 2-related factor 2                    |
| NSAIDs    | non-steroidal anti-inflammatory drugs                          |
| OCP       | oral contraceptives pill                                       |
| ONT       | oxford nanopore technologies                                   |
| OTUs      | operational taxonomic units                                    |
| PA        | physical activity                                              |
| PBS       | phosphate buffered saline                                      |
| PCA       | principal component analysis                                   |
| PCoA      | principal coordinates analysis                                 |
| PCR       | polymerase chain reaction                                      |
| PD        | phylogenetic diversity                                         |
| PDE4      | phosphodiesterase-4                                            |
| PERMANOVA | permutational multivariate analysis of variance                |

PERMDISP permutations, followed by a test for homogeneity of multivariate dispersion

PPIs proton pump inhibitors

PRISMA preferred reporting items for systematic review and meta-analysis

PUFA polyunsaturated fatty acids

RAGE receptor for advanced glycation endproducts

RCT randomized controlled trial

rDNA ribosomal DNA

RMSD root mean square deviation

RNA ribonucleic acid

ROS reactive oxygen species

S1P sphingosine-1-phosphate

SASA solvent accessible surface area

SCFA short-chain fatty acids

SD standard deviation

SIRT1 sirtuin 1

SMAD7 mothers against decapentaplegic homolog 7

SMD small molecule drugs

STAT signal transducers and activators of transcription

STZ streptozotocin

|                                      |                                       |
|--------------------------------------|---------------------------------------|
| TGF                                  | transforming growth factor            |
| TM-align                             | template modelling align              |
| TM-score                             | template modelling score              |
| TNBS                                 | trinitrobenzene sulfonic acid         |
| TNF $\alpha$                         | tumour necrosis factor alpha          |
| UC                                   | ulcerative colitis                    |
| VO <sub>2</sub>                      | volume oxygen                         |
| 1,25(OH) <sub>2</sub> D <sub>3</sub> | 1,25-dihydroxy vitamin D <sub>3</sub> |

## 2. Introduction

### 2.1 The global burden of Inflammatory bowel diseases: the “real” price of IBD

The term inflammatory bowel disease (IBD) stands for a group of chronic and lifelong diseases characterized by immune-mediated inflammation of the gastrointestinal tract and recurring periods of relapse and remission, including Crohn’s disease (CD) and ulcerative colitis (UC) (Yeshe et al., 2020). CD is characterized by discontinuous transmural lesions that can affect any part of the gastrointestinal tract (but most commonly it affects the large and small intestines). Instead, UC consists in continuous, diffuse, and superficial inflammation of the colon (Gohil & Carramusa, 2014; K. Hou et al., 2022a). IBD

is characterized by symptoms such as abdominal pain, fever, haematochezia, constipation or diarrhoea, and weight loss (O'Reilly et al., 2023). Both UC and CD can generate a range of complications, including anaemia, stenosis, abscesses, fistulas and an increased risk of colorectal cancer (S. Wang et al., 2024). Since the beginning of the 21st century, IBD prevalence has remained elevated in Western countries, while its incidence has increased in newly industrialized countries in Asia, Africa, and South America, emerging as a significant global public health challenge (S. Wang et al., 2024). A systematic review by Molodecky et al. summarised population trends and identified an increase in Crohn's disease incidence in three-quarters of studies; similarly, for ulcerative colitis they found an increased incidence for nearly two-thirds of studies and confirmed this trend to be a general phenomenon (Molodecky et al., 2012a). Incidence rates are heterogeneous in a defined geographical area. Urban population shows higher incidence than the rural one and incidence in urban populations preceded that in rural populations by one decade (E. Loftus et al., 2000; E. V. Loftus et al., 1998). It has been demonstrated also a north-south gradient, showing a higher incidence of Crohn's disease in northern latitudes than in southern latitudes (Khalili, Huang, et al., 2012; Nerich et al., 2006). A similar yet inverse relationship might exist in the Southern hemisphere, as a high incidence of IBD has been described in New Zealand (Gearry et al., 2006). The majority of studies show a peak of IBD incidence in the second to fourth

decade of life. Some cohort studies have proposed also a bimodal incidence rate with a modest second peak in the sixth and seventh decades of life (Cosnes et al., 2011; E. V. Loftus, 2004; Molodecky et al., 2012a). Incidence is similar between females and males in established populations but is influenced by race and ethnicity. Actually, the risk of IBD is threefold higher in the Jewish population than in non-Jewish populations, and in particular, the risk is higher for European Jewish individuals compared to those living in Israel (Bernstein et al., 2006). Furthermore, a first estimation suggested a considerably lower prevalence in African-American or Hispanic ethnicity than white populations, but the gap is smaller considering the incidence between white and non-white populations in comparable phenotypes (Mahid et al., 2008; Reddy & Burakoff, 2003). For what concerns Asian population, there is less data available; however, Japan, Hong Kong and Korea all demonstrated an increasing incidence of IBD between 1980 and 2003 (Thia et al., 2008). Curiously, in most of these communities the incidence of UC is greater than CD and often precedes its rising incidence of a decade. A study conducted on Asia-Pacific populations revealed that the incidence of ulcerative colitis and Crohn's disease were 0.76 and 0.54 per 100,000 inhabitants respectively, which is considerably lower than for Western countries (i.e. 0.6–24.3 per 100,000 for UC and 0.3–12.7 per 100,000 for CD in Europe) (Molodecky et al., 2012a; S. C. Ng et al., 2013). IBD still remains rare in Africa and South America, although some case series suggest

an increase in its incidence (Ukwenya et al., 2011). It is projected that by 2030, the number of people suffering from IBD in Europe and the United States of America will exceed 7 million, or the 0.3% of the population in North America, Oceania and many countries in Europe (Coward et al., 2019; Hammer & Langholz, 2020; Molodecky et al., 2012b). The incidence rate of IBDs is higher than the mortality rate and this is due to advancements in maintenance therapy (Kaplan, 2015; Kaplan & Windsor, 2021). However, thanks to medicine progress, population growth and aging, the burden of IBD is expected to continue rising until 2050 (S. Wang et al., 2024). As mentioned before, given the chronic nature of the disease characterised by frequent relapses and complications, it implies a significant burden on the healthcare system due to hospitalization and cost of medications, as well as on society in terms of wellbeing and productivity loss (G. Caviglia et al., 2020; G. P. Caviglia et al., 2023; Kuenzig et al., 2020). This emphasises the urgency of addressing research's effort towards a better understanding and management of the disease.

## **2.2 Risk factors**

### **2.2.1 Genetic predisposition**

Genetic predisposition to develop IBD was initially proved by studies on familial aggregation and twins from which it was concluded that there is an

important hereditary component (Ananthakrishnan, 2015). Patients with CD report a family history of the same disease in between 2% and 14% of cases, whereas a small proportion of these patients have a family history of ulcerative colitis. Similarly, 8% - 14% of subjects with UC have already a family history of IBD, more commonly UC. Therefore, for first-degree relatives of a patient with Crohn's disease, the relative risk of developing IBD is estimated to be around 5% in non-Jewish and 8% in Jewish patients, and about 1.6% and 5.2% respectively, the respective risk of developing ulcerative colitis (H. Yang et al., 1993). If both parents are affected, the risk of the progeny getting IBD before turning 30 years is estimated to be as high as one-in-three (Halme et al., 2006). Linkage studies have demonstrated an initial association between a locus on chromosome 16 and the development of IBD (Hugot et al., 1996). Five years later, this locus was characterized as the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) locus with three common variants that influence susceptibility to Crohn's disease (Hugot et al., 2001; Ogura et al., 2001). A subject that is homozygote at NOD2 has a 20-40 fold increase in disease risk, whereas heterozygosity confers a minor elevation in risk, 2-4 fold, yet it remains the strongest among the loci identified so far (Hugot et al., 2001; Ogura et al., 2001). Later, international studies have identified 163 distinct risk loci resulting in a broader map of potentially associated genes. Loci's majority are shared between both UC and CD with similar directions of effect, even though

some loci (such as NOD2 and ATG16L1) are specifically associated with just Crohn's disease and others with ulcerative colitis alone (Jostins et al., 2012). Regarding cases like resistance to treatment, very-early-onset and severe perianal fistulization, they might have different monogenic origins (i.e. IL-10 receptor mutations) (Glocker et al., 2009). However, the loci linked risk account for less than one-quarter of the heritability of IBD, suggesting a more prevalent role for environmental factors (Khor et al., 2011).

### **2.2.1 Environmental influence**

#### Pollution and greenspace

Animal studies have demonstrated that dust and smoke ingestion alter gut microbiome and trigger intestinal inflammatory responses (Kish et al., 2013). Among pollutants, residential exposure to nitrogen dioxide and sulfur dioxide has been studied on subjects aged 25 or younger. A higher risk of CD (odds ratio 2.31) and a higher risk of UC (odds ratio 2.00) have been found respectively for the first and second compound (Kaplan et al., 2010). Nevertheless, a case-control study on Europeans did not find any correlation between air pollutants and IBD development (Opstelten et al., 2016). On the contrary, a protective effect seems to be exerted by greenspace, defined as areas of vegetation or bodies of water in urban spaces. A Canadian study demonstrated that residential closeness to greenspaces is linked to lower risk of paediatric IBD

(HR: 0.77; 95% CI, 0.74-0.81). This protective effect could be explained by cleaner air and healthier lifestyle factors like a greater chance to engage in physical exercise (Elten et al., 2021). In general, living in a city, rather than in country side, has been associated with IBD onset (OR for UC was 1.06 [95% CI 0.78, 1.45] and OR for CD was 1.26 [95% CI 1.03, 1.53]) (Soon et al., 2012). A Canadian study confirmed this with a cohort study demonstrating a lower IBD risk for subjects living in rural areas compared with those in urban areas (incidence rate ratio: 0.90; [95% CI, 0.81-0.99]). The same study found that the protective effect is stronger in the first 10 years of life (incidence rate ratio: 0.58; [95% CI, 0.43-0.73]) (Benchimol et al., 2017).

#### Latitude and vitamin D

Vitamin D regulates a range of mechanisms among which calcium and phosphate metabolism, modulation of gut mucosal immunity and integrity of intestinal barrier. Immunity regulation seems to be mediated by an inhibition of lymphocytes T helper Th1/Th17, and promotion of lymphocytes T regulator (Treg cells) producing the anti-inflammatory IL-10 (Vernia et al., 2022). A part of vitamin D is synthesized in the skin through ultraviolet light, so its production is influenced by latitudinal variations. In fact, a US study on women at 30 years of age reported a reduced risk of developing CD (OR: 0.48; [95% CI, 0.30-0.77]) and UC (OR: 0.62; [95% CI, 0.42-0.90]) for females living in southern latitudes compared to northern latitudes (Khalili, Huang, et al., 2012). These

data have been confirmed by a systematic review on paediatric incidence of CD, latitude and sunlight exposure. The incidence was of 0.23 new cases per 100.000 habitants in higher latitudes, in particular in regions with longer periods of low sunlight (Holmes et al., 2015).

### Appendectomy

Undergoing an appendectomy implicates divergent effects on Crohn's disease and ulcerative colitis. A large cohort of 212,963 patients who had appendectomy before the 50<sup>th</sup> year of age showed that inflammation of the appendix, rather than the actual organ removal, might be responsible for a markedly lower incidence of UC (Andersson et al., 2001). In particular, for patients with perforated appendicitis, the adjusted hazard ratio (HR) was 0.58 [95% CI, 0.38 to 0.87]; for those with nonperforated appendicitis it was 0.76 [95% CI, 0.65 to 0.90]; and for those with mesenteric lymphadenitis it was 0.57 [95% CI, 0.36 to 0.89]. This protective effect is, however, limited to appendectomy before the age of 20. In fact, an increased risk of Crohn's disease for the successive 20 years after the surgery was found in the same cohort. Yet, if patients were operated before the age of 10, the risk of Crohn's disease was actually reduced (incidence rate ratio 0.48, [95% CI, 0.23-0.97]) (Andersson et al., 2003). Generally, appendectomy is followed by increased risk of CD, even though, in different studies, surgery for abdominal pain in Crohn's disease happened before formal diagnosis and this meant bias in the association of increased risk. The reason

leading to appendectomy seems to be relevant in determining outcomes. For example, appendectomy for perforating appendicitis has been related to increased risk of subsequent intestinal resection (IRR (incidence rate ratio) 2.11 [95% CI 1.21-3.79]), IRR 1.85 [95% CI, 1.10-3.18] after nonspecific abdominal pain, IRR 2.15 [95% CI, 1.25-3.80] after mesenteric lymphadenitis, 2.52 [95% CI, 1.43-4.63] after other diagnoses (Andersson et al., 2003).

### Tonsillectomy

Similar to appendix, tonsils are part of the lymphoid system, contributing to immune modulation and their removal can impair immune tolerance and increase IBD risk (Cesta, 2006). A recent meta-analysis showed a higher risk of both CD and UC after tonsillectomy (respectively OR: 1.93; 95% CI, 0.96-3.89 and OR: 1.24; 95% CI, 1.18-1.30) (R. Amin et al., 2024).

### Breastfeeding

Breast milk contains a variety of bioactive compounds such as immunoglobulins, cytokines and oligosaccharides. These components shape gut microbiota in a healthy biodiversity, modulate immune responses and reinforce intestinal barrier, countering the development of inflammatory states in the gut (Stewart et al., 2018). Differences in gut microbiota persist beyond 6 months of age, as reported by a meta-analysis comparing the gut microbiomes of infants exclusively breastfed with those who were not breastfed (Ho et al., 2018). A study on breastfeeding and the relative risk of developing IBD

conducted by Klement et al. concluded an odds ratio of 0.62 (95% CI, 0.67-0.86) for UC and 0.77 (95% CI, 0.61-0.96) for CD (Klement et al., 2004). These findings have been supported by another meta-analysis showing a significant negative association between breastfeeding and IBD (OR: 0.74; 95% CI, 0.66-0.83) (L. Xu et al., 2017). In the same study, the importance of the duration of breastfeeding was also noted: a longer duration, of 12 months compared to 3 or 6 months, was associated with a lower IBD risk. In particular, for Crohn's disease (OR 0.20, [95% CI 0.08 – 0.50]) and ulcerative colitis (OR 0.21, [95% CI 0.10 – 0.43]) as compared to 3 months odds ratio 0.62 [95% CI 0.39 – 0.97] or 6 months OR 0.46 [95% CI 0.31 – 0.69].

### Asthma

Among diseases, asthma has been found to be a risk factor for UC and CD (class II evidence) (Kuenzig et al., 2018). It should be noted that both IBD and asthma are immune-mediated pathologies sharing genetic and environmental risk factors. Barrier function impairment in gastrointestinal epithelium and in the lung, along with aberrant immune responses to pathogens and environmental triggers, are common features in both conditions (de Souza & Fiocchi, 2016). Also the hygiene hypothesis applies to both diseases, suggesting that living in sterile environments during childhood predisposes to developing chronic immune-mediated diseases later in life (T. Wu et al., 2025).

### Autism

A study conducted by Kim et al. discovered an association between autism spectrum disorder and risk for IBD, any IBD, OR = 1.66, [95% CI = 1.25-2.21],  $p < 0.001$  (J. Y. Kim et al., 2022). There have been numerous hypotheses trying to explain the correlation. The putative gut-brain connection for example, links the intestinal tight junction dysfunction in spectrum disorder to an altered intestinal permeability commonly found in IBD (D'Eufemia et al., 1996). These data have been actually confirmed by several other observational studies that documented a statistically significant correlation between autism and IBD (Alexeeff et al., 2017; Butwicka et al., 2019; Doshi-Velez et al., 2015; M. Lee et al., 2018).

### Infections

A putative association between infections and IBD has been investigated for a long time. Certainly, as said before, the factors involved in the etiopathogenesis are multiple and it is not easy to draw a direct association. A high presence of *Mycobacterium avium sb paratuberculosis* was found in patients with CD in some studies but not in others and a randomized controlled trial showed no clinical benefit using antimycobacterial treatment (Bernstein et al., 2004; Chacon et al., 2004; Feller et al., 2007; Selby et al., 2007). An association between IBD and *Salmonella* or *Campylobacter* infection has been postulated from a study conducted in Denmark (Gradel et al., 2009), whereas others, like the one about US military population (Porter et al., 2008) as well as the UK primary care

database, have demonstrated an increase in risk of IBD after episodes of gastroenteritis characterized by unclear bacterial or viral aetiology (HR 2.4 [95% CI 1.7-3.3]) (Rodríguez et al., 2006). Regarding patients with established IBD, infections are frequently a trigger of relapse and the most common is *Clostridium difficile*. This one is especially associated with hospitalized patients and unfortunately, often associated with relevant mortality and morbidity (Ananthakrishnan et al., 2009). Systemic infections might also act as triggers for relapse in those with established disease, even though supporting data are less powerful (Ananthakrishnan, 2015). On the contrary, infection by *Helicobacter pylori* seems to have a protective effect against IBD development. A meta-analysis on Asian population established that subjects with IBD had a significantly reduced proportion of *H. pylori* infection (X.-W. Wu et al., 2015). Another meta-analysis reinforced the similar trend across many ages and ethnicities, showing that *H. pylori* infection was actually associated with a lower probability of IBD (OR: 0.43; 95% CI, 0.36-0.50) (Castaño-Rodríguez et al., 2017). The reason for this protective effect could be the *H. pylori* genotype carrying the Cytotoxin-associated gene A (CagA). Indeed, another study showed that patients who were seronegative for *H. pylori* CagA did not show a decreased risk for IBD compared to healthy individuals without *H. pylori* infection (OR: 0.74; 95% CI, 0.46-1.17) (Tepler et al., 2019).

### Antibiotics

Exposure to antibiotics in early childhood can perturb microbiota composition and influence susceptibility to IBD. A case-control study on paediatric IBD cohort concluded that 58% of paediatric patients took an antibiotic in their first year of life, compared with just 39% of the controls (S. Y. Shaw et al., 2010). A study conducted on Finnish children confirmed that antibiotic-induced gut microbiota alteration in early life is associated with a higher risk of developing IBD (age 10–40 years, IRR 1.28, [95% CI 1.25 to 1.32]; age 40–60 years, IRR 1.48, [95% CI 1.43 to 1.54]; age  $\geq 60$  years, IRR 1.47, [95% CI 1.42 to 1.53], specifically slightly greater for CD than UC. (Virta et al., 2012). The same study concluded that the relationship between CD and antibiotic use was stronger in males vs females and that cephalosporins had the strongest association (Virta et al., 2012). The number of antibiotic courses is also relevant in fact, disease risk is greater for multiple courses of antibiotics than a single one (Kronman et al., 2012). However, the majority of the association studies on antibiotics were conducted on Western populations who usually experience low exposure to early-life infections or have good sanitation. In contrast to this data, a large population-based study in Asia found that antibiotic exposure demonstrated a protective association with both Crohn's disease and ulcerative colitis (Gevers et al., 2014).

### Medications

Besides antibiotics, other drugs have been associated with IBD development, such as aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), oral contraceptives pill (OCP), postmenopausal hormone therapy and proton pump inhibitors (PPIs). Regular use of aspirin was investigated in a prospective study on both European men and women, which found that regular intake was positively associated with risk of developing CD (OR = 6.14, 95% CI = 1.76–21.35), but no association was found for UC (Chan et al., 2011). In contrast to this data, a prospective study on US women didn't find any clear association between aspirin use and disease; yet, they found an association for NSAIDs frequent use, stronger for higher doses and longer duration and similar for CD and UC (Ananthakrishnan, Higuchi, et al., 2012). With respect to OCP, an association has been established between its use and IBD development: the relative risk for CD was 1.51, 95% CI 1.17-1.96,  $p = 0.002$  and 1.53 for UC 95% CI 1.21-1.94,  $p = 0.001$ . Nevertheless, the OCP cessation reverted the risk to that of the nonexposed population. (Cornish et al., 2008). Similarly, in a large prospective study, postmenopausal hormone therapy has been associated with a higher hazard ratio for UC (but not for CD) compared to women who never used hormones. Moreover, UC risk appeared to increase along with the duration of hormone use and decrease in time since suspension. Additionally, there was no difference in risk between the hormone type used. Factors like age, body mass index or smoking did not influence the risk of disease (Khalili,

Higuchi, et al., 2012). Lastly, PPIs seem to increase IBD risk by disrupting intestinal permeability through tight junctions' disruption, increasing susceptibility to gastrointestinal infections (like *C. difficile*) and also altering gut microbiota composition (Liang et al., 2024). A confirmation of that came from an observational study's meta-analysis concluding an increased risk of IBD for subjects exposed to or currently taking PPIs: the risk was definitely higher for CD (adjusted OR: 3.60; 95% CI, 1.10-11.74) than for UC (adjusted OR: 1.50; 95% CI, 0.5-4.5) (Shastri et al., 2022).

### BMI

Obesity involves a chronic low-grade inflammation status due to adipocytes-produced pro-inflammatory cytokines including TNF $\alpha$  and IL-6 (Singh et al., 2017). An association between high body mass index (BMI) and CD (HR: 1.42; 95% CI, 1.18-1.71), but not for UC (HR: 0.96; 95% CI, 0.80-1.14) was found in a meta-analysis (Rahmani et al., 2019). Interestingly, the relation between CD risk and BMI seems to follow a U-shaped direction. In fact, a study conducted by Mendall et al. confirmed that low BMI had a stronger hazard risk of developing CD (adjusted HR: 1.8; 95% CI, 0.9-3.7) compared to obesity (HR: 1.5; 95% CI, 0.8-2.7) (Mendall et al., 2018).

## **2.2.2 Lifestyle and physical activity**

### Smoking

The effects of smoking in IBD are controversial and not uniform. A meta-analysis quantified the risk of developing CD to be twofold if the subject is a smoker. Equally, the same risk weight has been found for former smoking and UC, but not for current smoking, which instead showed a strong inverse association (OR 0.58 [95% CI 0.45–0.75]) (Mahid et al., 2006). A prospective cohort study on female nurses concluded that after smoking cessation, the risk of UC increased within 2 – 5 years and remained high for twenty years (Higuchi et al., 2012). Consistently with higher incidence, smoking is also associated with a more aggressive disease course in Crohn's disease (Cosnes et al., 1999, 1996; Cosnes, 2008; Lakatos et al., 2007). In addition, smokers are more likely to require surgery and immunosuppression treatment and have a greater relapse probability after ileocecal resection. On the contrary, smoking interruption is frequently a flare precipitant for UC within a year of stopping. Also, UC current smokers have a milder disease course and require fewer surgeries and immunosuppressants (Cosnes, 2004, 2008). Although various theories have been formulated, none have been satisfactory enough to explain such divergent effects of smoking in IBD (Cosnes, 2004; Lakatos et al., 2007). For a long time nicotine was believed to be the trigger, but studies with nicotine replacement therapy in UC led to equivocal results (Birrenbach & Böcker, 2004). Furthermore, in Crohn's disease no association was found with oral tobacco use, suggesting that other tobacco molecules might also play a role. It has to be

considered also that smoking can alter smooth muscle tone, influence endothelial function, affect the integrity of mucous barrier and is also associated with changes in microbiota (Biedermann et al., 2014; Hatoum et al., 2006; McGilligan et al., 2007; Parkes et al., 2014). Although smoking is one of the most important risk factors taken into consideration in studies, susceptibility studies have to consider gender, ethnic characteristics and the complex gene–environment interaction.

### Hygiene hypothesis

Strachan et al. first proposed the hygiene hypothesis, trying to explain the increasing incidence of autoimmune diseases in the western world (Strachan, 1989). Several studies supported this theory recognising various protective factors such as number of siblings, larger family size, drinking unpasteurized milk, living on a farm and exposure to pets (particularly in early childhood) (Bernstein et al., 2006; Radon et al., 2007; Timm et al., 2014; Van Kruiningen et al., 2005). Nevertheless, most of these studies were case-control and could not exclude recall bias or confounders. In developing countries, hygiene habits did not present the same inverse association observed in Western countries and have been in fact associated with a higher risk of developing UC (Sood et al., 2014).

### Diet

Epidemiological studies of diet in IBD have been designed as case-control, being susceptible to bias and limitations (Chapman-Kiddell et al., 2010; J. K. Hou et al., 2011). Despite this and heterogeneity in design, the dietary fibre remains the most consistent macronutrient with inverse association to IBD (multivariate HR for CD, 0.59; [95% CI, 0.39-0.90]). (Amre et al., 2007; Ananthakrishnan, Khalili, et al., 2013). Paediatric patients newly diagnosed with CD consumed markedly less fruit and vegetables than non-IBD controls (Amre et al., 2007). Also, in a prospective study, women in the highest quintile of long-standing fibre intake had a risk reduction of 40% in developing CD (OR 0.59, 95% CI 0.39–0.90) (Ananthakrishnan, Khalili, et al., 2013). This lower risk was not detected for whole grains or cereals but was strong for fruit and vegetables fibre. The explanation for this association could be given by the fact that soluble fibre is metabolized by gut microbiota into short-chain fatty acids (SCFA) and these molecules can inhibit the transcription of pro-inflammatory factors (Galvez et al., 2005), fuel the epithelial cells reinforcing the intestinal barrier (P. Liu et al., 2021), and modulate immune and neural signalling pathways that contribute to the maintenance of intestinal homeostasis (Silva et al., 2020; Vinolo et al., 2011).

### Stress

For a long time IBD has been associated with psychological diseases and patient-reported triggers like neuroticism, obsessive-compulsive behaviour,

dependency, perfectionism and psychosocial anxiety (Bernstein et al., 2010a). Stress can trigger gut inflammation through various mechanisms via the hypothalamus–pituitary–adrenal axis and the autonomic nervous system. These activations result in proinflammatory cytokines production, which in turn leads to macrophages activation and alteration of intestinal permeability and gut microbiota (Bonaz & Bernstein, 2013). In a mouse study, the exposure to a stressful stimulus reduced microbiota diversity and increased levels of IL-6: curiously, this effect on IL-6 levels was alleviated by antibiotics (Bailey et al., 2011). These data have found support in observational studies revealing a possible association between IBD risk and major life stressors, anxiety and depression (Ananthakrishnan, KHALILI, et al., 2013; Bernstein et al., 2010b; Bitton et al., 2008; J. R. Goodhand et al., 2012; J. Goodhand & Rampton, 2008; Lerebours et al., 2007; Levenstein et al., 2000; J. Li et al., 2004; Maunder, 2005). Also, established depression or anxiety in these subjects is associated with hospitalization, surgery, less reactivity to immunosuppressive therapy, higher risk of relapse and reduced quality of life. Due to the complexity and intricate relationship of both kinds of disease, it still remains challenging to determine the relative contribution of one on the other in this bidirectional association (Ananthakrishnan, KHALILI, et al., 2013; Bernstein et al., 2010b; Bitton et al., 2008; J. Goodhand & Rampton, 2008; Levenstein et al., 2000; Maunder, 2005; Mawdsley & Rampton, 2005; Rampton, 2009).

## Sleep

It is unfortunately common to find sleep disorders in society, but they are also more frequent in IBD patients, especially with active disease (Ananthakrishnan, Long, et al., 2013). Most likely, the link might be bidirectional, given that a major disease activity may disturb sleep and poor sleep can exacerbate inflammation (Kinnucan et al., 2013; Swanson et al., 2011). A prospective study found that getting less than 6 hours (HR: 1.51; 95% CI, 1.10-2.09) or more than 9 hours (2.05, 95% CI, 1.44-1.92) of sleep per day increased the risk of developing UC, but not for CD (Ananthakrishnan, Khalili, Konijeti, Higuchi, de Silva, Fuchs, Richter, et al., 2014). Both duration and quality of sleep are important: low quality entails histological worsening and higher risk of clinical relapse (T. Ali et al., 2013; Ananthakrishnan, Long, et al., 2013).

## Physical activity

The association between physical exercise and IBD was first investigated by Sonneberg et al. This study discovered a higher risk in IBD development for people with a sedentary occupation like office work, mechanics, blacksmiths and locksmiths, meanwhile heavy manual labour such as carpenter, bricklayer, cleaning and maintenance were associated with a low risk of IBD (Sonnenberg, 1990). In accordance with the previous data on stress, results from a mouse model revealed that forced exercise caused inflammation and increased proinflammatory gene expression, while voluntary wheel training alleviated

colitis symptoms and reduced the same gene expression (Cook et al., 2013). Other evidences also show that prolonged, intense exercise bouts can cause debilitating gastrointestinal complaints including cramps, diarrhea and gut inflammation most likely due to gut ischemia and/or mechanical trauma (Bi & Triadafilopoulos, 2003; Packer N., 2010) This preliminary data have found support in several studies like the one that showed that rigorous physical activity entails a 44% of risk reduction for Crohn's disease (Khalili et al., 2013). A more recent meta-analysis confirmed that physical exercise was linked to a lower probability of getting CD in both cohort and case-control studies. Differently, for UC physical activity has been associated with a reduced risk in observational cohort studies, but not in case-control ones (Tiong et al., 2024). Therefore, several environmental factors modulate IBD risk with often conflicting effects: smoking increases Crohn's risk but seems to protect (paradoxically) from UC. Hygiene hypothesis suggests a protective role of early exposures to farm, animals and larger family size typical of Western countries. A high-fibre diet, regular and adequate sleep, and low levels of psychological stress are protective, as stress and sleep disturbances act through the HPA axis and alterations in the intestinal microbiota/permeability increase inflammation. Moderate/voluntary physical activity is associated with a lower risk of Crohn's disease (less clear for ulcerative colitis), while forced or excessive exercise may

prove deleterious. A comprehensive summary of IBD risk factors is illustrated in figure 1.

### Summary of Key Risk Factors for Inflammatory Bowel Disease (IBD)

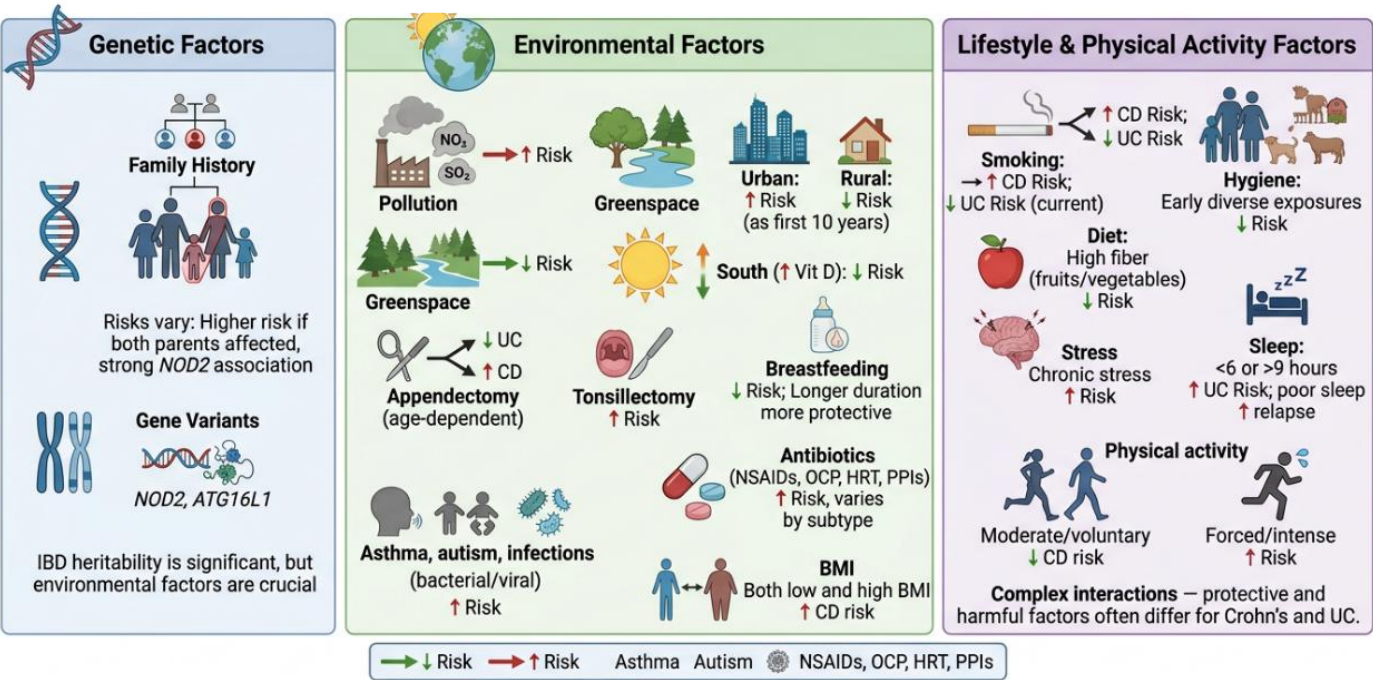


Figure 1: IBD risk factors comprehend not modifiable factors like genetic family history, not easily modifiable environmental factors and modifiable factors like lifestyle.

### 2.3 Gut microbiota and IBD: causation or correlation?

As discussed above, the aetiology of IBD is complex and multifactorial, involving genetic, environmental, dietary, immune and neuronal factors, all of which are also interlinked with a complex microbial community of bacteria, yeasts and viruses collectively known as gut microbiota (K. Hou et al., 2022b). This latter co-exists in a symbiotic relationship with the host, contributing to physiological processes, including digestive and metabolic function, regulation of immune responses, protection against pathogen colonization and

modulation of neuronal functions. When the host-microbiota interplay is altered, specific microbes and their metabolites can result in aberrant immune response, which contributes to the disease process in pathological conditions, such as cancer, diabetes, IBD, heart diseases and others (Devkota et al., 2012a; K. Hou et al., 2022a; M. Lee & Chang, 2021). In particular, IBD has been associated with gut dysbiosis, defined as a decrease in microbial diversity owing to a shift in the balance between commensal and potentially pathogenic microorganisms (Ni et al., 2017). Although studies about IBD and microbiota are advancing, a key question remains unclear: is dysbiosis a cause or consequence of immune activation and inflammation in IBD or both? There have been paediatric studies attempting to address the temporal relationship between dysbiosis and inflammation. Newly diagnosed, treatment-naive children with Crohn's disease showed dysbiotic changes in stool and mucosal biopsies, suggesting that dysbiosis might precede clinical disease and develop independently of long-standing inflammation and/or medical therapy (Gevers et al., 2014). Another prospective study on paediatric patients with Crohn's disease similarly concluded that dysbiosis reflected the presence and severity of inflammation; however, this study also found an independent association between dysbiosis and other factors such as diet and use of antibiotics (Lewis et al., 2015). Hence, these studies cannot prove that dysbiosis indeed precedes the disease, yet they can suggest that changes in microbiota might occur early

in IBD and perhaps contribute to the disease onset. Moreover it is known that faecal microbiota transplantation (FMT), that introduces screened donor stool into the gastrointestinal tract of a patient, has the potential as a viable treatment option for IBD. In fact, a recent review concluded that FMT has certain clinical and endoscopic benefits on UC patients (Gefen et al., 2025), while another one reported that it helped achieve clinical remission in 57% of adult patients with active Crohn's disease (Zhou et al., 2023). Conversely, a study demonstrated that FMT from IBD patients to mice altered the immune response causing IBD-like colitis in the transplanted animals. These findings clearly highlight the strict connection existing between microbiota composition and IBD. Likewise, IBD-related dysbiosis improves with treatment but nonetheless persists even in patients that have achieved full mucosal healing (K. A. Shaw et al., 2016). Such observations suggest that dysbiosis, as defined by our current understanding of disease-associated microbiota, is a fundamental feature of IBD. Furthermore, environmental factors, including inflammation itself, probably contribute to dysbiosis over time by altering the metabolic conditions in the gut (Colgan et al., 2023; Ni et al., 2017). Gut microbes can stimulate immune responses either by functioning as adjuvants or antigens. As adjuvants, they trigger innate immune responses through dendritic cells and other antigen presenting cells (APCs), and as antigens they stimulate the clonal expansion of T cells (Leone et al., 2013). It has been reported that genetically susceptible hosts (like IBD

patients and genetically engineered mice) have aggressive T cell responses to luminal commensal bacteria (Hoentjen et al., 2005; Lodes et al., 2004). Moreover, mechanistically, microbiota can impact inflammation as well as the intestinal barrier. A study conducted by Kleessen et al. reported a major bacterial mucosa invasion in IBD patients compared to controls (Kleessen et al., 2002). Another study documented a significantly increased abundance of adherent-invasive *E. coli* in CD patients, suggesting that the pathogenic bacteria may affect the permeability of the gut barrier, overcome the composition of resident microbiota, and eventually induce intestinal inflammation (I. Ahmed et al., 2016). The proposed mechanism through which pathogenic bacteria can cause inflammation is a disruption of the mucosal integrity (Chorawala et al., 2021). The impairment of intestinal epithelium's tight junction proteins leads to increased permeability of the barrier. This compromise eventually leads to translocation of luminal antigens, toxins and microbial metabolites to lamina propria (Dunleavy et al., 2023; Ghosh et al., 2021). These antigens, once in contact with macrophages and dendritic cells, trigger an immune response that leads to cytokines secretion such as IL-6, IL-23 and TNF $\alpha$  (Strober & Fuss, 2011). These pro-inflammatory cytokines further stimulate the T helper (Th) cell differentiation, particularly Th1 and Th17 cells, thereby exacerbating mucosal inflammation and tissue injury (Kou et al., 2025). Certainly, it has been assumed that the composition of gut microbiota is different between IBD subjects and

healthy controls. Indeed, IBD patients often exhibit reduced diversity of commensal bacteria and overrepresentation of pathogenic taxa like *Enterobacteriaceae* (I. Khan et al., 2019). In healthy subjects, over 90% of gut microflora is constituted by four main phyla i.e. *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. Other phyla like *Verrucomicrobia* and *Fusobacteria* are present in smaller proportions (Quigley, 2013; K. Yang et al., 2025). Among these phyla, *Firmicutes* accounts for almost 49–76% of the overall gut population, and within this phylum, *Clostridium* clusters (*Ruminococcaceae* family) and (*Lachnospiraceae* family) are essentially important in preserving intestinal homeostasis (Matsuoka & Kanai, 2015). The so-called beneficial bacteria with anti-inflammatory properties, including *Faecalibacterium prausnitzii*, *Bifidobacterium* spp, *Eubacterium rectale*, *Lactobacillus* spp and *Roseburia intestinalis* are notably reduced in CD and UC patients, alongside an increase of inflammatory bacteria such as *Escherichia coli*, *Bacteroides fragilis*, *Ruminococcus gnavus* and *Fusobacterium nucleatum* (Forbes et al., 2018; Hall et al., 2017; Su et al., 2020; Z.-K. Wang et al., 2014; Zuo & Ng, 2018). Also, the ratio of *Bacteroidetes* to *Firmicutes* is greater in healthy subjects (Morgan et al., 2012). However, the intestinal microbiota composition is highly variable from subject to subject so it is not possible to reduce it to a general approximation: differences in gut microbiota composition have actually been documented even between members of the same family (including twins) who are discordant for IBD

(Joossens et al., 2011; Willing et al., 2010). This suggests that dysbiosis is mainly associated with the degree of disease rather than environmental or genetic factors.

With regard to viruses, there is less data available and it shows that gut virome is mainly composed of bacteriophages (Breitbart et al., 2003). In particular, paediatric patients with CD have shown an increase in *Caudovirales* bacteriophages in ileal biopsies and intestinal washes (Norman et al., 2015; Wagner et al., 2013). This increase was associated with a reduction in bacterial diversity, but it remains uncertain whether bacteriophages have an implication in IBD or just reflect the underlying dysbiosis.

Similarly, when it comes to mycobiome, relatively few studies have investigated the role of fungi in IBD (Nilsson et al., 2006). A study on stool samples by Lewis et al. showed an overall increased representation of fungi in paediatric patients with Crohn's disease compared with healthy individuals, however the same five taxa accounted for the majority of fungi in both groups (Lewis et al., 2015). Yet, a study on adults with IBD described differences in the relative abundance of certain fungi comparing patients with healthy controls. In particular, fungal dysbiosis in IBD was associated with a decreased proportion of *Saccharomyces cerevisiae* and an increased abundance of *Candida albicans* (Sokol et al., 2017). Some animal model studies suggest that fungi might influence intestinal health suppressing the outgrowth of potential pathobionts

and promoting immunoregulatory pathways (Chiaro et al., 2017; Iliev et al., 2012; Lamas et al., 2016). Given few data and confounders, establishing a direct causal relationship between fungi and health or disease remains complicated. Ultimately, it is of notable importance to consider microbial metabolites. These latter can be classified as microbial products derived from diet, such as vitamin K, or de novo synthesised metabolites such as SCFAs. A third category includes molecules that were initially synthesised by the host and secreted into the lumen where they undergo modification by gut microbes such as secondary bile acids (Kho & Lal, 2018). A modification in microbial composition can consequently lead to metabolite alterations. As a matter of fact, a study comparing the composition and function of the gut microbiota of healthy individuals vs IBD patients showed that 12% of metabolic pathways were markedly different, compared with just 2% of genus-level clades. The pathway differences regarded amino acid biosynthesis and carbohydrate metabolism that were reduced in IBD microbiome in favour of nutrient uptake, virulence and secretion pathways (Morgan et al., 2012). These findings support a role for gut microbiota in the pathogenesis of IBD, although the underlying mechanisms that link gut dysbiosis to disease are not fully understood.

### **Interaction between microbiota and intestinal epithelial barrier**

The underlying mechanisms in IBD dysbiosis are very complex and still a matter of study. The proposed pathways of damage include interaction

between microbiota and receptors like the Nod-like receptor X1 (NLRX1). This intracellular receptor is a mitochondria-related NLR and is essential to retain a balanced glutamine metabolism and barrier function. The overgrowth of IBD-associated pathogens like *Veillonella* spp and *Clostridium* spp may activate NLRX1 which in turn triggers the sirtuin 1 (SIRT1) signalling pathway, resulting in dysbiosis and inflammation (Leber et al., 2018; Wellman et al., 2017). Another mechanism involves the glycosylation dysregulation of intestinal epithelial cells. This metabolic alteration leads to increased expression of truncated O-glycans. This change in glycan composition can disrupt the mucosal layer, trigger immunity and ultimately contribute to the onset of IBD (Kudelka et al., 2020). A mechanical way of damaging involves the destruction of the tight junction protein. This epithelial breach leads to *Enterobacteriaceae* intrusion, colonization and last to intestinal inflammation caused by factors released by apoptotic epithelial cells (C. J. Anderson et al., 2021; Coyne & Comstock, 2019). A dysfunction of mitochondrial work seems also to play a pivotal role in the intestinal epithelial barrier integrity. Indeed, a mitochondrial respiratory dysregulation forces epithelial cells to switch to abnormal phenotype of Paneth cells, causing metabolic imbalance and inflammation. This respiratory impairment in CD patients is also related to an increase in the relative abundance of hydrogen sulfide (H<sub>2</sub>S) producing bacteria and a reduction of H<sub>2</sub>S detoxification (Foppa et al., 2024). A final mechanism of

epithelial barrier destruction is via immune cells. In CD, over 50% of patients exhibit adherent-invasive *E.Coli* (AIEC) intestinal mucosa colonisation (Shawki & McCole, 2017). AIEC colonisation leads to inflammation through TNF- $\alpha$  release by immune cells (Palmela et al., 2018). Likewise, the invasion of *Klebsiella pneumoniae* causes macrophages activation and release of interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF) (Read et al., 2021). *Fusobacterium nucleatum* can cause intestinal inflammation promoting IL-17F/NF-kB signalling pathway, upregulating caspase recruitment domain 3 (CARD3) by nucleotide-binding oligomerization domain-containing protein 2 (NOD2) in colonic epithelial cells (Chen et al., 2020). Ultimately, Immunoglobulin A (IgA) is the most common antibody subtype in the intestine. In a stable environment, it plays a pivotal role for the dynamic balance within the gut microbiota, but if pathogenic bacteria secrete immunosuppressive protein, they can lead to the intestinal immune barrier destruction. There is, indeed, evidence about a higher presence of IgA-coated bacteria like *Prevotellaceae*, *Helicobacter*, and Segmented filamentous bacteria in IBD patients than in healthy controls (Kayama et al., 2020). A study conducted by Shapiro et al. also suggested that the analysis of IgA response to

microbiota can be used as a biomarker in IBD treatment (Shapiro et al., 2021).

All those mechanisms are illustrated in figure 2.

### Key Pathways of Microbiota–Intestinal Epithelial Barrier Interaction in IBD Dysbiosis

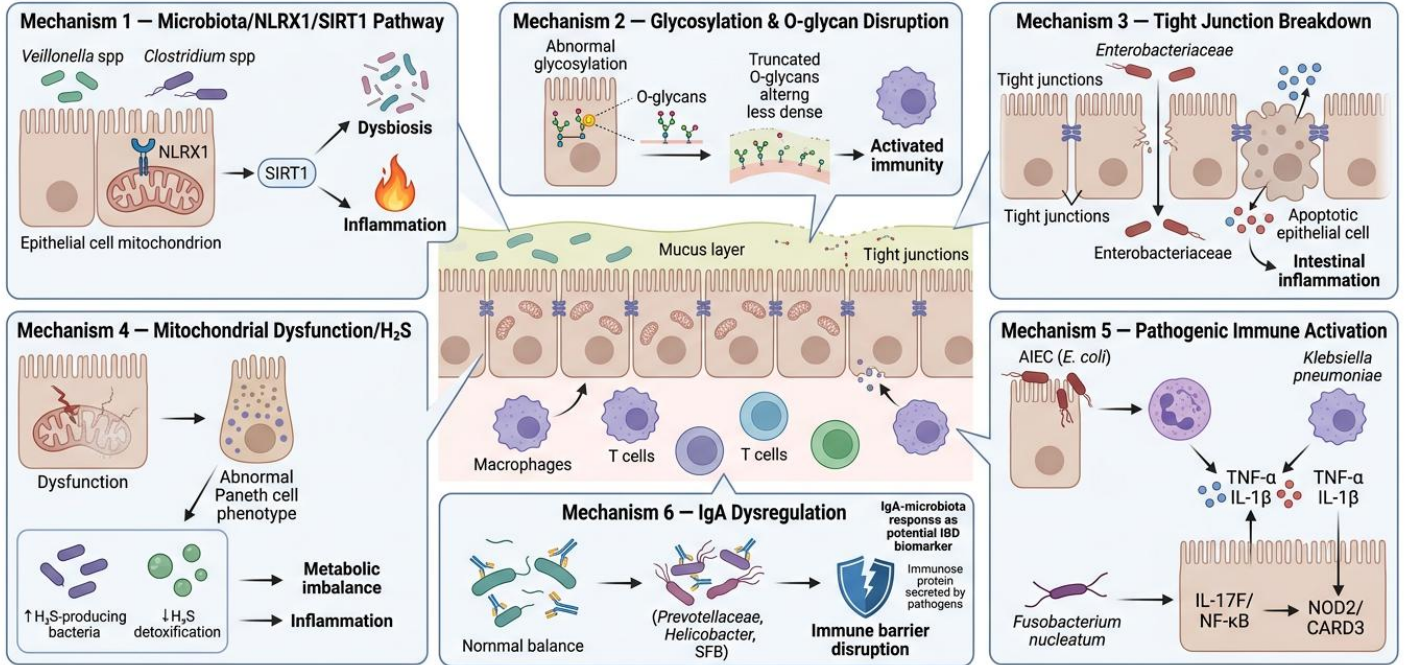


Figure 2: Summary of the multifactorial interactions between gut microbiota and the intestinal epithelial barrier in IBD, highlighting key pathogenic mechanisms leading to barrier dysfunction and inflammation.

#### 2.3.1 Interaction between microbiota, immune cells and gut-brain axis

The gut microbiota can shape the intestinal microenvironment inducing the differentiation of T lymphocytes cells, mainly in T helper 17 (Th17) and T regulatory cells (Treg) (Foppa et al., 2024). The inflammatory response of Th cells protects the host from injurious bacteria, but the overactivation of these cells is related to intestinal inflammation. In fact, IBD patients present a stronger antibody and T-cell response to microbial antigens compared to healthy subjects (Britton et al., 2020; Castellanos et al., 2018; Moayyedi et al., 2015). Another difference lies in the fact that IBD patients present an imbalance in

Th17/Treg ratio. The lack of Treg cells causes an exaggerated immune response in the intestine, leading to mucosal injury (Sun et al., 2017). Notably, gut-brain-axis communication can be modulated by gut microbiota via multiple mechanisms. Microbial-derived metabolites such as SCFAs,  $\gamma$ -aminobutyric acid (GABA), tryptophan derivatives, 5-hydroxytryptamine (5-HT), and catecholamines act as key signalling molecules that regulate intestinal resident cells (Cryan & Dinan, 2012; Forsythe & Kunze, 2012). These metabolites can also influence distant organ systems acting through endocrine pathways linking intestinal microbial dynamics to systemic neuroimmune regulation (C. Yang et al., 2026). The influence is bidirectional, since the enteric nervous system (ENS), acting through the major cellular components, glia, can affect microbiota composition. In fact, a study reported that ileal explants from glial-depleted mice secreted less functional lysozyme than controls with corresponding effects on faecal microbial composition (Prochera et al., 2024).

## **2.4 Treatment in IBD: is it time for new strategies?**

The IBD burden affects not only the individual but also the healthcare systems and global economies. The economic part embraces hospitalization costs, loss of work productivity, premature retirement and of course the cost of ongoing medications (Ananthakrishnan et al., 2020; Burisch et al., 2023). Therefore, any

strategy aiming at reducing the incidence of IBD has the potential to alleviate this burden both for the individual patient and for the healthcare system. A European observational study has demonstrated the lifestyle and dietary potential in preventing the onset of IBD (Lopes et al., 2023). Specifically, the incidence of CD could be reduced by 61.1% and UC by 42.2% for individuals who adhere to at least 7 of the following healthy living indexes: (1) minimal alcohol consumption ( $\leq 1$  drink/day for women, and  $\leq 2$  drinks/day in men); (2) maintaining a BMI between 18.5 and 25 kg/m<sup>2</sup>; (3) engaging in  $\geq 7.5$  metabolic equivalent of task hours per week of physical activity; (4) never smoking; (5) eating  $\geq 8$  servings of fruit or vegetables per day; (6) ingesting  $\geq 25$  g of fibre per day; (7) consuming  $\geq 0.5$  servings of nuts or seeds per day; (8) limiting red meat intake to  $< 0.5$  servings per day; and (9) eating  $\geq 2$  servings of fish per week (Lopes et al., 2023). Unfortunately, interventional or randomised controlled studies that could support such observational studies are currently lacking. Moreover, environmental influence on a subject may change according to ethnicity, race, or geography, as we can see by regional differences in smoking and CD risk (Molodecky et al., 2011; Piovani et al., 2021). Despite the variability, observational data are promising and, at the moment, they constitute a healthcare guidance for potential risk modulation (Chhibba et al., 2025).

#### **2.4.1 Current therapies**

Given the complexity of these multifaceted disorders, there is no single pharmaceutical treatment and above all, none is definitive. Currently, available treatments can only lead to remission, but unfortunately many patients experience relapses (B. Zhang et al., 2020). IBD cure initially involved anti-inflammatory aminosalicylates (ASA), corticosteroids, immunosuppressants and anti-tumor necrosis factor (anti-TNF) agents. 5-aminosalicylic acid (5-ASA) was initially used to treat rheumatoid arthritis, but then was also found to alleviate IBD symptoms. Its mechanism involves NF- $\kappa$ B inhibition, scavenging oxygen free radicals and interfering with pro-inflammatory metabolic products of arachidonic acid (J. Liu et al., 2023). As a result, 5-ASA can prevent intestinal epithelial cells apoptosis and suppress inflammation in the intestinal mucosa (MacDermott, 2000). The most common 5-ASA in IBD treatment are mesalazine, olsalazine, balsalazide, sulfasalazine and they are available as oral, suppository, enema and foam forms (Murray et al., n.d.). The side effects associated with this treatment include pancreatitis, cardio- and hepatorenal toxicity and sexual dysfunction (J. Liu et al., 2023). For what concerns corticosteroids, they have been one of the backbones in IBD therapy. They suppress inflammation downregulating the NF- $\kappa$ B pathway but are not effective in maintaining remission in the long term. The most commonly used are hydrocortisone, prednisone, prednisolone, budesonide and beclomethasone dipropionate. Yet, prolonged treatment with corticosteroids has numerous side

effects such as weight gain, hypertension, moon face, osteoporosis, diabetes, stretch marks, venous thromboembolism and others (Yeshi et al., 2024). Lastly, among conventional therapies, there are immunosuppressants that block the lymphocyte activation and proliferation. The most used are azathioprine (AZA), its metabolite 6-mercaptopurine (6-MP) and methotrexate (MTX). These are used in patients who are refractory to corticosteroids or, on the contrary, dependent and want to reduce their use (Chande et al., 2015). They have the advantage of low cost and good compliance being orally administered, but they also have notable adverse effects. Among others, MTX can cause bone marrow toxicity, hepatotoxicity and is absolutely contraindicated in pregnancy due to its embryo-toxicity (Lichtenstein et al., 2006a). Other immunosuppressants used are cyclosporine, used in specific UC clinical situation, and the better absorbed tacrolimus (Protic et al., 2013). These drugs are safer and better tolerated than corticosteroids in long therapy, but their side effects are still notable and require blood test monitoring (J. Liu et al., 2023). Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) has been proven to be overexpressed in intestinal laminae propria and submucosal tissues of Crohn's patients by Murch et al. in 1993 (Murch et al., 1993). Activating T cells and triggering inflammation, TNF- $\alpha$  plays an important role in the etiopathogenesis of CD and hence, TNF-  $\alpha$  blocking antibodies can alleviate inflammatory symptoms in CD patients (Deventer, 1997). Infliximab was the first TNF-  $\alpha$  antagonist adopted to treat moderate to severe active CD

and UC in patients who are refractory to traditional corticosteroids or immunosuppressive agents (Lichtenstein et al., 2006b). Other examples are adalimumab, certolizumab pegol and golimumab; these kinds of pharmaceuticals present adverse effects such as fever, urticaria, swelling and itching at injection site, vision disturbances, numbness and infections of upper respiratory and urinary tract (Berns & Hommes, 2016; Lichtenstein et al., 2006b). In the historical treatment of IBD, anti-TNF- $\alpha$  antibodies have played a key role thanks to their low cost, as well as the possibility of dose escalation in the treatment of acute severe UC episodes; for this, they remain the preferred treatment for IBD patients and infliximab and adalimumab also have biosimilars (D'Haens & Deventer, 2021). Due to their numerous side effects and limited efficacy, the conventional therapies are being substituted by more recent treatment known as small molecule drugs (SMD) and biologics. The novel small molecule drugs include Janus kinase inhibitor, sphingosine-1-phosphate receptor modulator, oral SMAD7 antisense oligonucleotide and PDE4 inhibitors. They represent a new IBD treatment option thanks to their convenient administration method, non-immunogenicity, short half-life and low cost (Baumgart & Le Berre, 2021). Janus kinase (JAK) is a non-receptor tyrosine kinase that operates cytokine signalling interacting with signal transducers and activators of transcription (STAT) (Ghoreschi et al., 2009). The inhibition of the JAK-STAT pathway reduces the synthesis of inflammatory

cytokines, inhibits the proliferation of CD<sup>+</sup> 4 T cells, and blocks the synthesis and secretion of various inflammatory cytokines (including IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 and IFN- $\gamma$ ) (Sandborn et al., 2017). Tofacitinib, Upadacitinib and Filgotinib are JAK-STAT inhibitors examples. Nonetheless, involving more than 50 cytokines signalling, their non-selective mechanism have raised safety concerns among regulators and require more clinical trials (Verden et al., 2018).

SMAD are mothers against decapentaplegic homologs, that are a regulator of the immunosuppressor TGF- $\beta$ 1 cytokine. Since SMAD7 has been shown to be overexpressed in gut submucosa of CD patients, this results in TGF-  $\beta$ 1 suppression and consequent burst of inflammation. The SMAD7 antisense oligonucleotide can impair the SMAD7 activity attenuating the clinical condition in CD patients (Troncone et al., 2018). Mongersen is an oral SMAD7 antisense oligonucleotide. Unfortunately, it concluded its path of development in phase III trial due to lack of efficacy (Bewtra et al., n.d.).

With regard to sphingosine-1-phosphate receptor modulators they act binding the sphingosine-1-phosphate (S1P) lysophospholipid. This last one is a metabolite signalling molecule derived from bioactive membranes which binds to T, B cells, dendritic and endothelial cells and is associated with differentiation migration and proliferation of these immunity cells (Marsolais & Rosen, 2009).

Ozanimod and Etrasimod are two oral S1P modulators (the latter still currently in phase II studies) (Sandborn et al., 2020). Phosphodiesterase-4 (PDE4)

inhibitors are molecules that can block the action of the enzyme phosphodiesterase-4. This protein facilitates the breakdown of cyclic adenosine monophosphate (cAMP) in inflammatory cells. Since the elevated levels of cAMP can suppress inflammation and tissue damage by leukocytes, blocking the PDE4 can help increase intracellular cAMP and quench inflammatory responses (Karuppuchamy et al., 2020). Apremilast and tetomilast are two PDE4 inhibitors which show as main adverse effects nausea, vomiting, diarrhea and headache (Bickston et al., 2012; Keating, 2017).

In addition to small molecule drugs, recently IBD treatment has entered a new era of biologics. Compared with older biologics, the newest can reduce hospitalization rate and surgical rate presenting the advantage of good efficacy, low incidence of adverse reactions and high safety (Neurath, 2017). The first group of biologics include the IL-12/IL-23 inhibitors: the IL-12 family is a set of cytokines including IL-12, IL-23, IL-27 and IL-35. IL-12 and IL-23 are pro-inflammatory cytokines which induce naive cluster of CD4+T cells to differentiate into Th1 and Th17 perpetuating inflammation (Moschen et al., 2019). Ustekinumab, risankizumab, guselkumab, brazikumab and mirikizumab are monoclonal antibodies binding IL-12 and IL-23 cytokines reducing the over-inflammation related to the etiopathogenesis of IBDs. Up to now, only ustekinumab has been approved for treating both CD and UC, and risankizumab has been approved for the treatment of CD (Verstockt et al., 2023).

In clinical trials, these new biologics have shown the possibility of maintaining the remission with an excellent safety profile. The second category comprise the integrin inhibitors. Integrin is a transmembrane adhesion receptor on leukocytes surface responsible for the migration of leukocytes from the blood to inflamed tissue (Zundler et al., 2017). The immunity cells, firmly sticking to the surface of endothelial cells, enhance the immune defence but aggravate the destruction of intestinal tissue as well. Natalizumab, vedolizumab, etrolizumab, and ontamalimab can specifically bind to the integrin expressed by lymphocytes and avoid their migration to inflammatory tissues. Moreover, they have a high intestinal selectivity and so they prove better clinical therapeutic effect on IBD patients (J. Liu et al., 2023).

#### **2.4.2 Adjuvant therapy**

In order to improve the therapeutic effect and life quality of IBD patients, some supporting factors have been investigated, among which was the use of probiotics, polyphenols, faecal microbiota transplantation, mesenchymal stem cell therapy and dietary intervention. Probiotics have some clinical data supporting the efficacy in IBD preventing postoperative recurrence, and pouchitis treatment (Glassner et al., 2020). More in particular, specific probiotic strains like *Lactobacillus plantarum* CKCC1312 and *L. fermentum* CKCC1858 have

proven beneficial for UC promoting mucosal integrity (A. Li et al., 2024). Another example brought by *Bacteroides thetaiotaomicron* showed improved clinical symptoms of dextran sulfate sodium (DSS)-induced colitis in mice: it could promote the differentiation of Treg/Th2 cells and suppress Th1/Th17 cell development (K. Li et al., 2021). Polyphenols and their microbial metabolites are documented to reduce the secretion of inflammatory cytokines like TNF- $\alpha$ , IL-6, IL-8, and IL-1 $\beta$  protecting gut barrier and modulating the immune function (H. Li et al., 2020). Last, dietary interventions can correct nutrient deficiencies but also improve clinical symptoms modulating intestinal microbiota and influencing epithelial barrier functions (Green et al., 2019).

#### **2.4.3 The role of physical activity**

Exercise training covers actually a heterogeneous group of activity like endurance, resistance, aerobic exercise training and others that vary in intensity, frequency, and duration (Bilski et al., 2014). In contrast to structured exercise training, physical activity is defined as any bodily movement produced by skeletal muscle contractions resulting in increased energy expenditure (Caspersen et al., 1985). Literature confirms that physical activity is inversely related with systemic inflammation. This beneficial effect could suggest a helpful tool especially in cases of chronic disease (Wilund, 2007). The intensity and duration of activity are also relevant, since the intensive exercise may induce a transient systemic inflammation and increased level of cytokines

suggesting a delicate balance between exercise intensity and its health benefits. In fact, prolonged and strenuous exercise causes impairment of the immune system function for several hours (Hoffman-Goetz & Pedersen, 1994; Pedersen & Nieman, 1998). On the contrary, regular moderate training has been proven to increase resistance to infections and so, immunity defence (Bartram et al., 2006; Fitzgerald, 1988; Walsh et al., 2011, 2011). In literature, the majority of studies have examined the beneficial effects of exercise in terms of quality of life and general fitness but relatively few have explored effects on IBD pathogenesis and measures of inflammation (Bilski et al., 2014). Among the few that explored benefits at molecular level, the proposed mechanisms comprise, for example, the anti-inflammatory effect through visceral fat mass reduction and/or by secretion of anti-inflammatory muscle-derived peptides, the so-called “myokines” (Pedersen & Febbraio, 2012). This means that exercise could stand as a first preventive tool against diseases that come from chronic inflammation. Other benefits besides the anti-inflammatory action, are improved flexibility, strength, reduced pain of the joints, prevention of osteoporosis (common in IBD), and in paediatric patients, especially with CD, improvement of growth failure, weight loss, and anemia (Bilski et al., 2013; Siffledeen et al., 2007; Uhrin et al., 2000). The fatigue is a common symptom in CD, even in quiescent state of the disease, and it’s probably mediated, at least in part, by cytokines (Minderhoud et al., 2007). The exercise proved to be helpful in such cases, but

also in quality of life and stress improvement (Minderhoud et al., 2004, 2007; Pedersen & Saltin, 2006; van Langenberg & Gibson, 2014).

The effects of physical activity on the gastrointestinal tract, particularly on gut microbiome, has been explored in animals and in humans, even though little. A study by Mika et al. observed a group of rats divided into two groups: a voluntary exercise group, having a wheel in the cage and a sedentary control group without access to a wheel. The voluntary aerobic exercise group showed an increased abundance of beneficial bacteria like *Lactobacillus* and *Bifidobacterium* compared to control group (Mika et al., 2015). Studies on humans reported similar results. In a review conducted by Varghese et al. interventions based on multiple exercise routines such as aerobic and resistance training, had more significant influence on bacterial diversity than, for instance, resistance training alone (Varghese et al., 2024). Moreover, just like the anti-inflammatory effects, low/medium intensity exercise may have a limited positive impact on gut microbiota diversity compared to high intensity. Moreover, the relationship between physical activity and gut microbiome appears to be bidirectional. In fact, some studies showed probiotic supplementation is associated with decreased inflammation, enhanced sports performance, and fewer gastrointestinal disturbances (Varghese et al., 2024). The proposed mechanisms through which the gut microbiota enhances muscle metabolism and health are various. For example, gut microbiota significantly

influences pathways involving AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin(mTOR): these are indeed pivotal in regulating muscle synthesis, but also glucose, protein and lipid synthesis (Bakker & Jaspers, 2015; Marcondes-de-Castro et al., 2023). Also, SCFAs produced by bacteria, can directly activate AMPK in muscles promoting its development and growth (Thomson, 2018). A specific SCFA, butyrate, can increase oxidative phosphorylation, thus boosting mitochondrial activities and supporting aerobic metabolism in skeletal muscles (S. Rose et al., 2018). Gut microbiome can impact also hormones production like insulin and androgens that are fundamental for protein synthesis and muscle growth (Qi et al., 2021). Elevated levels of *Bacteroides*, known for their pro-inflammatory actions like insulin resistance, lipogenesis and others, are commonly observed in subjects with high levels of androgens, such as polycystic ovary syndrome (Lin et al., 2021). Last, excessive exercise can cause a sort of stress that stimulate the hypothalamic–pituitary–adrenal axis. Corticotropin-releasing factor, in fact, alters gastrointestinal function, influences inflammatory processes, mucosal secretory functions and barrier functions causing increased intestinal permeability, which may in turn allow bacteria migration from the intestines and cause gastrointestinal issues (Chatoo et al., 2018).

For what concerns the preventive aspect, fewer studies are available than for the therapeutic aspect and, above all, they do not align. It is also worth

mentioning that some studies have explored the support given by meditation and mindfulness interventions in IBD disease management. As reported by Naude et al. review, mindfulness interventions produced more efficacious results in terms of stress, C-reactive protein and health-related quality of life compared to controls (Naude et al., 2023). Moreover, a very helpful tool for patients with chronic inflammatory diseases like IBD can be a Meditation in Motion discipline that fuses physical activity and meditation. In fact, the analysis conducted on some aikido practitioners found an increase of *Bacteroides*, *Faecalibacterium*, *Firmicutes* and a promotion of a balanced *Bacteroidetes/Firmicutes* ratio (Volpini et al., 2026). Despite these promising findings, the role of physical exercise in IBD and its interaction with the microbiota remain difficult to assess due to different experimental design conducted and different types, intensity, and most of all because of non-unique results.

#### **2.4.4 The role of the diet**

Studying methodologically the role of diet in IBD is not easy since there is a limitation in accurately capturing dietary intake as well as the potential for complex interactions between foods (Willett, 2013). Further, differences in food metabolism can vary among individuals. The same food products can even differ, based on preservation, processing, packaging, and preparation (D. Lee et al., 2015). Moreover, it is extremely difficult to recruit newly diagnosed patients

to clinical trials. Therefore, these dietary trials are impacted by the influence of medical therapies. Anyway, there are sufficient data to assert guidelines regarding the aetiology and management of IBD. Talking about the last, human studies about food and IBD can be categorized into elimination diets, exclusion of specific inflammatory mediators, inclusion of anti-inflammatory mediators, and inclusion of prebiotics. Of these, only exclusive enteral nutrition (EEN) dietary intervention with elemental, semi-elemental, or polymeric formula diets has been rigorously, widely studied and shown to induce remission of CD (Borrelli et al., 2006). In addition to reducing symptoms of CD, EEN has been associated also with mucosal healing, which may be a superior predictor of long-term outcome (Fell et al., 2000; Frøslie et al., 2007). The restriction diets such as the specific Carbohydrate and Paleolithic diet, exclude respectively all sugars other than monosaccharides: glucose, fructose, and galactose and processed meats, and emphasize intake of lean meats from non-domesticated animals as well as fruits and vegetables. However, these diets are shared without guidance from the medical community and there are insufficient well-conducted studies to prove their efficacy (D. Lee et al., 2015).

Regarding diet as prevention, as anticipated in previous chapters, the scientific community agrees on an increased risk of IBD among people who consume greater amounts of meat and fats—particularly polyunsaturated fatty acids and omega-6 fatty acids—and lower risk among people with diets high in fibre,

fruits, and vegetables (J. K. Hou et al., 2011). Diet alone is insufficient to cause IBD, but clearly a balanced diet rich in fibre and fruits represents a valid risk-lowering tool. Indole-3-carbinol, present in cruciferous vegetables and fruits, binds to the aryl hydrocarbon receptor and can attenuate colitis in mice (Ananthakrishnan, Khalili, et al., 2013). In an in vitro study fibre has proven to reduce *E.Coli* translocation across Peyer's patches, contributing to maintain epithelial barrier's integrity (Roberts et al., 2010). About dietary fat, an in vivo study on mice revealed that a high-saturated-fat diet promotes the growth of a sulphite-reducing pathobiont, *Bilophila wadsworthia*, and was associated with an inflammatory response (Devkota et al., 2012b). In small clinical case-control studies saturated fats have been associated with IBD risk, but prospective cohort studies did not identify such an association, offering a more complex effect overview (Amre et al., 2007; Ananthakrishnan, Khalili, Konijeti, Higuchi, de Silva, Fuchs, Willett, et al., 2014; Cabré & Domènech, 2012; Chapman-Kiddell et al., 2010; Hart et al., 2008). Further, a higher intake of n-6 polyunsaturated fatty acids (omega-6 PUFA) with respect to n-3 PUFA consumption has been associated with an increased risk of both UC and CD, but this association can be modified by polymorphism in fatty acid metabolising enzymes (Costea et al., 2014). Moving to carbohydrates, refined sugars and animal protein there is no consistent association with IBD (Chan et al., 2014; Chapman-Kiddell et al., 2010; J. K. Hou et al., 2011; Jantchou et al., 2010). In addition to direct interaction with

immune cells and receptors, diet has an influence on inflammation via gut microbiota modulation (Gill et al., 2022). Actually, macro- and micronutrients all contain components that can act as substrates for microbiota. Species such as *Roseburia* and *Faecalibacterium* are more abundant in subjects who follow a diet rich in fibre (e.g. legumes, cereals, fruits and vegetables) and they have been associated with lower intestinal inflammatory markers. This suggests that diet composition may indirectly regulate inflammation via gut microbes (Bolte et al., 2021). Ultimately, laboratory studies have given biological plausibility to a relationship between IBD and micronutrients, for example vitamin D seems to have a role in the pathogenesis and course of IBD (Cantorna et al., 2004; Cantorna & Mahon, 2004, 2005). It has been documented that, in a mouse model, mice knockout for the vitamin D receptor or deficiency of 1,25-dihydroxy vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) is associated with a higher risk of colitis. Administering 1,25(OH)<sub>2</sub>D<sub>3</sub> (vitamin D active form) improves inflammation and suppress proinflammatory gene expression (Cantorna et al., 2000; Froicu et al., 2003, 2006; Froicu & Cantorna, 2007). Patients that have just been diagnosed with IBD have commonly a vitamin D deficiency and it is also more common than in healthy individuals (Mouli & Ananthakrishnan, 2014). Such evidence has been reinforced by prediction through validate regression model: women in the highest quartile of predicted vitamin D (median 32.2 ng/ml) had a significantly lower risk for CD than those in the lowest quartile (OR 0.54, 95%

CI 0.30–0.99) (Ananthakrishnan, Khalili, et al., 2012). Zinc can inhibit pro-inflammatory mediators transcription in the NF- $\kappa$ B pathway and reduce myeloperoxidase activity; specifically in CD, intracellular zinc is relevant for autophagy, bacterial clearance, reduces intestinal permeability and, in a small study, seems to reduce relapses probability (El-Tawil, 2012; Haase & Rink, 2014; Lahiri & Abraham, 2014). On the contrary, dietary iron might cause colonic inflammation through IL-6–IL-11–STAT3 signalling (Chua et al., 2013) and increment of oxidative stress (Carrier et al., 2001). However, oral iron replacement studies in established disease do not give clinical support to an adverse effect of dietary iron. In conclusion, given its benefits, rich in fibre and fruit, low in red meat and processed food, and already a commonly recommended pattern for IBD patients, the Mediterranean diet (MD) meets all these requirements. MD has been associated with increased microbial diversity, and increased abundance of butyrate-producing bacteria in healthy subjects (Godny et al., 2022; Liao et al., 2024; D. D. Wang et al., 2021), patients with quiescent ulcerative colitis (Haskey et al., 2023), and first-degree relatives of patients with IBD (Turpin et al., 2022). MD is also associated with a variety of outcomes in patients with IBD, including reversing disease activity indices, decreasing inflammatory markers and improved metabolic status and quality of life (Godny et al., 2025).

#### 2.4.5 Natural products

There are some natural products and derivatives that have shown protection properties against colonic inflammation through restoring epithelial tight junctions, increasing mucin secretion, preventing luminal microbial dysbiosis, and reducing oxidative stress in the gut (Q. Xu et al., 2023). For instance, curcumin isolated from *Curcuma longa* in combination with artesunate (isolated from *Artemisia annua*) have proven promising results against IBD and they are currently under phase II clinical trial for CD (Iyengar et al., 2024). Berberine, epigallocatechin-3-gallate (isolated from *Camellia sinensis*) and triptolide (isolated from *Tripterygium wilfordii*) are three other compounds that showed anti-inflammatory properties applicable to IBD (Yeshe et al., 2024). Beyond plants, there have been studies using helminths and their products showing an improvement in inflammation in IBD animal models. In fact, mice infected with *Schistosoma mansoni* or its eggs showed reduced colonic inflammation after colitis induction via trinitrobenzene sulfonic acid (TNBS) (Shi et al., 2022). Similarly, low-molecular-weight metabolite from *Ancylostoma caninum* secretions proved reducing IL-23, TNF, and IL-1 $\beta$  cytokines protecting against TNBS-induced colitis in mice (Jamtsho et al., 2024). Also fungal kingdom showed potential in treating IBD: *Auricularia polytricha* and *Flammulina velutipes* seem to control NF- $\kappa$ B and Keap1/Nrf2 pathways, and alter the gut microbiota

making them candidates as probiotics for gut health (Nguepi Tsopmeji et al., 2022). Also, Dai et al. isolated 27 compounds from the marine *Penicillium* sp. ZYX-Z-143 including indole-terpenoids with significant anti-inflammatory activity (Dai et al., 2024).

#### **2.4.6 S100B protein: an interesting perspective**

S100B is a calcium-binding protein identified in 1965 that owes its name to its characteristic solubility in 100% saturated ammonium sulphate solution (Moore, 1965). It is part of the S100 protein family that comprises more than 20 calcium-binding proteins exhibiting structural similarities. S100B is composed of two homologues beta subunits, each of 9-14 kDa, constituting the core of the protein fraction originally isolated from brain extracts (Michetti et al., 2019). In nervous tissue, this protein has been shown to be concentrated in astrocytes and other glial cell types, such as oligodendrocytes, Schwann cells, ependymal cells, retinal Muller cells, and also enteric glial cells (Brookes et al., 1979; Didier et al., 1986; Ferri et al., 1982; Ludwin et al., 1976; Rickmann & Wolff, 1995). There are also some studies showing its presence in other interesting and non-neural tissues like immune cells, endocrine cells, stem cells and adipose tissue (Cocchia et al., 1981; Michetti et al., 1985; Stefansson et al., 1982; Tubaro et al., 2010). Its intracellular function does not appear clear: it seems to intervene in cell proliferation, survival, differentiation and to participate in calcium homeostasis regulation and enzyme activities (Michetti et al., 2019). On the other hand, it

seems easier to imagine its function in the extracellular compartment. Indeed, its presence in biological fluids has been widely investigated: it has been first recognized at elevated levels in the cerebrospinal fluid of multiple sclerosis patients in acute phase (but lower levels were found in the stationary phase) (Michetti et al., 1979). Other biological fluids where the protein has been detected were peripheral blood, cord blood, urine, amniotic fluid, saliva and human milk (Gazzolo, Bruschetti, Corvino, et al., 2001; Gazzolo, Bruschetti, Lituanica, et al., 2001; Gazzolo et al., 2000, 2003; Gazzolo, Florio, et al., 2005; Gazzolo, Lituanica, et al., 2005; KATO et al., 1983). All these matrices investigated revealed increased levels under various pathological conditions such as neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis), congenital/perinatal disorders like Trisomy 21, pre-term and full-term asphyxiated newborns, intrauterine growth retarded fetuses, and psychiatric disorders like schizophrenia and mood disorders. To date, S100B is considered a well-established biomarker also for malignant melanoma, and useful in assessing tumour load, stage and prognosis for those patients (Gambichler et al., 2018; Zarogoulidis et al., 2015). Out of the cell S100B can interact with surrounding cell types through the Receptor for Advanced Glycation Endproducts (RAGE) (Hofmann et al., 1999; Huttunen et al., 2000). This receptor initiates a complex intracellular signalling pathway which may include also the neuroinflammation. Extracellular S100B

biological activity has been curiously associated with its concentration. Many studies reported, at low (nanomolar) concentrations, neurotrophic effects such as promotion of neurite extension, sustain neuron survival, neutralization of neurotoxicant insults and increased reactive oxygen species (ROS) scavenger activity (Ahlemeyer et al., 2000; Barger et al., 1995; Bhattacharyya et al., 1992; Businaro et al., 2006; Clementi et al., n.d.; Haglid et al., 1997; Iwasaki et al., 1997; Kligman & Marshak, 1985; 'Neurite Extension and Neuronal Survival Activities of Recombinant S100 Beta Proteins That Differ in the Content and Position of Cysteine Residues', 1989; Nishiyama et al., 2002; Reali et al., 2005). In contrast, high (micromolar) concentrations have been associated with toxic/pro-inflammatory effects like up-regulation of inducible nitric oxide synthase (iNOS), NO release, NO-dependent death of neurons and glia, glutamate-induced neuronal death, cyclooxygenase-2 (COX-2) up-regulation, increased ROS production, perturbation of lipid homeostasis and oligodendrogenesis perturbation (Bernardini et al., 2018; Bianchi et al., 2010; Esposito et al., 2007; Hu et al., 1996, 1997; Huttunen et al., 2000; Petrova et al., 2000; Reali et al., 2012; Santos et al., 2018; Shanmugam et al., 2008; Villarreal et al., 2011). The association between high S100B concentration and inflammation has been proven also in the gut district: a study conducted by Esposito et al. demonstrated that S100B overexpression is associated with upregulation of iNOS and increased NO release in human ulcerative colitis biopsies (Cirillo et

al., 2009; G. Esposito et al., 2007). The involvement of S100B in colitis was proved using the antiprotozoal drug pentamidine, a selective inhibitor of S100B function, in a colitis animal model, resulting in a decrease of the severity and symptoms of the illness (G. Esposito et al., 2012). An intriguing aspect that contrasts these findings is that S100B serum and feces levels in patients with ulcerative colitis have been found lower than healthy controls (Celikbilek et al., 2014; Di Liddo, 2020). The peculiar gastroenteric compartment and/or possible interactions with microbiota might explain this discrepancy. Actually, the possible interactions of S100B with microbiota have been investigated in an *in silico* study conducted by Orsini et al. where proteins putatively interacting with S100B domains were found reduced in IBD patients with respect to healthy subjects (Orsini et al., 2020). Moreover, an *in vitro* study showed that S100B in cultured enteroglia cells was differentially expressed after exposure to probiotic or pathogen bacteria. This result suggests that enteric glia may actively respond to microbes by modulating their S100B levels (Turco et al., 2014). Based on these outcomes, an *in vivo* descriptive study found a positive correlation between microbiota biodiversity and the levels of S100B in whole colon tissue (mucosa and lumen content). Three distinct profiles were identified: the eubiotic genera were mainly concentrated in the group that had associated nanomolar concentrations of S100B. Potentially harbouring genera instead, often appeared in groups where S100B concentrations were very low

or high (Romano Spica et al., 2023). This association was confirmed in mice treated with Pentamidine where the decreased S100B activity was associated with a significantly reduced microbiota heterogeneity. Recently, the studies aiming to better understand this protein are increasing. A particular branch of them, starting from its presence in human milk, wondered if it would be present in atypical samples and they got very interesting outcomes. In fact, first S100B-like immunoreactive material has been detected in some invertebrates animal like the planarian (*Dugesia gonocephala*), the silkworm (*Bombyx mori*), the earthworm (*Pheretima communissima*) and the cockroach (*Periplaneta americana*) (Endo & Endo, 1988; Michetti & Cocchia, 1982). Afterwards, the protein was searched and found also in vegetable matrix such as spinach (*Spinacia oleracea*), but also in human milk, other mammalian milks like donkey's, goat's, sheep's and cow's and in dairy products (Galvano et al., 2009; Gazzolo et al., 2003; Michetti et al., 1992). Its very high conservation during phylogeny suggests a key role in different species. This finding is of singular interest, as considering S100B as a natural component of different foods, opens up the prospect towards a potential role in diet. These premises set up a study by our group pointing at estimating the quantity of S100B in different dietary patterns. This comparative research collected data from RCTs and observational cohort studies documenting dietary patterns and relative chronic disease risk (Ghaffar et al., 2025). The study concluded that dietary patterns rich in S100B, such as

Mediterranean and Italian diets, are correlated with higher alpha-diversity and lower estimated risk of inflammatory conditions such as Crohn's disease. These results support the biological plausibility of S100B as a modulator of the gut ecosystem, potentially acting through immunomodulatory and trophic mechanisms. It is also relevant to remind the key role played by breastfeeding as first source of nutrition of an individual. It is known in literature that breast milk contains a large number of agents protecting against infection, including anti-microbial, anti-inflammatory, and immunomodulatory agents that could potentially influence the development of chronic diseases (Rodriguez-Palmero et al., 1999). It also exerts a strong influence on intestinal microbial composition, leading to higher abundances of *Firmicutes* and *Actinobacteria* when compared to infants fed with infant milk formula (Azad et al., 2013; Hill et al., 2017). Furthermore, it has been documented that formula fed infants have a greater abundance of *Peptostreptococci* including *Clostridia*, which are associated with allergic sensitization and atopic response (Penders et al., 2006). Its protective feature has been reported also in a mouse model of IBD: animals were IL-10 deficient and the group that received breast milk resulted in minimal colonic inflammation compared with control group (Madsen et al., 2002). Afterwards, studies on humans have examined and proved its protective role in rheumatoid arthritis, celiac disease, type 1 diabetes (even though this last two findings are less robust) (Pieścik-Lech et al., 2017; Silano et al., 2016) and IBD. In addition to

this, the presence of a high concentration of S100B in breast milk should come as no surprise, given its putative neurotrophic role and correlating it to the milk's already known stimulating effect on newborn brain maturation (S. B. Amin et al., 2000; Gordon, 1997).

### **3. Aim of the study**

As discussed above, current pharmacological therapies for IBD have significantly improved disease management; however, important challenges remain, including primary non-response, loss of therapeutic efficacy over time, adverse effects, and long-term safety concerns. These limitations highlight the need to identify complementary strategies that can improve disease prevention and management. Increasing evidence suggests that lifestyle factors, particularly diet and physical activity, play a pivotal role in shaping the gut microbiota and influencing intestinal health. Therefore, integrating personalized nutritional interventions, microbiota-targeted strategies, and healthy lifestyle habits with conventional pharmacological treatments may represent a promising approach to improve clinical outcomes and patients' quality of life.

The overall aim of this thesis is to investigate the relationship between lifestyle factors, gut microbiota, and intestinal health in IBD. Specifically, this work seeks to better understand how physical activity, dietary patterns, and microbiota-modulating factors contribute to disease prevention, progression, and recovery, while exploring novel nutritional strategies that may complement current therapeutic approaches.

To address this objective, the thesis is organized into five complementary research areas.

First, a systematic review evaluates the current evidence regarding the role of physical activity in the prevention and management of IBD.

Second, an observational clinical study investigates the associations among adherence to the Mediterranean diet, physical activity, psychological status, gut permeability, dysbiosis, and gut microbiota composition in patients with IBD compared with healthy controls.

Third, the influence of maternal diet and lifestyle on maternal microbiota composition and vertical microbial transmission is explored, together with the relationship between breast milk S100B levels and maternal nutritional status.

Fourth, the potential of S100B as a novel dietary bioactive molecule is investigated by evaluating its presence in plant-derived food matrices, with the goal of identifying new nutritional sources capable of supporting gut health.

Finally, an in vivo experimental study examines whether orally administered physiological concentrations of S100B can modulate gut microbiota composition, while also assessing its intestinal fate and optimizing faecal sample preservation methods for microbiome analyses.

Together, these studies aim to provide a comprehensive understanding of how lifestyle and microbiota interact in maintaining intestinal homeostasis and influencing IBD, ultimately contributing to the development of innovative, microbiota-centered preventive and therapeutic strategies.

## **4. Materials and methods**

It is worth noting that all the statistical analyses, coding skills and metagenomic data management here conducted have been acquired during the six-month period abroad at the APC Microbiome Ireland.

### **4.1 Literature review**

A first review of literature limited to physical activity (PA) and IBD was carried out to investigate the role of physical exercise in prevention and management of the chronic disease. The relevant data literature was collected through a systematic search on the following digital databases: PubMed, Scopus, Web of Science and Cochrane Library. The combination of the keywords (“physical activity” OR “exercise\*”) AND (“IBD” OR “Inflammatory bowel disease\*” OR “gastrointestinal inflammatory disease\*” OR “Crohn’s disease” OR “ulcerative colitis”) was used for the research in the aforementioned databases. Specifically, the research on PubMed was carried out by searching title, abstract and MeSH terms; on Scopus, Web of Science and Cochrane Library it included topic by title, abstract and keywords. The search was performed from 10 October 2023 to 31 October 2023. The review was conducted according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines (Page et al., 2021). The protocol was registered in PROSPERO (CRD42023446860). The review question was framed using the PICO

framework and the following eligibility criteria (a) Population: adult individuals of any gender; (b) Intervention: any type of PA, including exercise and sport; (c) Comparison: age-, gender- and condition-matched control group (if present); (d) Outcome: assessment of the effects of PA in prevention or treatment of IBD; (e) Study: observational studies, semi-experimental and experimental studies. The review focused on the role of PA in prevention and therapy of IBD in adults of any gender. Only studies which measured PA and its effects on human IBD development and progression were considered eligible. Only those studies which specifically analysed the role of PA in prevention or treatment of IBD among adult patients of any gender were considered. Furthermore, the possible presence of confounding factors was also considered. All the studies which included individuals under the age of 18 years who were affected by any chronic conditions other than IBD or did not concern PA were excluded. Only articles presenting observational studies, semi-experimental and experimental studies were considered eligible. Reviews, meta-analyses, case studies, proceedings, qualitative studies, editorials, commentary studies and any other type were excluded. Reviews and meta-analyses were examined to identify further articles in their references that may not have been included in the baseline research results. Only articles published in English and Italian were included. Titles and abstracts acquired from the four databases were transferred to the reference software Zotero systematic review

manager for the relevance assessment process. In the next step, potentially eligible studies were screened by title and abstract, following the previously stated inclusion criteria; the screening was conducted by four authors independently. Then, full texts were read independently by the same four authors and disagreements were mediated by the evaluation of three other authors. The following data were extracted: author, publication year, country, sample characteristics (size, age, gender, ethnicity, socio-economic status, education, anthropometric parameters), type of control, type of IBD and disease status, PA-related information (type, frequency and duration of PA/exercise), main outcomes, confounding factors assessed and main findings. The quality assessment was performed using the Newcastle–Ottawa Quality Assessment Scale (NOS) for cohort, case–control studies and clinical trials, then adapted from cohort and case control studies to perform a quality assessment for cross-sectional studies (Wells et al., 2021). An overall rating of “poor”, “fair” or “good” quality was assigned to each eligible article according to the proportion of NOS criteria met. The assignment was done by four authors independently. The database search yielded a total of 3129 records. Of these, 1147 duplicates were removed and 1982 were screened by title and abstract. The eligible articles were 68, and after the full-text assessment, 29 out of 68 were then included in the review.

## **4.2 Observational study on IBD cohort**

In collaboration with the University of Rome, “Campus Bio Medico”, clinical data about lifestyle, psychological status and stool microbiota composition from a cohort of subjects affected by IBD were collected. The study protocol was approved by the Ethics Committee of Campus Bio-Medico University of Rome (Protocol number PAR: 001.23(109.22) OSS, accepted:18/05/2023), in accordance with the Declaration of Helsinki. The study population selected was subjects with stable weight belonging to the Gastroenterology UOC clinics of the Policlinico Universitario Campus Bio-Medico of Rome affected by inflammatory bowel disease, (both Crohn’s disease and ulcerative colitis) and healthy controls. Consecutive enrolment began in January 2023. These were divided into two groups “IBD” or “Control” based on their health status. The inclusion criteria for patients with IBD are (1) Diagnosis of IBD for at least 3 months according to the Montreal classification (Satsangi et al., 2006), in subjects never treated with biological therapy (naïve) or in maintenance therapy with drugs belonging to the anti-TNF class such as Infliximab, Adalimumab and Golimumab (2) Patients affected by IBD with colonic, ileal or ileocolic localization. (3) Patients aged between 18 and 60 years. (4) Residence at the same address for the last 5 years. Exclusion criteria: (1) Taking antibiotics and/or probiotics in the last 3 months. (2) Taking any biological therapy except infliximab. (3) Taking nonsteroidal anti-inflammatory drugs (NSAIDs) in the

last 3 months. (4) Recent diagnosis (in the last 3 months) of bacterial or parasitic infection of the gastrointestinal tract. (5) Cigarette smoking.

Inclusion criteria for controls: (1) Asymptomatic subjects for gastrointestinal tract diseases/disorders (selected using a questionnaire to exclude chronic diseases and each gastrointestinal tract symptom present). (2) Subjects aged between 18 and 60 years of age. (3) Subjects, if aged 45 years or older, who have undergone at least once in the 5 years preceding colonoscopy to screen for colorectal cancer that has documented the absence of macroscopic lesions (including the presence of colonic diverticula) and the absence of microscopic changes on histological examination of colonic biopsies. (4) Residence at the same address for the last 5 years.

Exclusion criteria: (1) Assumption of antibiotics and/or probiotics in the last two weeks. (2) Taking nonsteroidal anti-inflammatory drugs (NSAIDs) in the last 3 months. (3) Recent diagnosis (in the last 3 months) of bacterial or parasitic infection of the gastrointestinal tract (4) Coexistence of severe psychiatric illness as the predominant clinical problem. (5) Coexistence of other severe diseases and/or medical history of abuse of alcoholic beverages and/or narcotic substances. (6) Cigarette smoking. The clinical protocol provides that all enrolled subjects signed a written informed consent to participate in the study. Every participant underwent anamnesis data collection, physical examination, anthropometric measurement and blood samples were collected after fasting.

Dietary habits were assessed using the Food Frequency Questionnaire (FFQ), and a food diary was also completed in the three days preceding faecal sample collection. For each patient, a stool and urine sample were collected to test for zonulin and indican respectively. Psychological parameters like anxiety and depression status were evaluated with the Hospital Anxiety and Depression Scale (HADS). This self-assessment questionnaire consists of 14 questions divided into two subscales. Each subscale contains 7 items scored from 0 to 3, resulting in individual subscale scores ranging from 0 to 21. (Zigmond & Snaith, 1983). The score of each subscale was then interpreted non-depressed/non-anxious with a score from 0 to 10, while a score of 11 or more was considered depressed/anxious. Lastly, data about physical activity was collected through the validated International Physical Activity Questionnaire (IPAQ) (Craig et al., 2003).

#### **4.2.1 Data preparation**

From the total of 89 subjects three were eliminated due to lacking data. Based on the Food Frequency Questionnaire, in order to assess a level of adherence to the Mediterranean Diet dietary pattern, the Mediterranean Diet Serving Score (MDSS) was used: the MDSS is an index based on the latest revision of the Mediterranean food pyramid, which considers food groups based on the recommended intake frequency (Monteagudo et al., 2015). Foods were divided into three categories: those recommended at each meal: fruit, vegetables, extra

virgin olive oil, and cereals; those to be consumed daily such as dairy products and dried fruit; and those recommended weekly like potatoes, legumes, eggs, fish, white meats, red meats, and sweets. A score was awarded only if the frequency of consumption met the guidelines: 3 points for foods to be consumed at each meal; 2 points for those with daily frequency; 1 point for those with weekly frequency. The overall MDSS score could range from 0 to 24; a lower value than 16 indicated low adherence to the Mediterranean diet, while a score equal to or greater than 16 reflected good adherence to the Mediterranean nutritional model. Moreover, the answers given by patients to IPAQ were used to calculate levels of Metabolic equivalent per minute a week (MET-min/week) and relative IPAQ-scores through the R package IPAQlong, (version 0.1.0) (Ponce-de-Leon, 2022).

#### **4.2.2 rDNA extraction and metagenomic analysis**

DNA extraction from each faecal sample from IBD cohort participants was realized using QIAmp PowerFaecal Pro DNA kit (Qiagen, USA), in accordance with the manufacturer's instructions. Metagenomic analysis (DNA isolation and quantification; 16s rDNA amplification; illumina MiSeq sequencing and bioinformatic analysis) was performed on stool samples according to the following procedures. Briefly, after thawing, faecal samples ( $\leq 150$  mg) were aliquoted and transferred into 2 mL screw cap tubes containing glass beads and BashingBead™ lysis buffer (Zymo Research, Tustin, CA, USA). The tubes were

then vortexed for 30 min on an iSWIX System (Neuation Technologies, Gujarat, India), and up to 400 µL of the supernatant was used for DNA extraction using the Quick-DNA™ Faecal/Soil Microbe Miniprep Kit (Zymo Research, USA), according to the manufacturer's instructions. DNA was quantified using fluorescence spectroscopy (Quant-iT™ PicoGreen® dsDNA Assay Kit, Thermo Fisher, Waltham, MA, USA), and its integrity was checked using agarose gel electrophoresis. Dual-indexed universal primers (341F, CCTACGGGNGGCWGCAG, and 802R, GACTACHVGGGTATCTAATCC; Illumina, San Diego, CA, USA) were used for two-step PCR amplification of the V3–V4 hypervariable regions of the 16s rRNA gene (16S/ITS Nextera two-step PCR kit, Illumina Inc., USA), according to the procedure described by Filardo et al. (Filardo et al., 2022). The sequencing data were classified into operational taxonomic units (OTUs) using the software framework QIIME2 (version 2023.9-amplicon). Bioinformatic analysis of the OTUs was performed by normalizing all samples to the sample with the lowest read count for alpha diversity comparisons. Faith's phylogenetic diversity (PD) index, Shannon's Diversity Index and Observed Features Diversity Index were used as metrics for alpha rarefaction analysis.

#### **4.2.3 Determination of indican in urine samples**

Indican levels were assessed in urine samples from IBD cohort by colorimetric quantitative assay kit (KA3944, Indican Assay Kit, ABNova Corporation,

Taipei, Taiwan) following the manufacturer's instructions. Quantification was performed using a five-point calibration curve in the range of 0.2–20 mg/dL.

#### **4.2.4 Determination of zonulin in faecal samples**

Faecal zonulin concentration was measured in triplicate using a competitive enzyme-linked immunosorbent assay (ELISA) (IDK® Zonulin ELISA Kit, Immunodiagnostik AG, Bensheim, Germany), following the manufacturer's instructions. Results were expressed as ng per mL. According to the manufacturer's specifications, the intra-assay and inter-assay coefficients of variation are 3.4% and 13.3%, respectively. A median faecal zonulin concentration of  $61 \pm 46$  ng/mL is reported by the manufacturer as the reference value for the faecal matrix.

#### **4.2.5 Statistical analysis**

All the following statistical analysis performed for this thesis were conducted in R Studio (version 4.5.2). Shannon and Simpson alpha diversity indices were given already calculated by third parties. Before each test, the assumptions of statistical applicability were verified. The correlation between microbiota biodiversity and physical activity was computed through Spearman's correlation test. To assess a difference in microbiota composition and in Mediterranean Diet Score, between groups, the t-test was used, while the Wilcox test was used for zonulin and indican levels. In the control group, the difference in microbiota biodiversity in anxious and non-anxious subjects was

assessed through the t-test. The Spearman's correlation test was used to calculate the following correlations: indican and physical activity (all the subjects and just IBD group), MDS and age and Faith's PD and age in control group. Lastly, linear regression models have been used to assess the effect of various explanatory variables on a continuous variable like the effect of sex and zonulin on MDS, microbiota biodiversity and sex on zonulin levels, sex and age on indican concentration and indican and pathology on Shannon values.

### **4.3 The nutritional intervention study on mother's cohort**

In a second work of collaboration with the University of Rome "Campus Bio-Medico" the effects of two different diets on a restricted number of mothers and their newborns were explored. The study population was dyads of pregnant women and their about to be born babies pertaining to the Gynecology and Obstetrics UOC clinics of the Fondazione Policlinico Campus Bio-Medico of Rome, in turn followed by the Gastroenterology and Digestive Endoscopy UOC and the Clinical Nutrition UOS of the Fondazione Policlinico Campus Bio-Medico of Rome. The study protocol was approved by the Ethics Committee of Campus Bio-Medico University of Rome (Protocol number PAR: 001.22(71.21)) in accordance with the Declaration of Helsinki.

The inclusion criteria were: (1) Adult women aged 18-40, (2) BMI between 18.5 and 34.9 (normal weight, overweight, obesity I° grade); (3) Healthy newborns

of both sexes, born at term ( $\geq 37^{\text{th}}$  week of gestational age) and preterm ( $< 37^{\text{th}}$  week of gestational age) by natural or caesarean delivery, without serious complications and/or congenital malformations; (4) Ability of the parent/legal guardian to sign the informed consent form to take part in the study, to respond to the interviews and questionnaires administered.

Exclusion criteria: (1) Twin pregnancy; (2) Presence of known transmissible infectious disease (e.g. SARS-CoV-2, HIV, Hepatitis, etc.), moderate/severe organic pathologies with effects on nutritional status and gastrointestinal (inflammatory, neoplastic, autoimmune, metabolic) and psychiatric (depressive, obsessive syndrome, eating disorders) function; (3) Newborns with severe congenital malformations and/or admitted to neonatal intensive care units and/or tube-fed; (4) Major gastrointestinal surgery, except cholecystectomy or appendectomy within the last 5 years; (5) Use of cortisone, antidepressant, chemotherapy, hypoglycaemic drugs, systemic or intestinal absorption antibiotics or drugs active on the gastrointestinal tract in the last 7 days before the collection of biological samples; (6) Patients who are already participating in other interventional study protocols and/or who are already following a targeted nutritional plan.

In order to see the effects of a diet on microbiota composition, the subjects were randomly assigned to two groups, diet or control. Both groups received nutritional counselling suggesting a healthy diet based on Mediterranean

model. Just the diet group, beside the basic Mediterranean pattern, were invited to enrich this diet with some particular vegetables.

#### **4.3.1 Mothers' enrolment, clinical and nutritional assessment**

Participants have signed written informed consent, also authorizing their children's participation in the study. Women enrolled were given structured interviews by authorized staff, in order to collect information about the health status of themselves and child, and to acquire clinical, anthropometric, demographic, socioeconomic, lifestyle, medical, obstetric and childbirth history, presence or absence of infections and symptoms of disease, as well as recent or current medications (including antibiotics). Participants were also asked to complete a 7-day food diary and complete specific questionnaires to investigate their eating habits and use of prebiotics, probiotics, and supplements (maternal and infant FFQ) and their levels of physical activity (IPAQ). The enrolled women were involved in a nutritional education intervention through food counselling sessions and the distribution of a brochure containing practical recommendations and suggestions, with the aim of promoting the Mediterranean diet as a healthy and sustainable lifestyle. Special kits for collecting biological samples were then delivered, including sterile containers and detailed instructions, to be used at the times required by the study protocol. Anthropometric measurements were performed in duplicate, using standard techniques and correctly calibrated instruments. The

mothers' body weight was measured in kilograms (kg) using a digital electronic scale, with the participant in light clothing and without footwear. Height was measured with an extendable stadiometer, calibrated daily. Pregnancy weight and weight gain during pregnancy were self-reported. Body mass index (BMI) was calculated as the ratio of weight (kg) to height squared ( $m^2$ ), while pre-pregnancy BMI and gestational weight gain were classified according to the Institute of Medicine guidelines. To assess adherence to the Mediterranean Diet dietary pattern, the Mediterranean Diet Serving Score (MDSS) was also used (Monteagudo et al., 2015).

#### **4.3.2 Faecal microbiota analysis**

A total of 57 faecal samples were collected, belonging to 13 mothers and 12 babies. The samples were collected at four different timepoints: from the mother in the third trimester of pregnancy (mT0); post-partum in the first 7 days from the mother (mT1) and from the newborn (bT0); three months after delivery from the mother (mT2) and from the baby (bT1) and from the baby at six months of life (bT2). Sequencing of the 16s rRNA gene has been performed using Oxford Nanopore Technologies (ONT) third-generation sequencing technology. In detail, genomic DNA from overnight cultures of putative lactic acid bacteria was extracted using a special extraction kit (NORGEN BIOTEK CORP). The degree of purity of the extracted DNA was evaluated by spectrophotometric reading of the absorbance ratios 260/280 and 260/230, using the

NanoDropThermo Scientific) spectrophotometer, and by analysing small sample volumes. The concentration of the extracted DNA was instead determined with the Qubit fluorimeter (Thermo Scientific). Bacterial DNA was then used to prepare the 16s rRNA genomic library using the "rapid sequencing DNA-16s Barcoding kit 24 V14" (SQK-16S114.24) (ONT) kit, following the manufacturer's protocol. Briefly 10 nanograms of DNA from each sample were added to a PCR reaction mix (50µl) containing the enzyme Taq polymerase (LongAmp Hot Start Taq) and the barcode sequence. The latter, by binding to the DNA of the sample, allows its identification in the subsequent phases of the analysis. PCR was conducted according to the following amplification protocol: initial denaturation at 95°C for 1 minute, followed by 25 cycles consisting of denaturation at 95°C for 20 seconds; annealing at 55°C for 30 seconds and extension at 65°C for 2 minutes. A final extension to 65°C was finally performed for 5 minutes. At the end of the PCR reaction, the concentration of amplified DNA was quantified using the Qubit fluorimeter and the samples were pooled, in equimolar amounts (60 ng per sample). The obtained genomic library was loaded onto a Flow cell (R10.4.1) and subjected to sequencing for approximately 24 hours at 37°C, using a MinION MK1C (ONT) sequencer. During sequencing, basecalling was performed in parallel, using the model "Fast accurate model" and a QSCORE threshold of 8. At the end of the analysis, the FASTQ format files, containing the 16s rRNA sequences, were processed with the Epi2me Lab

platform, using the NextFlow workflow/wf-16s pipeline. For taxonomic classification, the ncbi\_16s\_18s database and the Kraken2 and Minimap2 classifiers were used. For Kraken2 the default workflow/wf-16s settings were retained, while for Minimap2 the following parameters were applied: max\_len:2000, min\_len: 800, min\_percent\_identity: 97% and min\_ref\_coverage: 90%.

#### **4.3.3 S100B immunodetection in breast milk**

Human milk samples (n = 19) were collected and stored at -20°C until measurements. On the day of the assay, the samples were centrifuged at 500×g for 15 min, the aqueous fraction was collected and centrifuged again at 3000 x g for 15 min. The final aqueous part was diluted and assayed according to the instructions. The protein concentration was measured in all samples using two different commercially available ELISA kits: Abcam, ab234573, Amsterdam, the Netherlands and Antibodies, A77268, Stockholm, Sweden. The Abcam kit antibody expresses reactivity with mouse, rat and human S100B, while Antibodies kit exhibits high specificity for the detection of human S100B. No significant cross-reactivity or interference between S100 beta and analogues was observed. Each measurement was conducted in duplicate according to the manufacturer's recommendations and the blank- subtracted averages were reported. The limit of sensitivity of the assay was 139 pg/ml for Abcam and 18.75 pg/ml for Antibodies.com. Before the assay, the samples were centrifuged

at 1000 x g for 20 minutes: then the supernatant was collected and assayed. Both protocol's kits were followed to perform the respective enzyme-linked immunosorbent assay. Absorbance was measured on a microtiter plate reader (Bio Rad, iMark Microplate Absorbance Reader, Hercules, CA, USA) at 450 nm. The concentration of S100B ( $\mu\text{g/mL}$ ) in the samples was interpolated from the standard curve. The reported results of the protein are the means of the two used kits.

#### **4.3.4 Total protein quantification in breast milk**

The milk samples were also used for the quantification of the total amount of protein to have also a normalization of the S100B quantity. The samples were kept at  $-20\text{ }^{\circ}\text{C}$  until the assay. The Bradford assay was the method used for the quantification (Bradford, 1976) using the Bio-Rad Protein Assay Kit II (Bio-Rad Protein Assay Dye Reagent Concentrate and Bio-Rad Protein Assay Standard II Bovine Serum Albumin, Bio-Rad Laboratories, Life Science Group 2000 Alfred Nobel Drive Hercules, California 94547 USA). Following the manufacturer's instructions for microtiter plates, after the standard and dye preparation, the samples were diluted 1:10 and assayed in duplicate. After 5 minutes of incubation with dye reagent, absorbance was measured on a microtiter plate reader (Bio Rad, iMark Microplate Absorbance Reader, Hercules, CA, USA) at

595 nm. The results were then multiplied by the factor of dilution. The linear range of this microtiter plate assay was 0.05 mg/ml to approximately 0.5 mg/ml.

#### **4.3.5 Data preparation**

Provided microbiota data composition were already calculated relative abundances. The alpha indices Shannon and Simpson have been computed with the package *vegan* (version 2.7.2). For the Beta diversity the Bray-Curtis distance was computed with the *vegdist* function (*vegan* package). Later, a Principal Coordinates Analysis (PCoA) was conducted on such Bray-Curtis distances using the *cmdscale* function for ordination and visualization of sample clustering. In order to explore which taxa drive the difference between groups in mothers, microbiome relative abundance data were first transformed using the centered log-ratio (CLR) transformation using *clr* function from package *compositions* (version 2.0.9). Before transformation, taxa with zero abundance in all samples were removed and a pseudocount of  $10^{-6}$  was added to all abundances to avoid undefined logarithms. CLR-transformed abundances were then compared between diet and control using the Wilcoxon rank-sum test for each taxon. Multiple-testing correction was performed using the Benjamini–Hochberg false discovery rate (FDR), and taxa with FDR-adjusted p-values  $< 0.05$  were considered significantly differentially abundant.

#### 4.3.6 Statistical analysis

Before each test, the assumptions of statistical applicability were verified for each dataset. In all the analyses was considered statistically significant a p value  $< 0,05$ . The correlation between S100B levels and age of the subjects was computed through Spearman's correlation test. A difference in S100B levels between the IPAQ scores in control group was computed through the Kruskal-Wallis test. To assess if there is a difference in Mediterranean diet score before and after delivery a paired t-test was performed both in overall subjects and for control group only. The corresponding Wilcoxon paired test was conducted for comparing MDS before and after delivery in diet group. As for the microbiota analysis, changes over time, by group or by physical activity in alpha diversity were evaluated using linear mixed-effects models implemented with the lmer function from the package lme4 (version 2.0.1). Time point, group and IPAQ score were included as a fixed effect, while subject (mother or baby) was included as a random intercept to account for repeated measurements within individuals. Statistical significance of the fixed effects was assessed using the lmerTest package. Pairwise comparisons between time points were performed using estimated marginal means with multiple-testing correction. Differences in microbial community composition between groups and subjects were tested by permutational multivariate analysis of variance (PERMANOVA) using the adonis2 function (vegan package) with 9,999 permutations, followed by a test

for homogeneity of multivariate dispersion (PERMDISP). To identify taxa responsible for differences in microbial composition between diet group and control group, taxa with zero relative abundance in all samples were excluded (n=3 excluded taxa: *Proteus*, *Clostridioides\_A*, *Enterobacter\_B\_713587*). The relative abundances of the remaining taxa (n=71) were transformed according to the Centered Log-Ratio (CLR), after adding a pseudocount of  $1 \times 10^{-6}$  to handle null values. For each taxon, a Wilcoxon-Mann-Whitney test (stats package) was conducted comparing the CLR-transformed values between the two groups, followed by correction for multiple comparisons using the Benjamini-Hochberg (FDR) method. Taxa with corrected p-value  $< 0.05$  were considered significantly differential. All the significative values of the tests pertaining to this study were corrected through the Benjamini-Hochberg correction (FDR). The tests were grouped into one family on the basis of being conducted on the same sample and answering the question of association between microbiota/diet/S100B and lifestyle.

#### **4.4 In silico S100B investigation**

In order to explore if a different matrix from the ones already investigated (various human cells above mentioned, animal material, human, animal milk and derivatives) could have a similar structure to human S100B protein, first an *in silico* test on vegetables has been conducted. This pre-inspection had the aim

of being a guideline for the following experimental search of S100B-like immunomaterial in vegetable matrix.

#### **4.4.1 Data preparation**

The human S100B protein was compared with similar compounds by running blastp against the UniProtKB and the SwissProt databases (*UniProt's Blastp Page*, 2024). Only organisms belonging to the *Viridiplantae* clade were retained. The results were manually filtered to remove duplicates and low-quality items. The best 100 proteomes were selected and the corresponding FASTA sequences were downloaded from UniProt (Chiara et al., 2018). Subsequently, they were aligned using MAFFT (Katoh et al., 2002) (version 7.490) and trimmed with trimAl (version 1.4) (Capella-Gutiérrez et al., 2009). Finally, IQ-TREE (version 2.2.2.6) (Minh et al., 2020) was employed to infer the final phylogenetic tree, using the Le and Gascuel substitution matrix (Le & Gascuel, 2008) with a 4-category discrete gamma model (LG+G4) (Z. Yang, 1994). The results were compared with the phylogenetic tree built using the NCBI Common Taxonomy Tree (*NCBI Common Taxonomy Tree*, 2024) tool passing the Taxa IDs of the species we previously selected (Castrignanò et al., 2020).

#### **4.4.2 Molecular modelling**

To predict the three-dimensional structure of the proteins that manifested better sequence homology, the protein modelling software AlphaFold2 was used (version v2.3.0) (Jumper et al., 2021). The alignments required for this procedure

were generated using MMseqs2 (version 2) (Steinegger & Söding, 2017) and HHsearch (version 1.12) (Steinegger et al., 2019) software, which allowed us to obtain detailed sequence relations between the proteins of interest. The outputs generated from the structural prediction were used to calculate the root mean square deviation (RMSD) and TM-align (D. Zhang et al., 2007; Y. Zhang & Skolnick, 2005). Subsequently, the structures were incorporated into PyMOL to calculate the solvent accessible surface area (SASA) based on the original structures of the proteins (S. Ali et al., 2014; Castrignanò et al., 2007). TM-align is an algorithm used for comparing sequence independent protein structures. When confronted with two protein structures of unknown equivalence, TM-align undertakes the generation of an optimized residual-residual alignment through dynamic programming heuristic iterations, grounded in structural similarity. The algorithm subsequently furnishes an optimal superimposition of the two structures, accompanied by a TM-score value that quantifies the degree of structural resemblance. The TM-score, constrained within the range of (0, 1), attains a value of 1 for a perfect structural match. In accordance with established structural statistics from the Protein Data Bank, TM-scores falling below 0.2 are indicative of randomly selected, unrelated proteins, whereas scores surpassing 0.5 generally signify congruent folding in SCOP/CATH.

## **4.5 In field investigations**

### **4.5.1 Sample preparation**

In order to verify the presence of S100B-like immunoreactive material in plant matrix different ELISA kits from different manufacturers, with different antibodies (Abcam GR3360381-1, Cambridge, UK, Millipore Merck EZHS100B-33K, Billerica, MA, USA, MyBiosource MBS2503148, San Diego, CA 92195-3308 USA) have been used. Notably, the antibodies used, according to the indications offered by the manufacturers, reacted specifically with the beta subunit of the protein as present in humans, but also in other mammalian species, such as rat and mouse (as indicated for both antibodies) and hamster, ox, horse, monkey, pig, rabbit, (as indicated for Merck). No cross reactivity with other members of the S100 protein family (namely, S100A1, S100A6, S100A13) was observed using the Merck antibody, as tested by the manufacturer. The sensitivities declared by the manufacturers were 2,7 (Merck), 139 pg/mL (Abcam) and 18,75 pg/ml (MyBiosource). The samples, origin and quantity used for these experiments are resumed in table 1. Approximately 80–120 mg of vegetal organisms were weighted: the initial weights were gradually reduced because experimentally it was noticed an optimal extraction efficiency with the lowest quantity of material. Into every sample, 1 mL of Cell Extraction Buffer PTR 1X (Abcam, 6300003, lot R1874, Amsterdam, The Netherlands), glass beads (Sigma-Aldrich, G1145-10G, lot 019K5306, Saint Louis, MO, USA) and Protease inhibitor 1:100

(Sigma-Aldrich, P2714-1BTL, lot 0000116640, Saint Louis, MO, USA) were added. To obtain a homogenate, samples were vortexed briefly and then incubated on ice for 20 min. After this time, they were centrifuged at  $16,300 \times g$  for 20 min and the supernatants were transferred into clean tubes. The samples were then immediately assayed.

*Table 1: Sample type, specimen, quantity and origin of samples analyzed through Enzyme-Linked Immunosorbent Assay. ns = not specified*

| <b>Sample</b> | <b>Specimen</b>      | <b>Quantity (mg)</b> | <b>Origin</b>                                                                                |
|---------------|----------------------|----------------------|----------------------------------------------------------------------------------------------|
| Açaí          | Berries powder       | 119                  | Organic açai berries from Brazil. “NaturaleBio” – International Food Europe srl; Rome, Italy |
| Banana        | Pulp powder          | 110                  | Fruit from Ecuador “Purasana”- Biovita bvba Heulestraat; Gullegem, Belgium                   |
| Banana        | Fresh pulp           | 80                   | ns                                                                                           |
| Baobab        | Fruit pulp powder    | 153                  | Raw baobab from South Africa. “Cibocrudo srl”; Ciciliano, Italy                              |
| Broccoli      | Fresh inflorescences | 83                   | Sicily, Italy                                                                                |
| Cabbage       | Fresh leaves         | 84                   | ns Italy                                                                                     |
| Cocoa         | Fava beans powder    | 110                  | Organic fairtrade cacao from UK “Naturya” Bath, BA2 7PQ, UK                                  |
| Durian        | Fresh pulp           | 122                  | Thailand                                                                                     |
| Durian        | Freeze/Dry pulp      | 105                  | “Sunshine international Co.ltd” Songpinong Thamai, Chanthaburi 22120, Thailand               |
| Durian        | Pulp powder          | 80                   | Thailand                                                                                     |

|                             |                                    |     |                                                                              |
|-----------------------------|------------------------------------|-----|------------------------------------------------------------------------------|
| Graviola                    | Fruit and plant extract powder 4:1 | 144 | (graviola from Perù)<br>“AMTEC Trading GmbH”; Wolfsberg, Austria             |
| Jack fruit                  | Lyophilized pulp                   | 139 | Fruit from Tanzania. “Cibocrudo srl”; Ciciliano, Italy                       |
| Kiwi                        | Fresh pulp                         | 100 | ns, Italy                                                                    |
| Kombucha                    | Lyophilized tea powder             | 128 | Fermented tea from China. “Xi’an Le Sen Biotechnology Co.Ltd”<br>Xi’an China |
| Laurel                      | Fresh leaves                       | 106 | ns, Italy                                                                    |
| Mela Annurca ( <i>cps</i> ) | Apple Annurca dry extract          | 133 | Apple from Campania, Italy<br>“4TIFY”- GeneS; Rome, Italy                    |

#### 4.5.2 Enzyme-Linked Immunosorbent Assay for S100B

An enzyme-linked immunosorbent assay (ELISA) for S100B (Abcam, ab234573, Amsterdam, the Netherlands and Millipore Merck EZHS100B-33K, Billerica, MA, USA) was carried out on the supernatants on duplicates according to the manufacturer’s protocol. Briefly, after the reagents, samples, and standards preparation, the appropriate volume, according to kit’s protocol, of sample or standard were added to appropriate wells: both standards and samples were assayed in duplicate. The antibody cocktail was added to all wells and left for incubation at room temperature for the respective required time. In the washing process, supernatant was removed and approximately 350  $\mu$ L of Wash Buffer 1X was added; the process was repeated as many times as described. Subsequently, substrate solution was added to each well, incubated, and then

the staining reaction was stopped with the Stop solution. Absorbance was measured on a microtiter plate reader (Bio Rad, iMark Microplate Absorbance Reader, Hercules, CA, USA) at 450 nm. The concentration of S100B (ng/mL) in the samples was interpolated from the standard curve obtained by the standards.

## **4.6 *In vivo* assessment of oral S100B supplementation on gut microbiota**

A first preliminary experiment was set up in collaboration with the department of Pharmacology “Vittorio Erspamer” University “La Sapienza” in Rome.

### **4.6.1 Sample collection**

A total of 28 faecal murine samples were collected at six different timepoints from male mice (n = 6) of the wild type C57BL/6J strain (Charles River, Calco, LC, Italy) following a single orally administration (gavage) of a solution of S100B 1 mg/kg in PBS. Randomly, some of the stool samples will be collected in a vial with pure ethanol, while the most will be conserved with no liquid. The experiment was carried out according to Sapienza University’s Ethics Committee. Animal care was in compliance with the IASP and European Community (EC L358/1 18/12/86) guidelines on the use and protection of animals in experimental research.

#### **4.6.2 Quantification of S100B protein**

The protein quantification on faeces and urine samples has been realized using a SimpleStep ELISA® (enzyme-linked immunosorbent assay) kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. Approximately 30 mg of stool samples were resuspended in 1ml of PBS and homogenized. Samples were centrifuged for 10 minutes at 5000 × g, and the supernatant was collected for analysis. Urine samples were centrifuged at 2000 rpm for 10 minutes, and the supernatant was collected and assayed.

#### **4.6.3 DNA extraction and 16s rDNA sequencing**

Approximately 30 mg of murine faeces were collected in vials containing either air or pure ethanol, randomly assigned. Samples were stored at -20 °C until processing. DNA extraction was performed using the QIAmp DNA Mini Kit and DNeasy Blood & Tissue Kit following the buccal swab protocol with minor modifications and the use of NAO Baskets. Samples were placed into semipermeable NAO Baskets and incubated with 200 µL lysozyme solution (20 mg/mL lysozyme [Sigma-Aldrich], 20 mM TrisHCl at pH 8, 2 mM EDTA, and 1.2% Triton X-100 [Sigma-Aldrich]) at 37°C for 30 minutes. Following incubation 20 µL proteinase K and 400 µL buffer AL were added and samples were centrifuged at 10,000 × g for 1 minute, to elute the digestion solution. A second incubation step was performed at 56°C for 10 minutes and after which 400 µL of ethanol was added. Subsequent washing steps and DNA purification

were performed in accordance with the manufacturer's instructions. DNA was eluted in 60 µL elution buffer (10 mM Tris-HCl and 0.5 mM EDTA at pH 9.0).

Amplicon libraries targeting the V1 – V2 hypervariable region of the 16S rRNA gene were prepared for next-generation sequencing (NGS) according to the 16S Metagenomic Sequencing Library Preparation Guide (Illumina, part# 15044223 rev A; San Diego, CA, USA). The PCR amplicons were obtained using primers

Ba27F 5'-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGG

CTCAG-3' and Ba338R 5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCTGCCTCCCGTA

GGAGT-3', which include overhang adapter sequences (Giampaoli et al., 2017).

Tagged polymerase chain reaction (PCR) products were generated by two-step PCR using unique barcoded primer pairs. In this strategy, the adapter-containing target primers were used in the first PCR reaction to amplify the target gene (16S rDNA), and this product was then used in the second PCR using the barcode-containing primers to identify the individual sample. Each amplification reaction had a total volume of 25 µL, including 12.5 µL of KAPA HiFi Hot Start Ready Mix (Roche, Pleasanton, CA, USA), 5 µL of each primer (1 µM), and 2 µL of template deoxyribonucleic acid (DNA). Reactions were conducted on a Techne® TC-PLUS thermal cycler (VWR International, LLC, Radnor, PA, USA).

Following amplification, 5  $\mu$ L of PCR product from each reaction was used for agarose gel (1%) electrophoresis to confirm amplification. Libraries were prepared using the MiSeq Reagent Kit Preparation Guide (Illumina, San Diego, CA, USA). The final concentration of the cleaned DNA amplicon was determined using the Qubit PicoGreen dsDNA BR assay kit (Invitrogen, Grand Island, NY, USA). Then, the library was sequenced on an Illumina ISeq100 platform and 150 bp paired-end reads were generated.

#### **4.6.4 Sequencing data preparation and statistical analysis**

Removal of Illumina sequencing adaptors was performed by the built-in pipeline trimming during FASTQ file generation. Quality control and pre-processing of the raw sequences, as well as subsequent analysis was conducted in R (version 4.5.2) Quality filtering and downstream processing were performed using DADA2 (version 1.38.0). Forward and reverse reads were concatenated due to the expected insert size (300-400 bp) exceeding the read length. Both sequences were trimmed at the last 10 bases, and the maximum expected error rate was set to 2. Amplicon sequence variants (ASVs) were inferred using the dada function, yielding 22875 ASVs. 140 base pairs long have been identified. After chimeras removing using removeBimeraDenovo 2120 ASV remained. Taxonomic classification up to genus level was conducted using the assignTaxonomy function with the GreenGenes2 database (version 2024.09).

One sample (mouse 3 at 64h) was excluded due to very poor number of reads (< 150 at genus level).

Alpha diversity indices (Shannon, Simpson, and Chao1) diversity indices were computed with the iNEXT package (version 3.0.2). Prevalence filtering retained taxa present in at least two samples. Data were centred-log-ratio (CLR) transformed using ALDEx2 (version 1.42.0), and Euclidean distance (Aitchison distance) was computed with the `dist` function. Differences in alpha diversity (measure of the within-sample diversity) across time points were assessed using the Kruskal-Wallis test. Beta diversity (measure of between samples diversity) was assessed by a principal component analysis (PCA) on Aitchison distances. Permutational multivariate analysis of variance (PERMANOVA) was performed using the `adonis2` function (vegan, version 2.7.2) with permutations restricted within mouse to assess the overall effect of time point (2h, 4h, 8h, 16h, 32h, 64h), storage method (air/EtOH) and S100B detection (presence/absence) on microbial composition. Differential abundance over time was assessed using linear mixed-effect models with pairwise time point comparisons, fitted on CLR-transformed data using the `lmer` function from lmerTest (version 3.2.0). P-values were adjusted for multiple testing using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). Population trends were estimated with the `predict` function applied to linear mixed model with logarithmic time points.

## **5. Results**

### **5.1 Effect of physical activity on IBD patients: literature review**

The results brought up by the systematic review are not consistent: as for prevention, physical activity demonstrated good or null effectiveness. With regard to treatment, most of the studies reported improvements in IBD-related sarcopenia, osteoporosis, quality of life, fatigue and decreased risk of relapse. Nevertheless, many reported exercise-related increase in cytokine and inverse association between strenuous activity and mucosal healing. The database search provided a total of 3129 articles. Of these, 1147 duplicates were removed and 1982 were screened by title and abstract. After the full-text assessment, 29 of the 68 eligible articles were included in the review. Six of them (Furuya et al., 2022; Hlavaty et al., 2013; Khalili et al., 2013; Klein et al., 1998; Lautenschlager et al., 2023; Rasmussen et al., 2021) investigated the role of PA in developing the disease, while the others evaluated the potential beneficial effects of PA in individuals affected by IBD. The first group included three cohort studies, two case-control studies and a cross-sectional study, which were performed in Europe, Asia and the USA. Both genders were observed in all the studies, with a sample size ranging from 232 to 57,053 individuals. Both CD and UC were considered across all the studies. Regarding quality assessment, three studies

were considered “poor”, two “fair” and one “good”. Four studies showed an inverse association between PA and onset of IBDs or at least of CD. The study by Rasmussen et al. did not find any association between PA and risk of developing IBDs. However, in the same study, a higher time spent in do-it-yourself work appeared to be associated with IBD onset. The study by Furuya et al. registered a positive association between occupational PA level and UC risk.

Regarding the studies which investigated the role of PA in IBD treatment, nine of the selected articles described randomized controlled trials (Cronin et al., 2019; Elsenbruch et al., 2005; K. Jones et al., 2020; Klare et al., 2015; Lamers, de Roos, Bongers, et al., 2021; Lamers, de Roos, Koppelman, et al., 2021; Lamers et al., 2022; V. Ng et al., 2007; Watters et al., 2001), while the others reported observational studies. Seventeen studies were performed in Europe, four in the Americas and two in Asia. The sample size varied across the selected studies from 20 to 117 for RCTs and from 12 to 289,658 individuals for observational studies. Both genders were represented in all the studies but one which involved only men (D’Inca et al., 1999) and two which did not report this information (P. D. Jones et al., 2015; Watters et al., 2001). Of the 16 studies which reported the status of the disease for participants, all but three included participants with different clinical conditions. As for quality, 12 of these studies were considered “poor”, ten “fair” and one “good”. The results differ across the

selected studies. As for RCTs, the study by Cronin et al. showed a significant reduction in body fat percentage among patients who underwent 8 weeks of aerobic and resistance training, together with improvements in IBD-related sarcopenia and obesity-related metabolic disorders (Cronin et al., 2019). The study by Elsenbruch et al. reported a significant improvement in mental health, but this resulted from a multicomponent intervention, including stress management training, moderate exercise, Mediterranean diet, behavioural techniques and self-care strategies (Elsenbruch et al., 2005). Jones K et al. found significant improvements in bone mineral density and muscular function among IBD patients who underwent the 26-week exercise programme and not among those who did not exercise; quality of life and fatigue improvements were also registered in this study (K. Jones et al., 2020). Even Klare et al. registered an improvement in quality of life among patients who practiced moderate-intensity running (Klare et al., 2015). Lamers et al. observed an exercise-related increase in cytokine production among IBD-walkers and non-walkers and no changes in faecal calprotectin concentration; a significant increase in disease activity was registered among CD and not in UC walkers (Lamers, de Roos, Bongers, et al., 2021; Lamers, de Roos, Koppelman, et al., 2021). In the subsequent RCT by Lamers et al., significant improvements in impact of disease in daily life and fatigue were obtained after a multidisciplinary intervention, but they were related to changes in diet and not

to PA level, which remained the same (Lamers et al., 2022). Ng et al. reported a significantly higher improvement in quality of life and symptoms in patients involved in walking with respect to controls (V. Ng et al., 2007). The 12-month exercise intervention implemented by Robinson et al. led to significant improvements in bone mineral density among participants, which were not observed in controls (Robinson et al., 1998). Instead, the resistance training intervention applied by Watters et al. showed a positive relationship between exercise and wellbeing in IBD patients (Watters et al., 2001). As for the observational studies, the study by D'Inca et al. showed a post-exercise increase in oro-caecal transit time and neutrophils which did not differ from that observed in healthy controls. Henderson et al. registered similar changes among IBD patients and healthy controls in breath volatile organic compounds and cytokines production after repeated prolonged moderate exercise (Henderson et al., 2022). Holik et al. found a significant inverse relationship between daily PA and disease activity, which was independent by intensity level and type of IBD (Holik, 2019). Even in the study by Jones P et al., higher exercise level was associated with decreased risk of relapse or active disease for IBD patients (P. D. Jones et al., 2015). Kim et al. found an association between weekly PA level and quality of life (B. Kim et al., 2021). Lamers et al. found a significant association between disease activity and PA in CD but not in UC patients, and an improvement in general fitness, quality of life and self-image

was reported by the majority of participants. The findings of Lo et al. indicated a lower mortality rate related to PA in IBD patients (Lo et al., 2021). In the study by Mack et al., leisure-time physical activity has been associated with reduced risk of onset and management of IBD comorbidities such as osteoporosis and colon cancer (Mack et al., 2011). In the study by Ratajczak-Pawłowska et al., vigorous, moderate and total PA were found to be positively related to bone mineral density (BMD) and body mass among IBD patients, while only moderate PA was related to BMD in controls (Ratajczak-Pawłowska et al., 2023). Spijkerman et al. found an apparent suppression of the proinflammatory response in IBD patients after three consecutive days of walking (Spijkerman et al., 2021). In the study by Taylor et al., moderate-to-vigorous physical activity and walking were associated with physical and mental health-related quality of life (Taylor et al., 2018). Tew et al. found that PA was negatively and independently associated with depression, disease activity and perceived barriers to exercise in people with CD, and with depression and age in people with UC (Tew et al., 2016). Watanabe et al. showed that strenuous activity is significantly inversely associated with mucosal healing but not with clinical remission (Watanabe, 2021). The study by Wiestler et al. reported that disease activity and quality of life were significantly correlated with the duration of strenuous PA per day (Wiestler et al., 2019). Adverse events were reported in

four studies. Apart from one fall, they consisted of limitations to PA related to the disease.

In short, physical activity, especially moderate, brings significant benefits in IBD management (improving sarcopenia, bone density, quality of life and reducing risk of recurrence), while its preventive role remains uncertain. Intense/strenuous exercise in patients can be counterproductive, being associated in some cases with increased disease activity and worse mucosal healing.

## **5.2 Results of the observational IBD cohort study**

### **5.2.1 Characteristics of the population**

The examined cohort appears to be quite heterogeneous in all the aspects considered, especially regarding age differences. The characteristics here described are summarised in table 2. A total of 86 subjects were enrolled in the study, divided into 48 healthy controls and 38 patients with inflammatory bowel disease (IBD). Within the IBD group, 14 subjects were diagnosed with Crohn's disease (CD) and 24 with ulcerative colitis (UC). In the control group, 33 subjects were female and 15 males, with a median age of 24 years (IQR: 5). The IBD group showed a more balanced sex distribution, with 19 females and 19 males, and a higher median age of 42.5 years (IQR: 30.2) (Figure 3). This age imbalance is a limitation of the study and has to be taken in consideration while

interpreting results. Among CD patients, 5 were female and 9 males, with a median age of 26 years (IQR: 28); UC patients included 14 females and 10 males, with a median age of 48.5 years (IQR: 33.5). Adherence to the Mediterranean diet, assessed by the Mediterranean Diet Score (MDS), was higher in controls (mean  $\pm$  SD:  $12.0 \pm 5.17$ ) compared to IBD patients ( $9.42 \pm 4.84$ ). Within the IBD group, CD patients showed the lowest adherence ( $8.3 \pm 4.74$ ), while UC patients scored slightly higher ( $10.1 \pm 4.92$ ).

*Table 2: Anthropometric and clinical data of the study cohort*

|                                                            | <b>Controls</b> | <b>IBD</b>      | <b>Chron's Disease</b> | <b>Ulcerative Colitis</b> |
|------------------------------------------------------------|-----------------|-----------------|------------------------|---------------------------|
| <b>Total</b>                                               | 48              | 38              | 14                     | 24                        |
| <b>Female</b>                                              | 33              | 19              | 5                      | 14                        |
| <b>Male</b>                                                | 15              | 19              | 9                      | 10                        |
| <b>Age (median, Q1-Q3)</b>                                 | 24 (5)          | 42,5 (30,2)     | 26 (28)                | 48,5 (33,5)               |
| <b>Mediterranean Diet Score (mean <math>\pm</math> SD)</b> | $12 \pm 5,17$   | $9,42 \pm 4,84$ | $8,3 \pm 4,74$         | $10,1 \pm 4,92$           |
| <b>Anxiety (n)</b>                                         |                 |                 |                        |                           |
| yes                                                        | 4               | 7               | 2                      | 5                         |
| no                                                         | 18              | 14              | 7                      | 7                         |
| <b>Depression (n)</b>                                      |                 |                 |                        |                           |
| yes                                                        | 0               | 6               | 1                      | 5                         |
| no                                                         | 22              | 15              | 8                      | 7                         |
| <b>IPAQ score</b>                                          |                 |                 |                        |                           |
| low                                                        | 0               | 2               | 1                      | 1                         |
| medium                                                     | 7               | 6               | 3                      | 3                         |
| high                                                       | 30              | 19              | 7                      | 12                        |

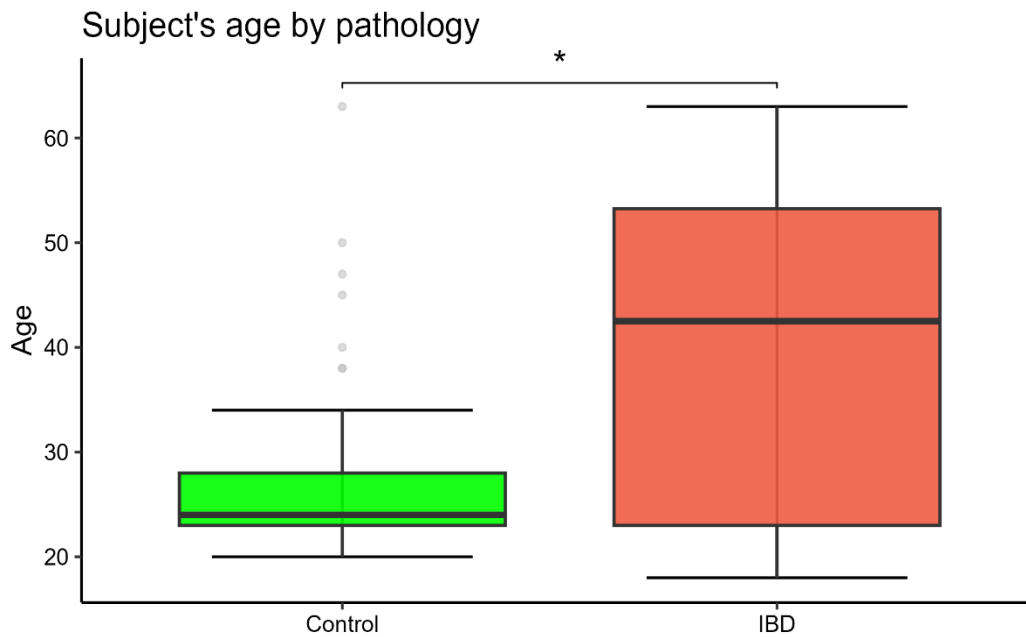


Figure 3: Representation of the subject's age in IBD vs control group (Wilcoxon rank-sum test,  $W = 629.5$ ,  $p = 0.019$ ,  $n = 85$ )

Regarding psychological comorbidities, anxiety was reported in 4 controls and 7 IBD patients (2 CD, 5 UC), while depression was absent in the control group but present in 6 IBD patients (1 CD, 5 UC). It should be noted that psychological assessment data were available only for a subset of participants (22 controls and 29 IBD patients). Physical activity levels, evaluated using the International Physical Activity Questionnaire (IPAQ), showed that most controls were classified as highly active ( $n = 30$ ), while the remaining were classified in the medium category ( $n = 7$ ) and none in the low category. In the IBD group, 19 patients scored as highly active, 6 moderately active, and 2 had low physical activity levels, with a similar distribution observed across CD

and UC subgroups. All the characteristics of the population are summed up in table 2.

A curious result came out performing analysis on the separated groups (IBD and controls). In healthy subjects' group, alpha diversity index Faith's PD, expressing biodiversity as the sum of the lengths of all the branches on phylogeny tree that span the members of the studied set, was higher in non-anxious subjects (mean  $4361 \pm 816$ ) than in anxious ones (mean  $3941 \pm 564$ ) ( $n = 14$ , Welch's t-test,  $t = 3.02$ ,  $p = 0.031$ ) (Figure 4). This difference does not surprise, as some studies have already demonstrated a bidirectional relation between anxiety and gut microbiota (Chen et al., 2019). Indeed, a study conducted by Hai-yin Jiang et al. showed that subjects with generalized anxiety disorders, compared to control group, had lower diversity and richness of microorganisms, exhibiting reduced levels of SCFAs-producing bacteria and an increased abundance of bacteria such as *Escherichia-Shigella*, *Fusobacterium*, and *Ruminococcus gnavus*. Interestingly, these changes in microbiome persisted even during generalized anxiety disorders remission, suggesting that the causality direction is probably two way (Jiang et al., 2018).

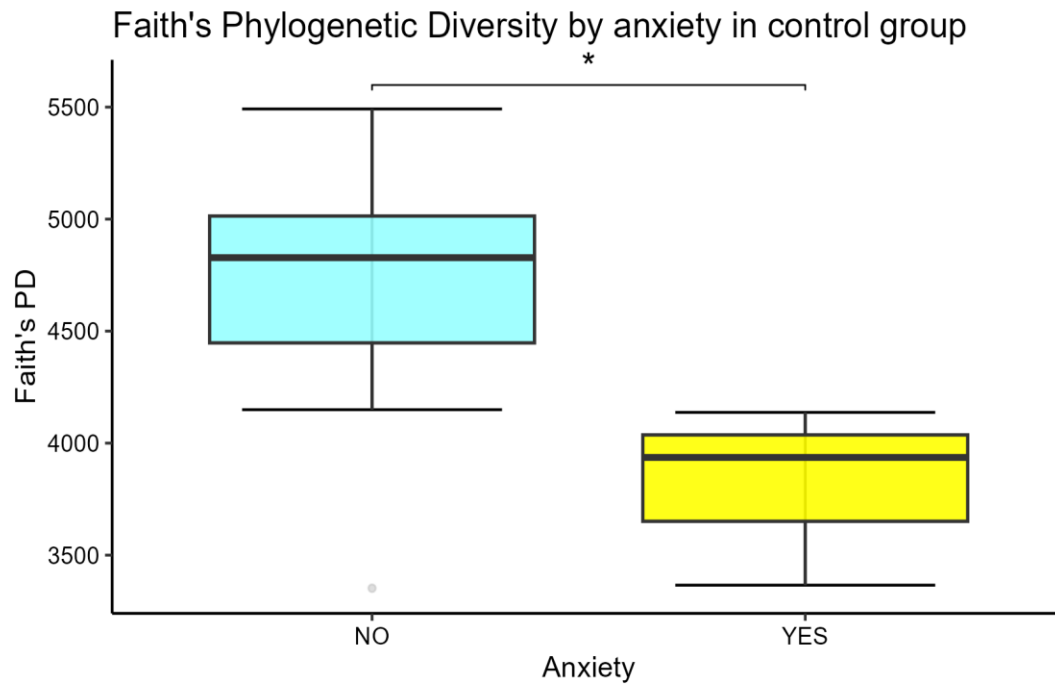


Figure 4: Alpha index Faith's Phylogenetic diversity in anxious and non-anxious subjects in control group ( $n = 14$ , Welch's  $t$ -test,  $t = 3.02$ ,  $p = 0.031$ )

### 5.2.2 Microbiota results and adherence to Mediterranean diet

The microbiota richness analysis yielded some interesting results in relation to mainly pathology, adherence to Mediterranean diet and age. The alpha index Faith's Phylogenetic Diversity was significantly different between healthy controls and patients. In fact, as it was expected to be, Faith's PD was lower in IBD patients ( $3975.6 \pm 821$ ) than in controls ( $4522.7 \pm 663$ )  $n = 43$ , Welch's  $t$ -test,  $t = 2.33$ ,  $p = 0.026$  (Figure 5). In fact, it is well-established that IBD patients

generally show lower overall microbiota diversity compared to healthy people, due to bacterial dysbiosis (Levy et al., 2017; Pittayanon et al., 2020).

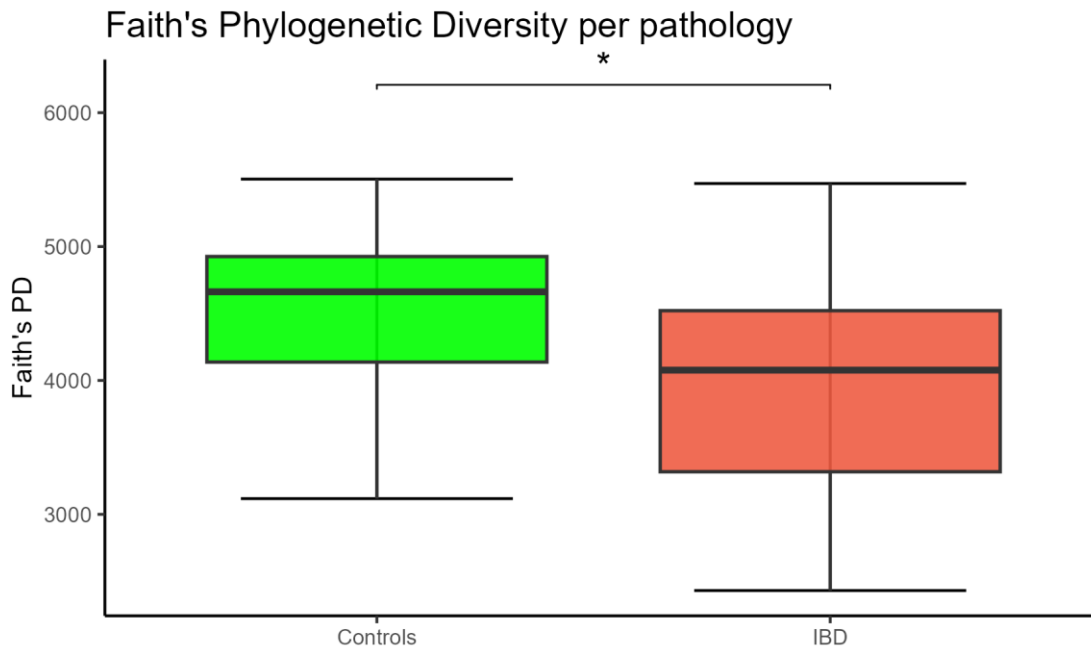


Figure 5: Faith's Phylogenetic Diversity differed statistically between healthy controls and IBD patients (Welch's t-test,  $t = 2.33$ ,  $p = 0.026$ ,  $n = 43$ )

In line with the previous result, the Mediterranean Diet Score (MDS) also was nominally higher in control group (mean  $12 \pm 5.17$ ) compared to patients ( $9.42 \pm 4.84$ ) ( $n = 65$ , Welch's t-test,  $t = 2.04$ ,  $p = 0.045$ ) (Figure 6). However, when applying the multiple comparison correction (Benjamini-Hochberg, FDR) this result did not exceed the corrected significance threshold ( $p = 0.051$ ) and should therefore be interpreted with caution. This observation confirms a trend already observed in some works about IBD patients who generally have a low

adherence to Mediterranean Diet (MD) (even if they live in Mediterranean Basin) (Fiorindi et al., 2021; Marsh et al., 2022; Vrdoljak et al., 2020).

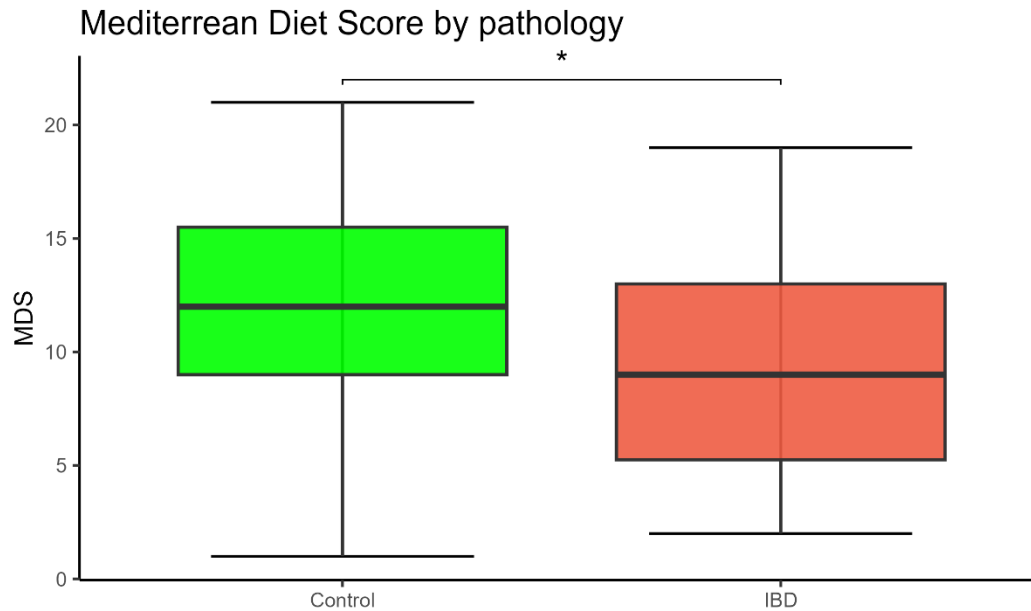


Figure 6 Mediterranean Diet Score is significantly higher in controls than in patients' group:  $n = 65$ , Welch's  $t$ -test,  $t = 2.04$ ,  $p = 0.045$

Moving on to the dysbiosis marker, urine indican, levels were significantly different when comparing patients with healthy controls: the median is higher in IBD group (7.37 mg/dl, IQR 4.41) than controls (3.30 mg/dl, IQR 3.01) (Wilcoxon rank-sum test,  $W = 121$ ,  $p = 0.004$ ,  $n = 45$ ) (Figure 7). This result aligns well with the use of indican as marker of dysbiosis. Indeed, it is part of the organic acid group, products of bacterial metabolism in abnormal overgrowth (Lord & Bralley, 2008).

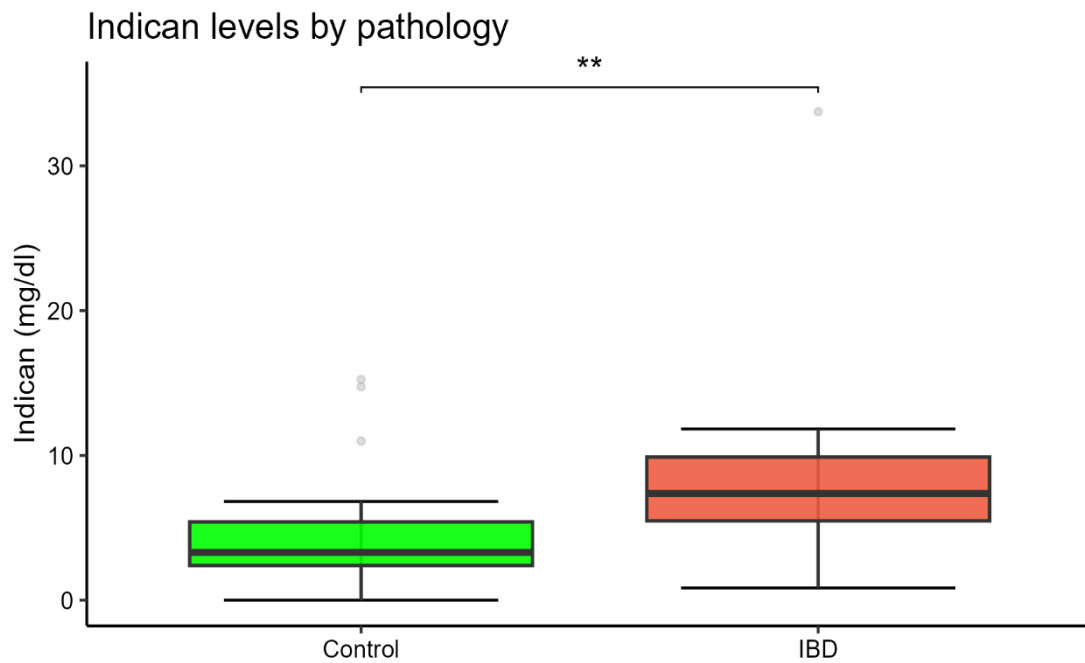


Figure 7: Urine indican levels in IBD group vs Control: significantly higher levels are detected in IBD group (Wilcoxon rank-sum test,  $W = 121$ ,  $p = 0.004$ ,  $n = 45$ ).

Regarding the adherence to Mediterranean diet, a positive correlation between MDS and age was found in the control group (Spearman's correlation  $\rho = 0.52$ ,  $p = 0.0006$ ,  $n = 39$ ) (Figure 8).

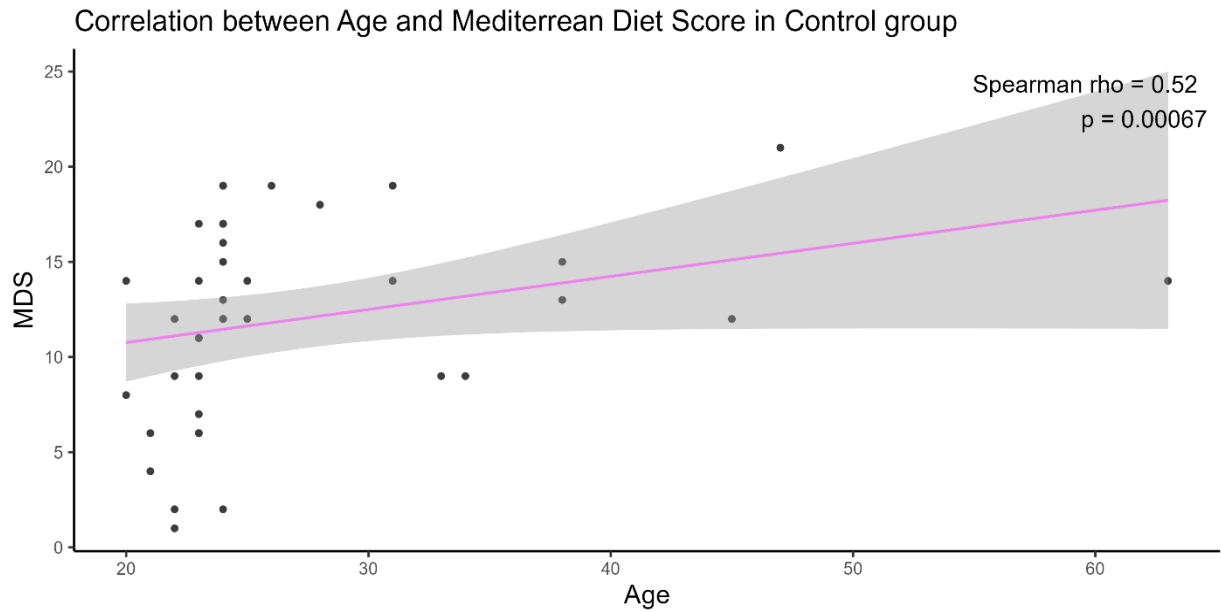


Figure 8: Positive significant relation between Mediterranean Diet Score and age in control group (Spearman's correlation  $\rho = 0.52$ ,  $p = 0.0006$ ,  $n = 39$ )

In this context, in line with the previous results, in the same group of healthy controls, a similar positive trend for Faith's PD index and age (Spearman's correlation  $\rho = 0.42$ ,  $p = 0.033$ ,  $n = 25$ ) came out (Figure 9). Investigating the various variables and their interaction, a linear regression model revealed a significant interaction between sex and faecal zonulin levels on MDS ( $\beta = 0.048$ , 95% CI [0.004, 0.091],  $p = 0.033$ ,  $n = 42$ ). Although the overall model did not reach statistical significance ( $F = 2.40$ ,  $p = 0.083$ ) a difference between zonulin and adherence to Mediterranean diet between males and females was observed (Figure 10). In females, zonulin levels decrease as the MDS increases, while on the opposite, male levels of zonulin augment as the MDS increases.

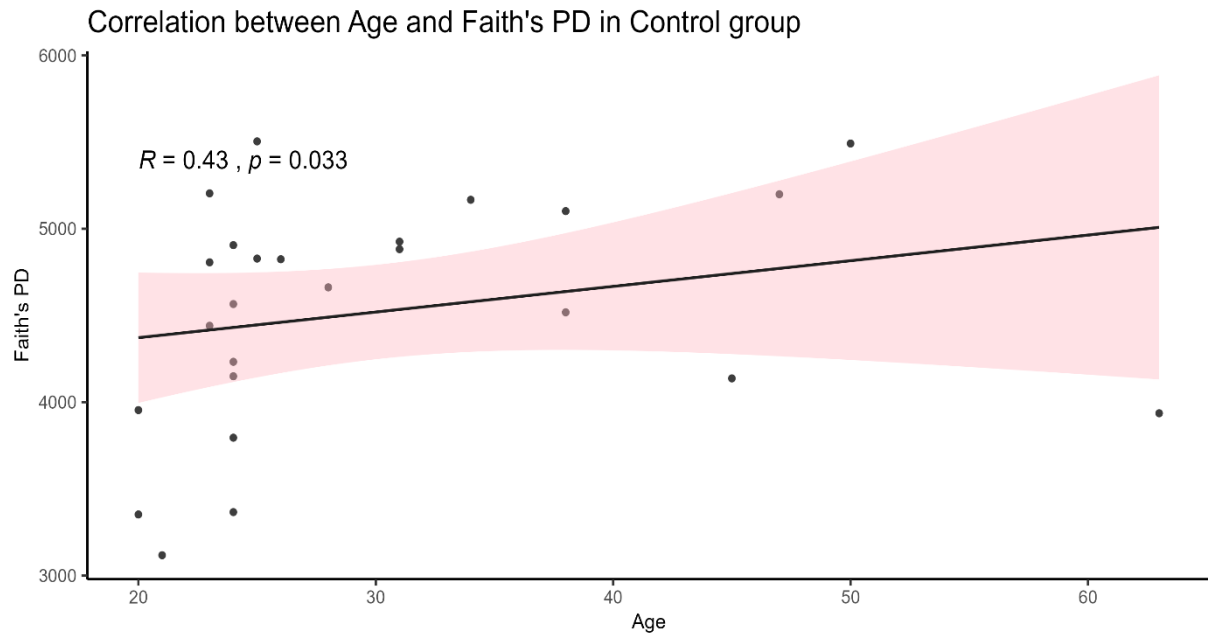


Figure 9: A positive significant correlation exists between Faith's Phylogenetic Diversity index and age in control group (Spearman's correlation  $\rho = 0.42, p = 0.033, n = 25$ )

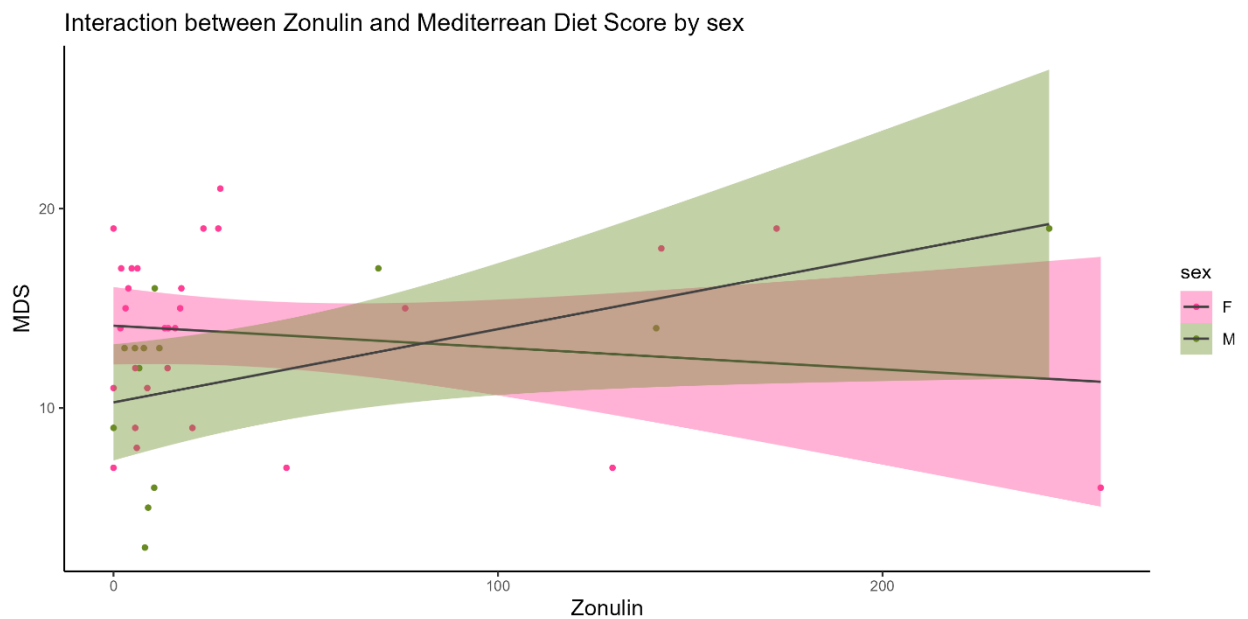


Figure 10: Gamma regression model showing a significant interaction between sex and MDS on zonulin concentrations ( $\beta = 0.048, p = 0.033, n = 42$ ). The  $p$  value of the interaction remained significant also after exclusion of Cook's influential values.

This suggests that the relationship between intestinal permeability and adherence to the Mediterranean diet may differ according to sex. Same reasons can explain another interaction found between sex, microbiota diversity and gut permeability. A very similar result has been obtained on a sex-by- Shannon interaction on zonulin levels ( $\beta = 1.55$ , 95% CI [0.12, 2.97],  $p = 0.034$ ,  $n = 43$ ). Higher Shannon index was associated with lower zonulin concentrations in females, whereas the relationship tended to be positive in males, reflecting probably the same sex-specific associations between gut microbial diversity and intestinal permeability (Figure 11). The interaction remained significant after exclusion of influential observations identified by Cook's distance ( $\beta = 1.44$ , 95% CI [0.20, 2.69],  $p = 0.023$ ), indicating that the finding is reliable.

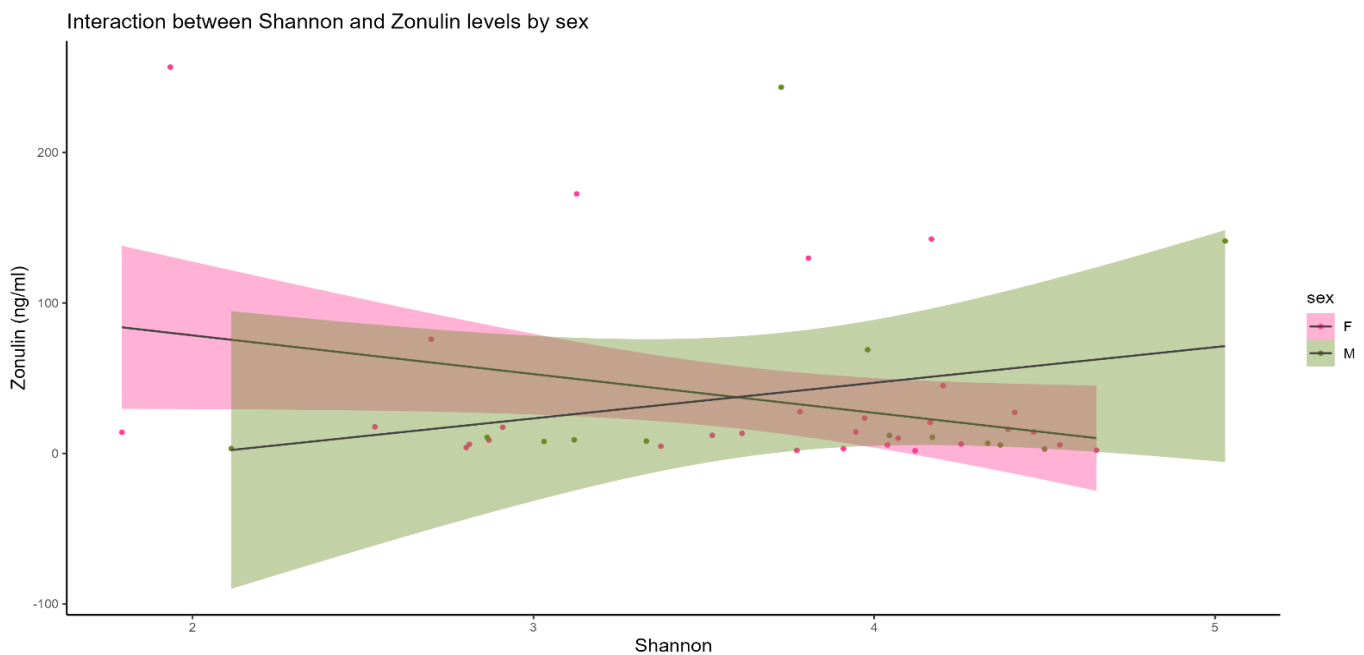


Figure 11: Gamma regression model showing a significant interaction between sex and Shannon index on zonulin concentrations ( $\beta = 1.55$ ,  $p = 0.034$ ,  $n = 43$ ).  $P$  value of the interaction remained significant also after exclusion of Cook's influential values.

One last linear regression unveiled a significant interaction between pathology and indican on Shannon index ( $\beta = 0.11$ , 95% CI [0.02, 0.21],  $p = 0.019$ ,  $n = 43$ ), confirmed by Cook's distance analysis ( $\beta = 0.04$ , 95% CI [0.005, 0.07],  $p = 0.027$ ) (Figure 12). The result in healthy controls confirms that increased protein fermentation and dysbiosis result in a reduced microbial richness. Conversely, this relationship was not observed in patients with IBD, suggesting that the disease itself may alter the physiological link between microbial diversity and bacterial tryptophan metabolism. This odd trend among patients could be also explained by the limited number of IBD subjects compared to controls (respectively  $n = 18$  vs  $n = 25$ ).

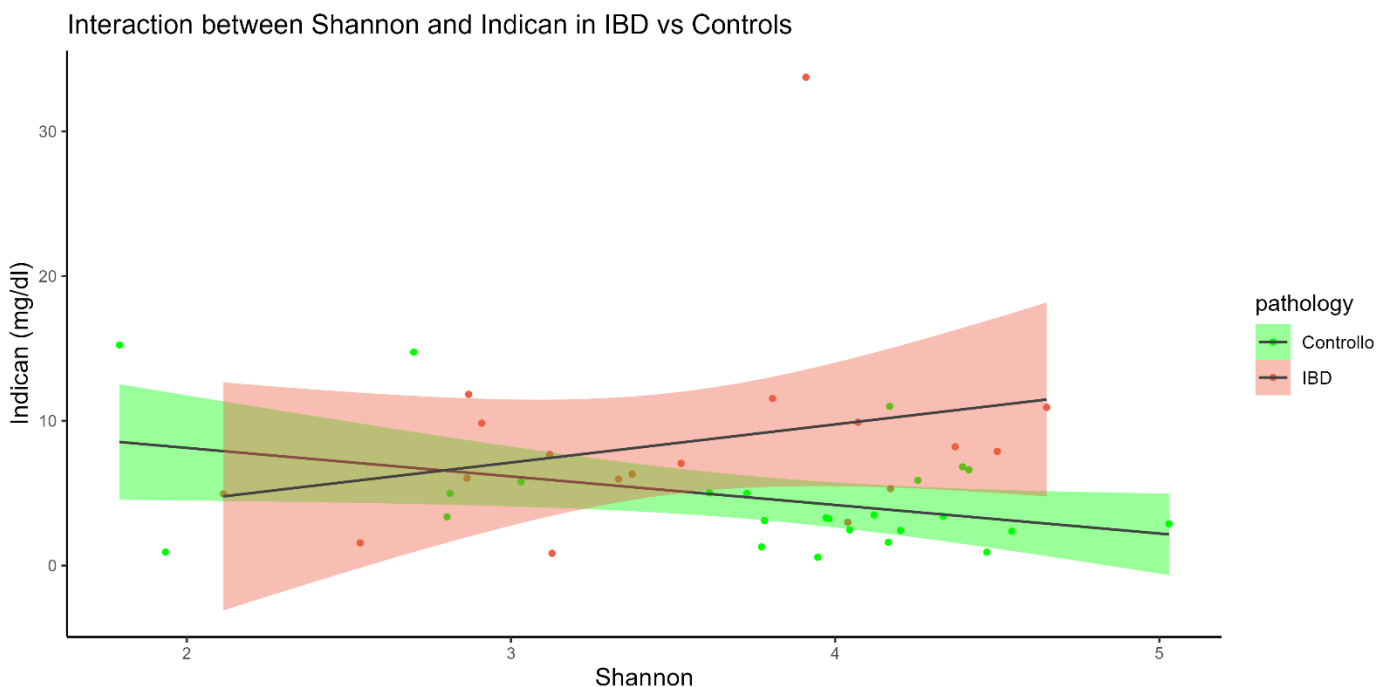


Figure 12: Linear regression model showing an inverse trend between controls and IBD patients in indican levels over Shannon index: in healthy subjects' urine indican levels decrease when Shannon increases, whereas in IBD patients indican levels tend to augment together with Shannon index ( $\beta = 0.11$ ,  $p = 0.019$ ,  $n = 43$ ).

### 5.2.3 Physical activity influence

Physical activity was not reflected by a richness difference in microbiota composition. Nevertheless, it showed an evident opposite effect between a

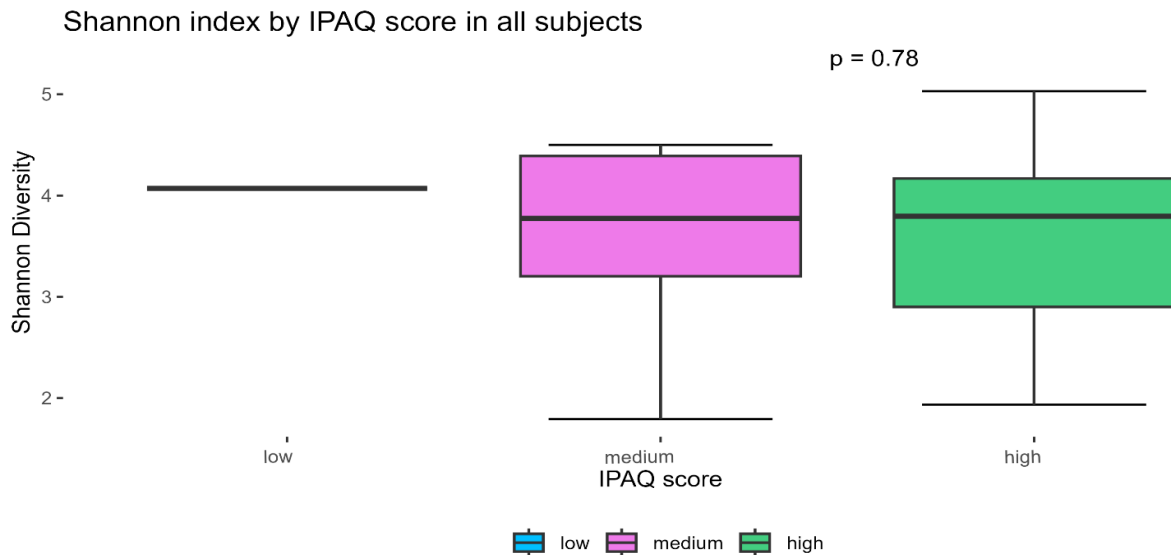


Figure 13 Alpha diversity index Shannon by IPAQ score in all subjects

healthy microbiota and disease-associated gut microbiota. The Shannon alpha diversity index did not show any significant differences between IPAQ scores testing all the subjects (Kruskal–Wallis test:  $\chi^2 = 0.50$ ,  $n = 36$ ,  $p = 0.780$ ) (Figure 13). Even so, considering IBD group and controls separately, an inverse trend emerges between IBD patients and control group (Figure 14). Specifically, for IBD patients, this results in a significant inverse correlation between microbial diversity and physical activity level expressed as Metabolic Equivalent of Task per minute per week (MET-min/week) (Spearman's correlation  $\rho = -0.62$ ,  $p = 0.016$ ,  $n = 15$ ).

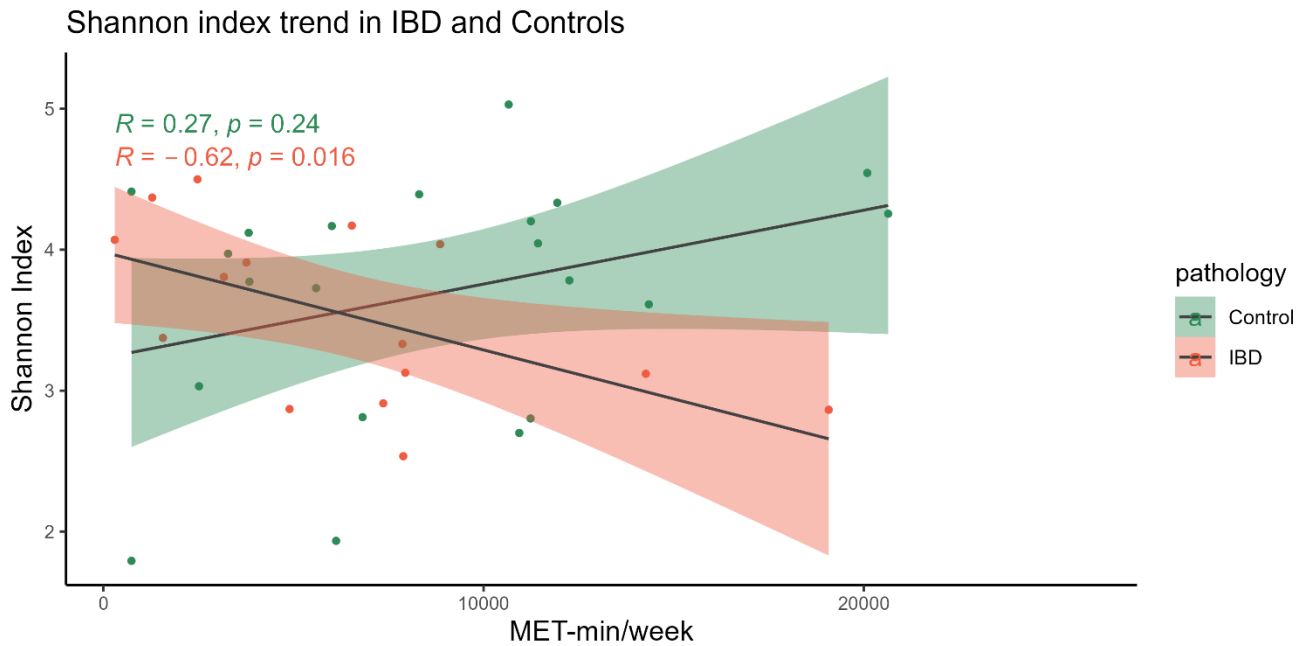


Figure 14 Shannon index inverse trend over physical activity levels (MET-min/week) between healthy controls and IBD patients. There is an inverse significant correlation in IBD group (Spearman's  $\rho = -0.62, p = 0.016, n = 15$ ).

In addition, physical activity resulted in a negative correlation with indican levels considering all the subjects (Spearman's correlation  $\rho = -0.38, p = 0.020, n = 37$ ) (Figure 15). Curiously, the same significant negative correlation persists when testing only IBD group (Spearman's correlation  $\rho = -0.57, p = 0.026, n = 15$ ) (Figure 16), but not in the control group (Spearman's correlation  $\rho = -0.24, p = 0.27, n = 22$ ) suggesting that the effect is driven by a pathology effect.

Hence, physical activity does not alter microbiota richness, but in IBD patients there is a trend reflecting a relation between higher levels of exercise with lower microbial diversity (unlike controls). At the same time, a beneficial effect on intestinal dysbiosis specific to the pathological condition is illustrated by a negative correlation between higher physical activity and lower indican levels.

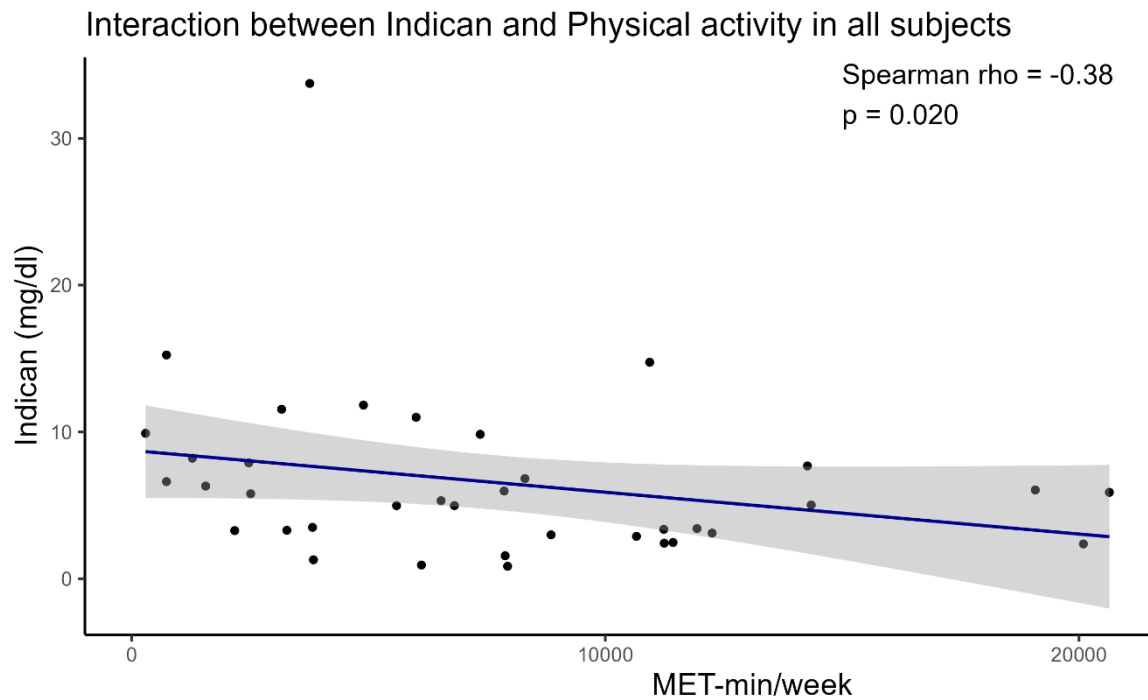


Figure 15: statistically significant negative correlation exists between urine indican level (mg/dl) and physical activity levels (MET-min/week) in all the subjects (Spearman's correlation  $\rho = -0.38$ ,  $p = 0.020$ ,  $n = 37$ )

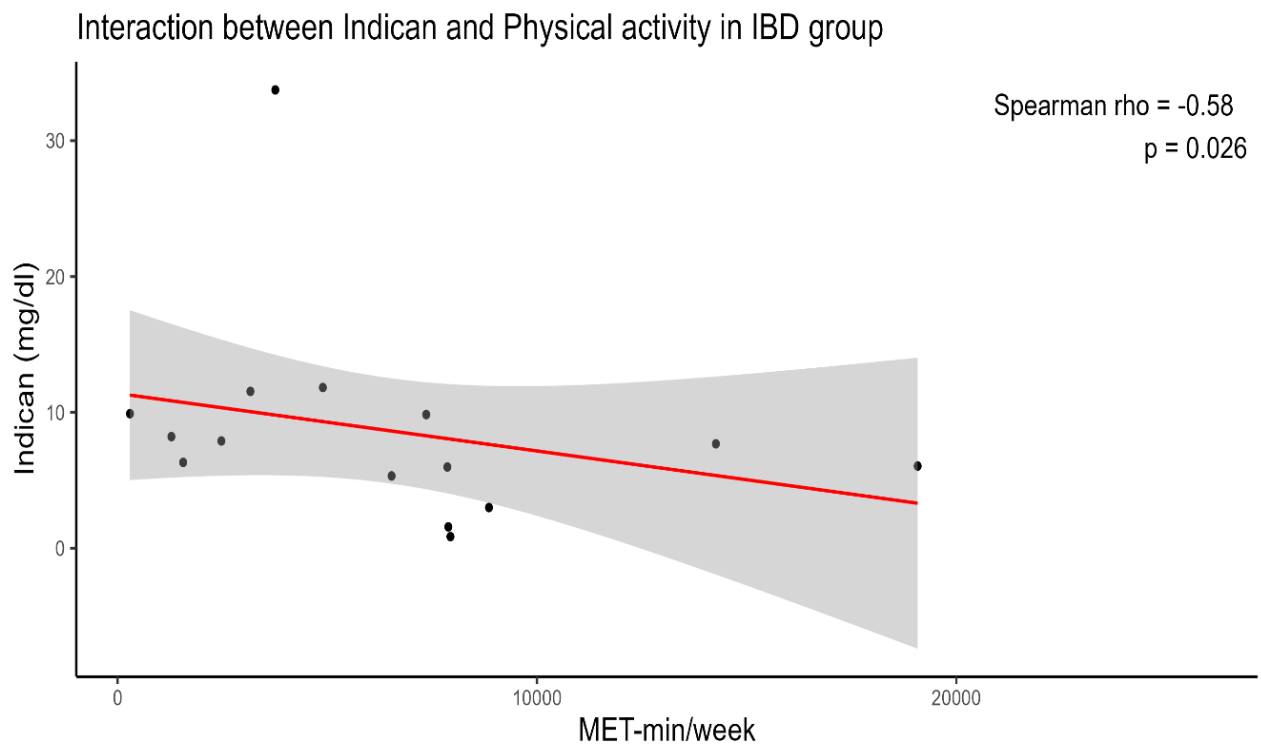


Figure 16 A significant negative correlation emerges between urine indican levels (mg/dl) and physical activity (MET-min/week) in IBD patients (Spearman's correlation  $\rho = -0.57$ ,  $p = 0.026$ ,  $n = 15$ )

## **5.3 Results of the mother's cohort observational study**

### **5.3.1 Cohort description**

The subjects enrolled in this cohort included 22 mothers, two of whom were not assigned to any group. The remaining 20 were divided into a control group (n = 11) and a dietary intervention group (n = 9). The two groups were comparable in age, with a mean  $\pm$  SD of  $34.5 \pm 3.24$  years in the control group and  $32.2 \pm 4.18$  years in diet group. Breast milk samples were collected from 11 mothers in control group and 8 from the diet group. Adherence to Mediterranean diet was assessed before and after nutritional counselling using the Mediterranean Diet Score (MDS). At baseline, the two groups showed very similar adherence levels (control:  $14.5 \pm 2.46$ ; diet:  $14.6 \pm 2.07$ ). Following the dietary intervention, both groups showed an increase in MDS, with a median post-counselling score of 16.5 (IQR: 2) in the control group and 17 (IQR: 2.5) in the diet group. Physical activity levels were evaluated using the IPAQ questionnaire. In both groups, the majority of mothers fell in the low-to-medium activity categories. In control group, 4 mothers were classified as low activity and 7 as medium, with none classified in the high category. The diet group showed a similar distribution, with 3 mothers in the low, 5 in the medium, and 1 in the high activity category. All the descriptive data are summarized in table 3.

Table 3: Descriptive statistics of the mother's cohort

|                                         | Control group | Diet group |
|-----------------------------------------|---------------|------------|
| <b>Mothers</b>                          | 11            | 9          |
| <b>Age</b>                              | 34,5±3,24     | 32,2±4,18  |
| <b>Milk samples</b>                     | 11            | 8          |
| <b>Mediterranean Diet Score</b>         |               |            |
| <b>Pre-counselling (mean ±SD)</b>       | 14,5±2,46     | 14,6±2,07  |
| <b>Post-counselling (median, Q1-Q3)</b> | 16,5 (2)      | 17 (2,5)   |
| <b>IPAQ score (n)</b>                   |               |            |
| <b>low</b>                              | 4             | 3          |
| <b>medium</b>                           | 7             | 5          |
| <b>high</b>                             | 0             | 1          |

### 5.3.2 Nutritional counselling increases adherence to Mediterranean diet

As expected, the adherence to Mediterranean diet, measured as Mediterranean Diet Score (MDS), was higher after nutritional counselling intervention than before in all together taken subjects but also just in diet and control group alone. Specifically, in all the subjects, the mean MDS before intervention is  $14.94 \pm 1.83$ , while after is  $17.2 \pm 1.51$  (paired t-test  $t = -7.58$ ,  $p < 0.001$ ,  $n = 18$ ) (Figure 17). In diet group median MDS pre counselling is 16, IQR 2 and after MDS is median 17, IQR 2.5 (Wilcoxon signed rank test  $V = 0$ ,  $p = 0.019$ ,  $n = 7$ ). In control group mean MDS before is  $15 \pm 2.05$  and mean MDS after is  $17.1 \pm 1.4$  (paired t-test  $t = -4.58$ ,  $p = 0.0013$ ,  $n = 10$ ) (Figure 18). Following a Mediterranean dietary pattern during gestation has been widely demonstrated to be beneficial for the health of both the mother and offspring: the benefits brought by MD are favourable

outcomes for both maternal and newborn health, particularly, reduced risk of gestational diabetes, preterm birth and abnormal fetal growth (Amati et al., 2019; S. Esposito et al., 2026; Scarpato et al., 2026). Promoting the MD in pregnancy has given the expected results.

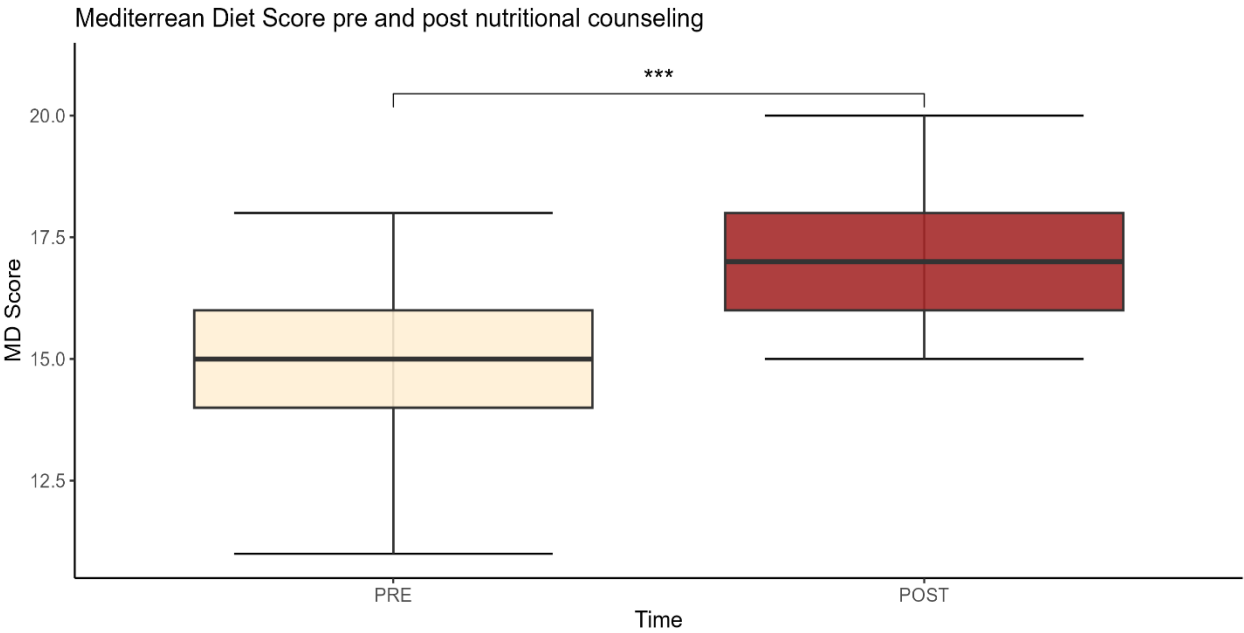


Figure 17 Mediterranean Diet Score is significantly higher in all the mothers after delivery compared to before delivery (paired *t*-test  $t = -7.58$ ,  $p < 0.001$ ,  $n = 18$ )

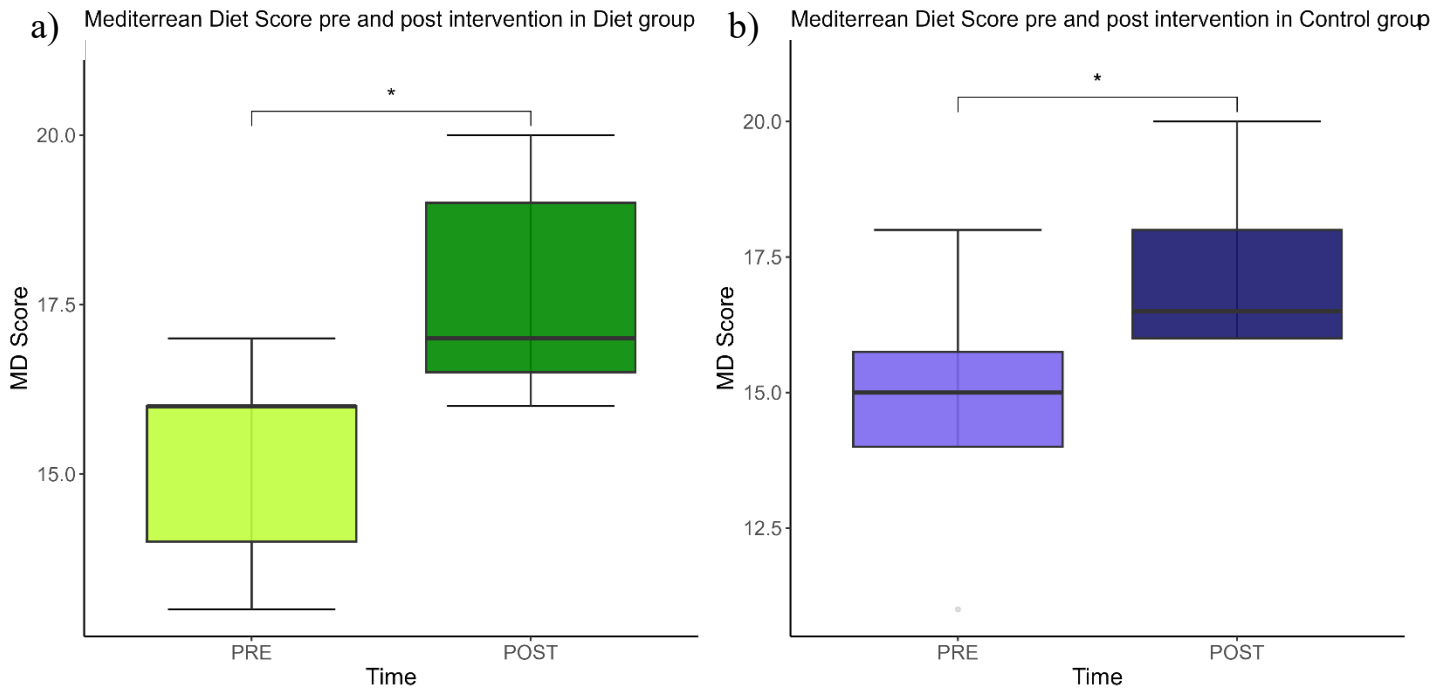


Figure 18: Boxplots illustrating the Mediterranean Diet Score in pre and post-dietary nutritional education in diet group (a) (Wilcoxon signed rank test  $V=0$ ,  $p=0.019$ ,  $n=7$ ) and in control group (b) (paired  $t$ -test  $t=-4.58$ ,  $p=0.0013$ ,  $n=10$ )

### 5.3.3 Microbiota results

The mothers' microbiota composition resulted to have very high inter-individual variability, yet it was different between the two groups and different from babies. The maternal microbiota is dominated by members of the genera *Bacteroides\_H*, *Phocaeicola\_A\_858004*, *Faecalibacterium*, *Alistipes\_A\_871400* and *Akkermansia* (Figure 19). There is a considerable between-subject variability in taxonomic composition across all timepoints mT0 (third trimester of pregnancy), mT1 (post-partum, within the first 7 days) and mT2 (3 months after delivery). A first glance at the microbial composition in mothers divided by IPAQ scores (inactive and sufficiently active) reveals a similar composition: yet,

in sufficiently active mothers there is a decrease of *Parabacteroides\_B\_862066*, *Gemmiger\_A\_73129* and *Acetifactor* genera, while there is an increment of taxa like *Akkermansia*, *Alistipes*, *Bacteroides\_H*, and *Faecalibacterium* (Figure 20).

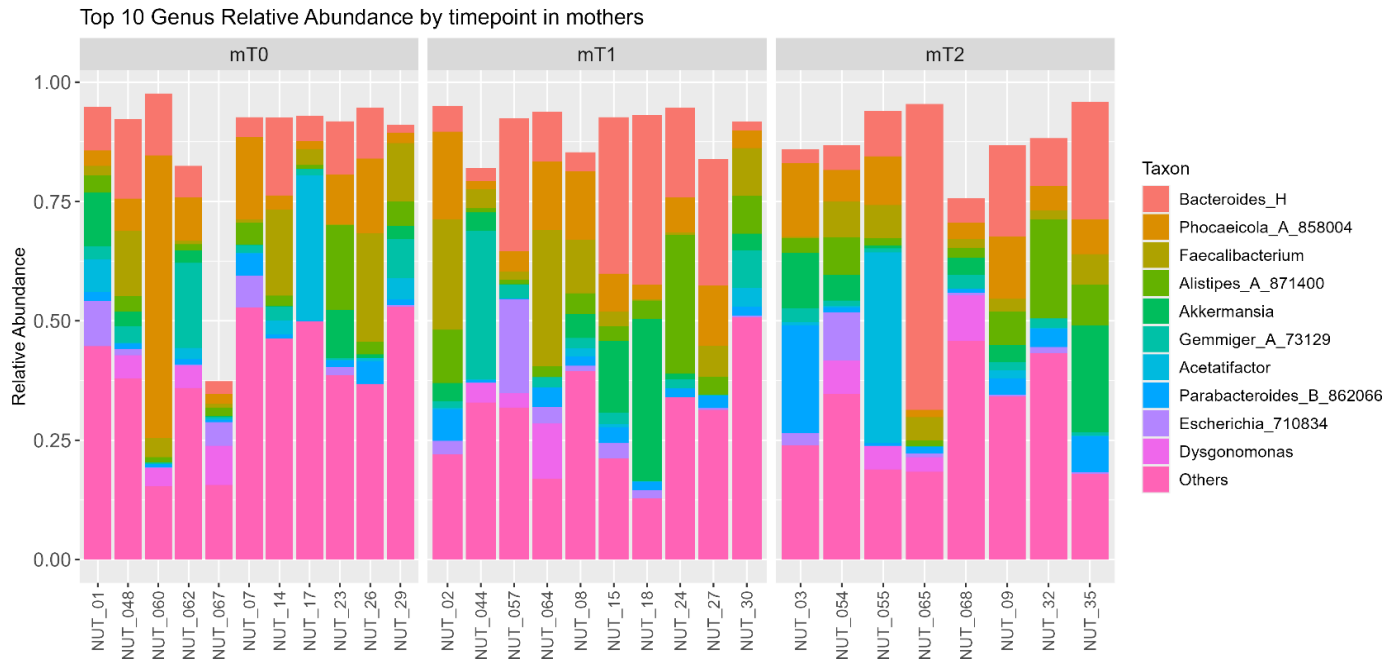


Figure 19 The first 10 genus relative abundance by timepoint in mother's microbial composition

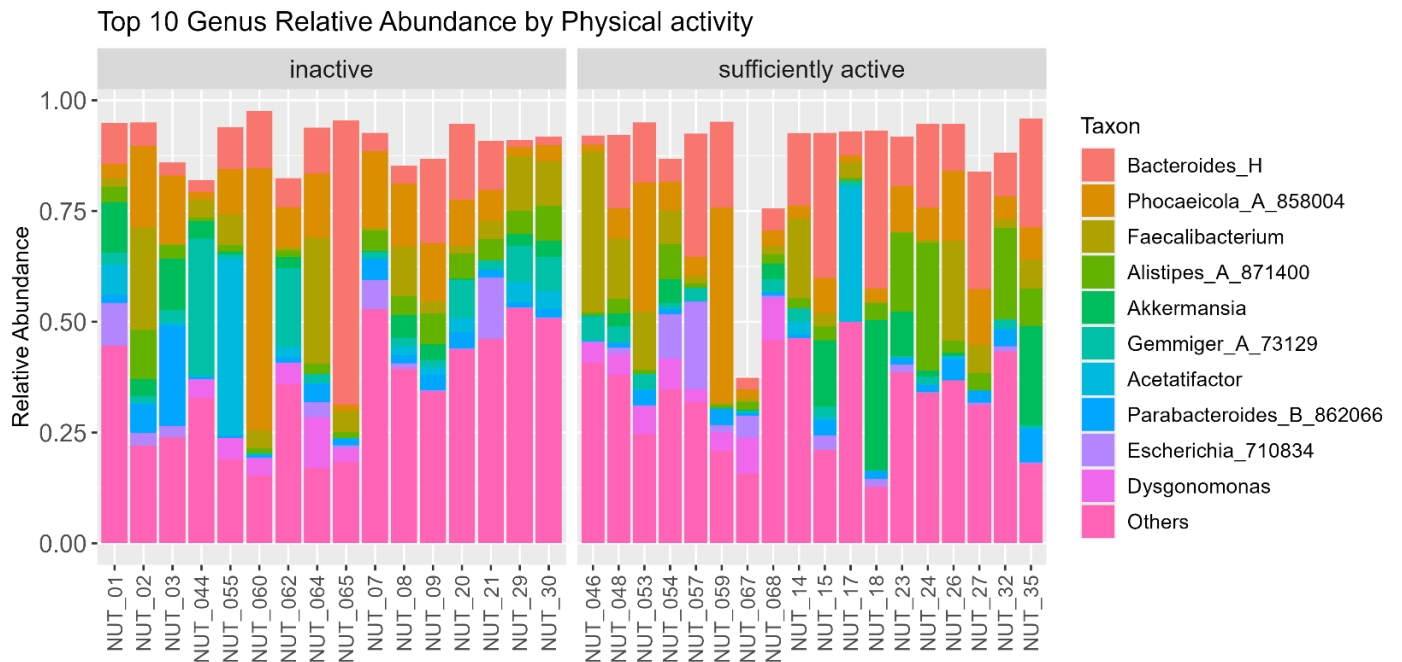


Figure 20: The most representative relative abundance of taxa in subjects divided by physical activity IPAQ score (inactive and sufficiently active)

The incremented genera have been already documented augmenting in physically active subjects (Aguiar et al., 2024; Aya et al., 2021; Morita et al., 2019), improving gut health, metabolism and giving protection against chronic inflammation. However, it is important to note that much of the literature observed this changes in athletes, healthy adults or obese subjects and much less studies observed pregnant women. Comparing the mother's microbiota composition with that of their newborns, it is evident a difference in taxa composition (Figure 21). The main representative taxa in infants are *Escherichia\_710834*, *Veillonella\_A*, *Phocaeicola\_A\_858004* and *Bifidobacterium\_388775*. Here also, there is a strong inter-individual variability.

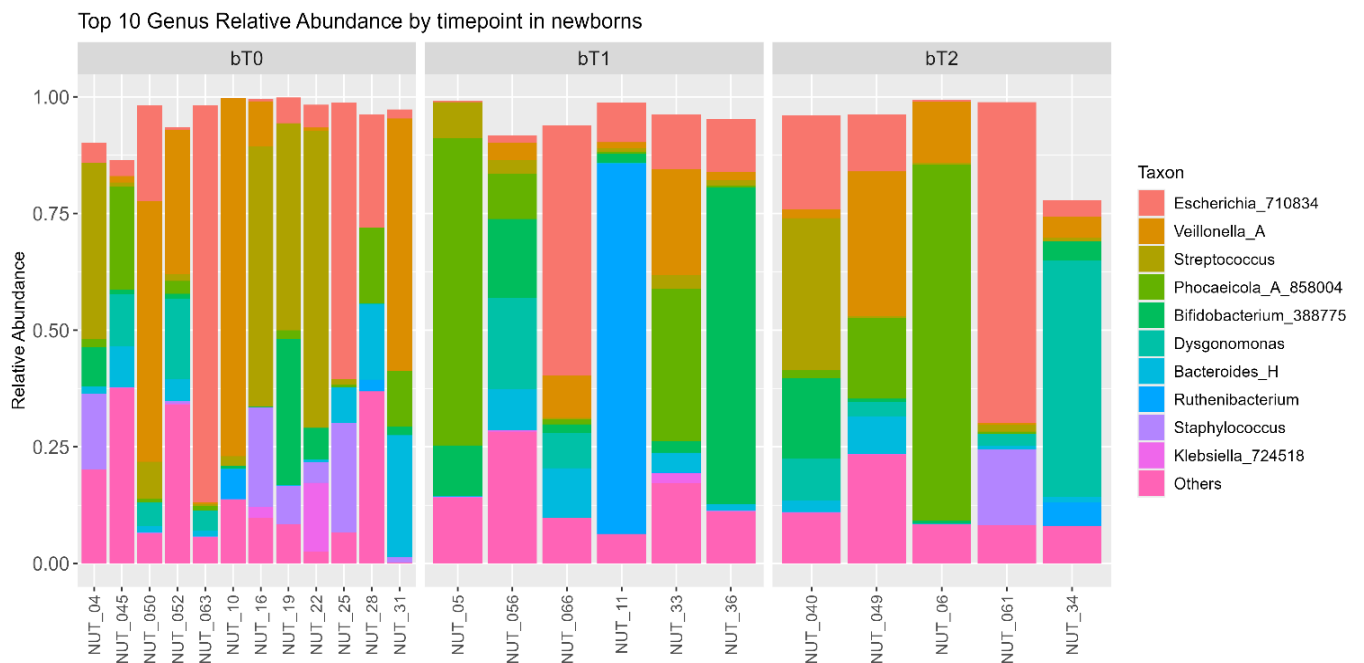


Figure 21: Top 10 relevant taxa in babies' microbial composition at the three different time points

This composition is aligned with literature data about newborn's microbiota: immediately after birth, and here represented as bT0, microbiota is

overpopulated by *Enterobacteriaceae* (to which *Escherichia\_710834* belongs) and *Staphylococcus* (Matsuki et al., 2016). Later it is replaced by *Bifidobacterium* (MITSUOKA, 2014) as it is reflected by representation of time bT2 (3 months old). This kind of microbiota *Bifidobacterium*-dominated, also known as “*Bifidus flora*”, keep on until the introduction of complementary food (Palmer et al., 2007; Vallès et al., 2014). As the infant approaches the weaning phase, the relative abundance of *Bacteroides* gradually increases, leading to the competitive exclusion of *Bifidobacterium* within the intestinal microbiota (Pantazi et al., 2023). Comparing the relative abundance of main taxa of babies’ microbiota between groups, there is a visual decrease in *Streptococcus*, *Staphylococcus*, *Ruthenibacterium* and *Bifidobacterium\_388775* and an increase of *Escherichia\_710834*, *Bacteroides\_H* and *Veillonella\_A* taxa in diet group compared to control (Figure 22).

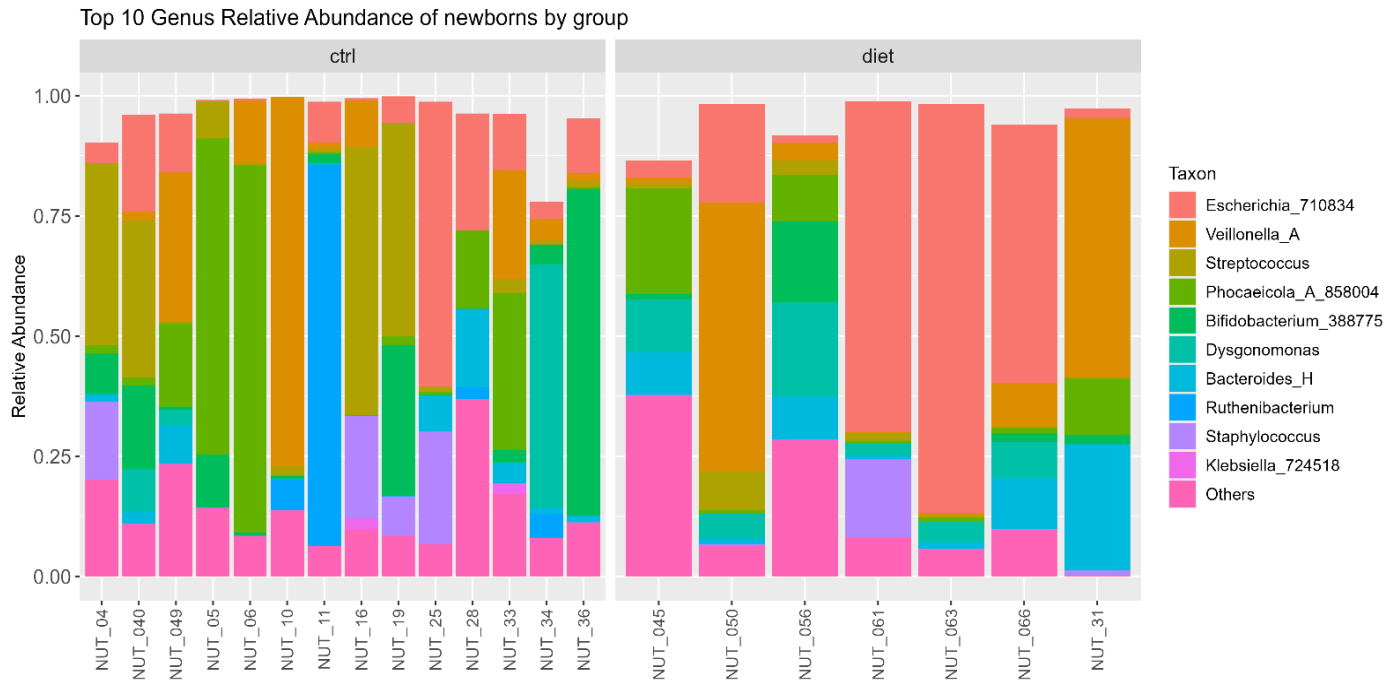


Figure 22: Most representative taxa in babies' microbiota composition divided by group

The statistical analysis of alpha diversity indexes Shannon and Simpson didn't show any significant differences between timepoints or groups, or by physical activity. In particular, Shannon index over time (Linear mixed model with mother as random effect  $F(2, 20.02) = 0.20$ ,  $p=0.82$ ,  $n=34$ , mothers=13) appears to be higher at T0 lowering at T1 and T2 and having a remarkable heterogeneity in particular at T2 (Figure 23). Similarly, there is no significant difference of the index between groups (Linear mixed model with mother as random effect  $F(1, 9.07) = 0.38$ ,  $p=0.54$ ,  $n=29$ , mothers=11), yet the diet group presents a wider dispersion of data compared to control. Even though not statistically relevant, this heterogeneity reflects the higher median value (2.86, IQR 0.58) of the group following a Mediterranean diet pattern enriched in some vegetables, compared

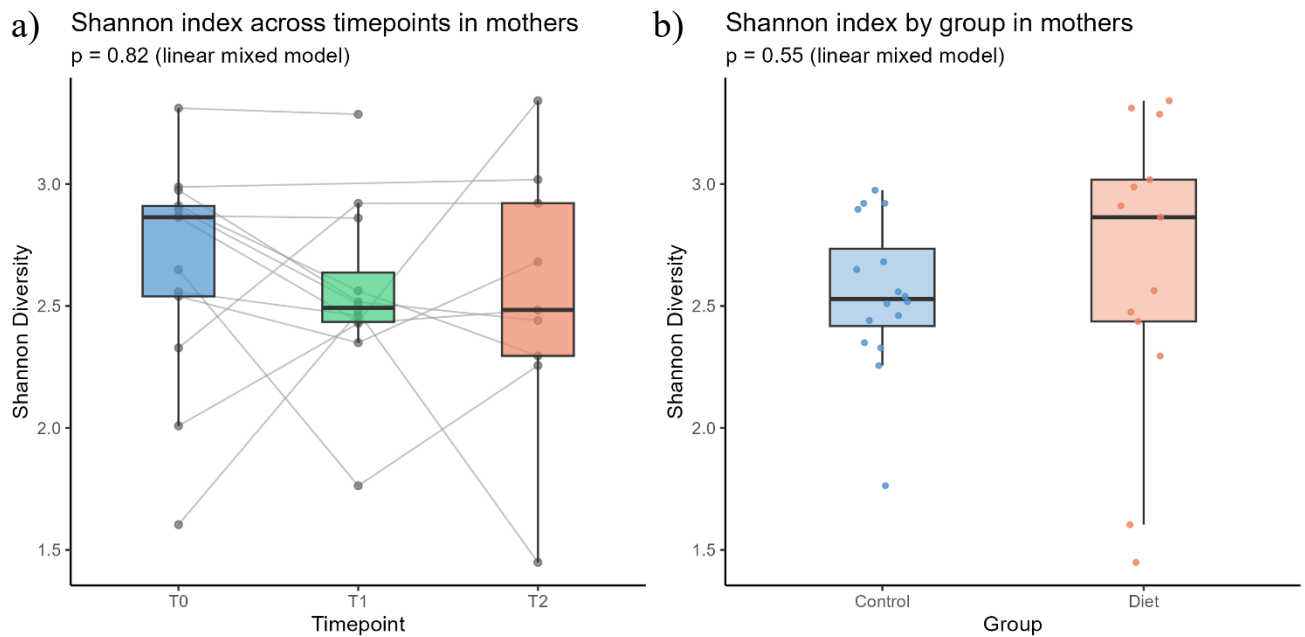


Figure 23: (a) Microbiota alpha diversity index Shannon trend over time (linear mixed model with mother as random effect  $F = 0.20$ ,  $p = 0.81$ ,  $n = 34$ , mothers = 13). The grey points represent the single Shannon values and the grey lines link the values of the same subject showing the trend of one subject over time. (b) mean Shannon index compared between groups (linear mixed model with mother as random effect  $F = 0.38$ ,  $p = 0.54$ ,  $n = 29$ , mothers = 11).

to control (median 2.53, IQR 0.31). Regarding beta diversity, a significant difference in microbial composition was found between mothers and infants (PERMANOVA:  $F(1,55) = 10.49$ ,  $R^2 = 0.16$ ,  $p < 0.001$ ). Nevertheless, the dispersion homogeneity test revealed significantly different intra-group variability between the two groups (analysis of variance:  $F(1,55) = 17.6$ ,  $p = 0.001$ ). Therefore, the observed difference may reflect also a different dispersion within group, rather than a real difference. The Principal Coordinates Analysis (PCoA) of the Bray-distanced data resulted in a clustered distribution between mother's and babies' microbial composition (Figure 24).

The analysis then conducted separating moms by their newborns didn't report any differences in microbiota composition over timepoints in either of the

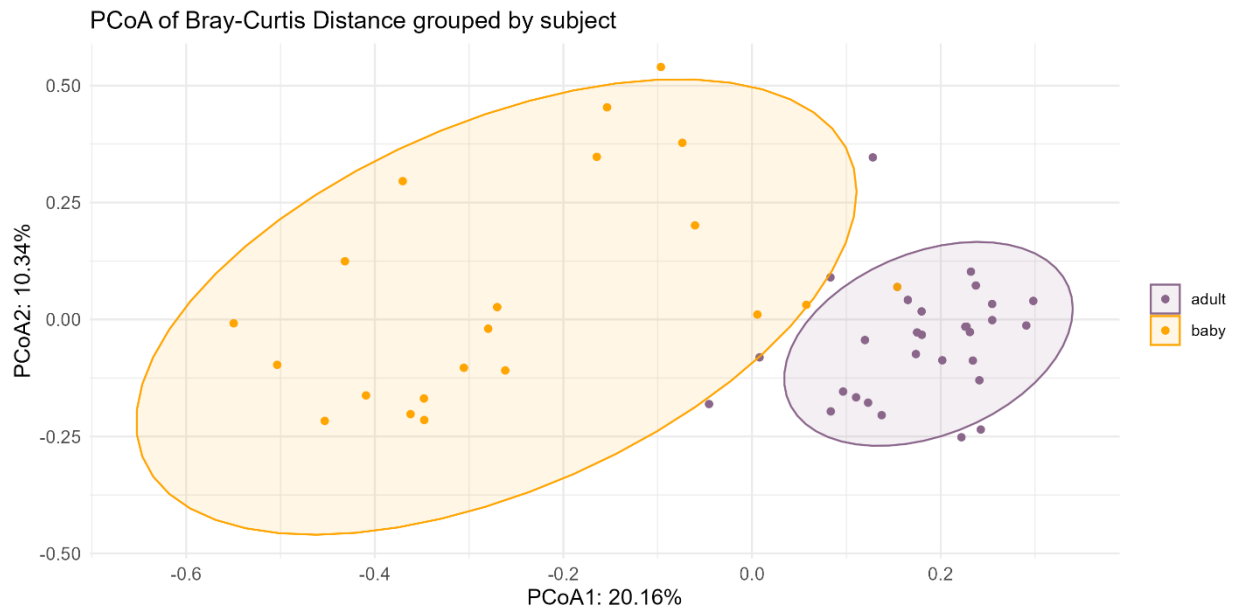


Figure 24: Principal Component Analysis of Bray-Curtis distanced microbiota composition by subject. The PCoA1 and PCoA2 explain respectively the 20.16% and 10.34% of the total variance.

groups (in mothers, PERMANOVA:  $F(2,31) = 0.60$ ,  $R^2 = 0.037$ ,  $p = 0.82$ ,  $n = 34$ ), (babies, PERMANOVA:  $F(2,20) = 0.73$ ,  $R^2 = 0.06$ ,  $p = 0.49$ ,  $n = 23$ ). This could be explained by the high inter-individual variability detected that characterizes gut microbial communities, which appeared to exceed the temporal variation within subjects. Moreover, the relatively small sample size (11 mother-baby dyads) and limited number of repeated observations may have reduced the statistical power to detect subtle longitudinal changes. Looking at the mother's microbiota composition between the two groups, a first visual analysis shows a reduction of the genera *Alistipes\_A\_871400*, *Akkermansia* and *Parabacteroides\_B\_862066* in diet group compared to control (Figure 25).

A consistent difference has been confirmed in mother's beta diversity comparing diet and control group (PERMANOVA:  $F(1,27) = 3.07$ ,  $R^2 = 0.102$ ;  $p <$

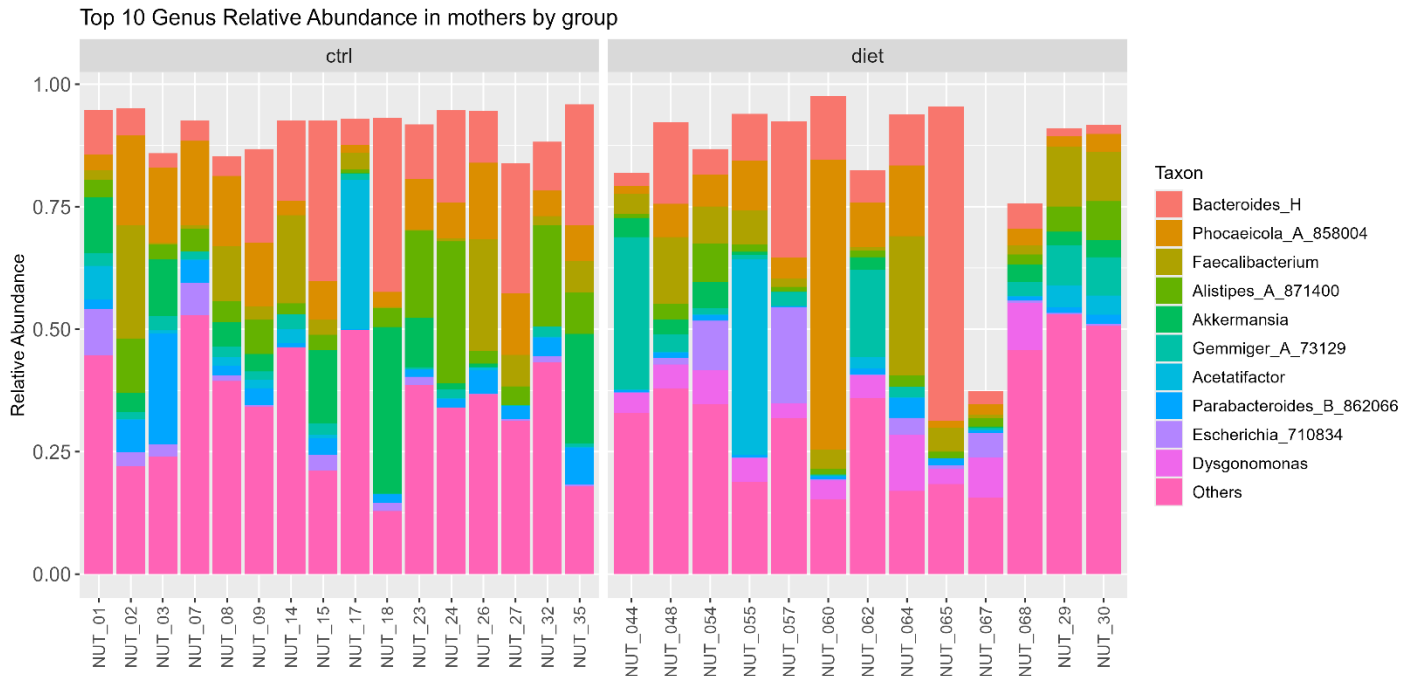


Figure 25: Genus's top 10 relative abundance representation of the mother's microbiota divided by group (0.001) indicating that approximately 10% of the variance in beta diversity is explained by the dietary intervention. A test for homogeneity of groups dispersions (analysis of variance  $F(1,27) = 0.34$ ,  $p = 0.56$ ) confirmed that PERMANOVA indeed reflected differences in microbial composition rather than intra-group variability, even though the relative PCoA on Bray–Curtis distances did not show a visual separation between diet and control groups, with substantial overlapping between samples (Figure 26). The same significant difference, however, did not occur in the group of babies (PERMANOVA:  $F(1,19) = 1.62$ ,  $R^2 = 0.07$ ,  $p = 0.11$ ).

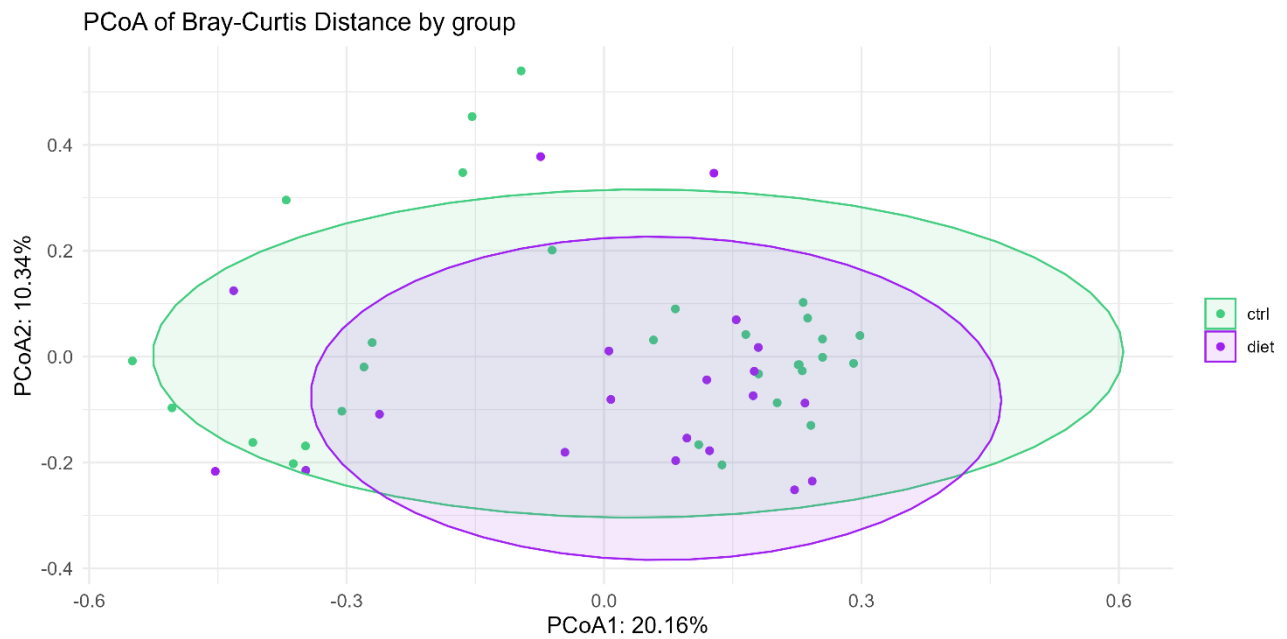


Figure 26: Principal Component Analysis of Bray-Curtis distanced microbiota composition by group. The PCoA1 and PCoA2 explain respectively the 20.16% and 10.34% of the total variance.

Eventually, based on mother's differences between groups, in order to identify the taxa contributing to the observed community differences, CLR-transformed abundances were compared between groups using Wilcoxon rank-sum tests with FDR correction. Twenty-three taxa showed significant differential abundance (adjusted  $p < 0.05$ ). Using the diet group as reference, the relevant taxa are reported in table 3.

Table 3: the twenty-three taxa that drive the difference between groups: CLR-transformed abundances compared using Wilcoxon rank-sum tests with FDR correction.

| Taxon                           | p <sub>adj</sub> | Trend  |
|---------------------------------|------------------|--------|
| <i>Streptococcus</i>            | 0.0010           | Diet ↓ |
| <i>Alistipes_A_871400</i>       | 0.0027           | Diet ↓ |
| <i>Parabacteroides_B_862066</i> | 0.0044           | Diet ↓ |
| <i>Alistipes_A_871404</i>       | 0.0044           | Diet ↓ |
| <i>Dysgonomonas</i>             | 0.0092           | Diet ↑ |

|                               |        |        |
|-------------------------------|--------|--------|
| <i>CAG-267</i>                | 0.0092 | Diet ↓ |
| <i>Gemella</i>                | 0.0092 | Diet ↓ |
| <i>Clostridium P</i>          | 0.0092 | Diet ↑ |
| <i>Faecalibacillus</i>        | 0.0098 | Diet ↓ |
| <i>Bifidobacterium 388775</i> | 0.0151 | Diet ↓ |
| <i>Phocaeicola_A_858004</i>   | 0.0162 | Diet ↓ |
| <i>Haemophilus D 735815</i>   | 0.0207 | Diet ↓ |
| <i>Veillonella_A</i>          | 0.0208 | Diet ↑ |
| <i>Barnesiella</i>            | 0.0208 | Diet ↓ |
| <i>Staphylococcus</i>         | 0.0265 | Diet ↑ |
| <i>Klebsiella_724518</i>      | 0.0289 | Diet ↑ |
| <i>Faecalimonas</i>           | 0.0315 | Diet ↑ |
| <i>Bacteroides_H</i>          | 0.0344 | Diet ↓ |
| <i>Enterococcus_H_360604</i>  | 0.0376 | Diet ↓ |
| <i>Enterococcus B</i>         | 0.0410 | Diet ↓ |
| <i>Ruminiclostridium_E</i>    | 0.0447 | Diet ↓ |
| <i>Akkermansia</i>            | 0.0467 | Diet ↓ |
| <i>ER4</i>                    | 0.0467 | Diet ↓ |

### 5.3.4 Effects of physical activity

Physical activity didn't result in significant differences among other parameters except for S100B. Indeed, it seems to increase the S100B concentration in breast milk. Among mothers in the control group, women classified as sufficiently physically active exhibited higher normalized S100B concentrations (median 0.0137 IQR 0.00) than inactive mothers (median 0.0102, IQR= 0.00) (Kruskal-Wallis test  $\chi^2 = 6.03$ ,  $p = 0.014$ ,  $n = 11$ ) (Figure 27). Although the biological significance of this finding is unclear, regular physical activity is known to influence inflammatory signalling, metabolic homeostasis and the production of several bioactive proteins (Leal et al., 2018; Pedersen, 2019; Pedersen et al., 2007). It is therefore conceivable that maternal physical activity may also affect the secretion of S100B into breast milk. As far as is known, no previous studies have specifically investigated the relationship between maternal physical

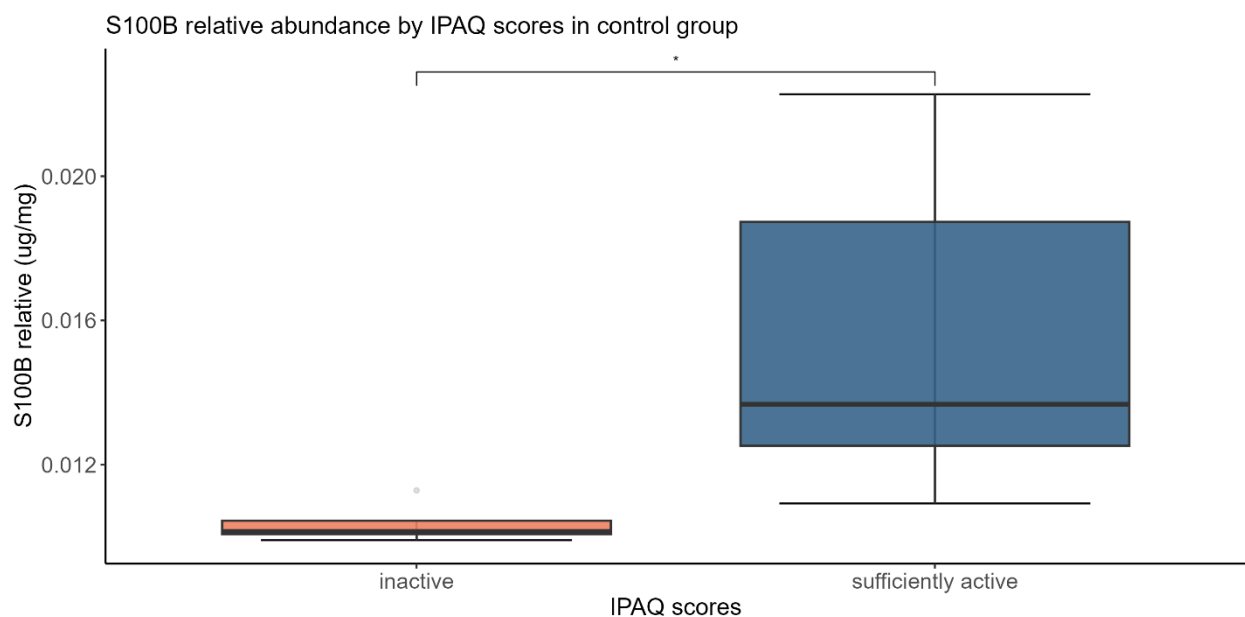


Figure 27: In control group S100B relative concentration appears to be lower in physically inactive mothers compared to sufficiently active mothers (Kruskal-Wallis test  $\chi^2 = 6.03$ ,  $p = 0.014$ ,  $n = 11$ ).

activity and S100B breast milk concentration. Consequently, also considering the limited number of subjects, this finding should be regarded as hypothesis-generating and requires confirmation in larger cohorts.

### 5.3.5 S100B concentrations negatively correlate to age

The already existing data about S100B protein being present in breast milk found confirmation in this study. All the samples tested positive and the values of the protein ranged from a minimum of 0.017  $\mu\text{g/ml}$  to a maximum of 0.084  $\mu\text{g/ml}$ . When investigating the relationship a moderate negative correlation was found between S100B (absolute value) levels and subject's age (Spearman's correlation test  $\rho = -0.63$ ,  $p = 0.0038$ ,  $n = 19$ ) (Figure 28). Interestingly, conducting the analysis separately on the diet and control groups, the S100B levels persist

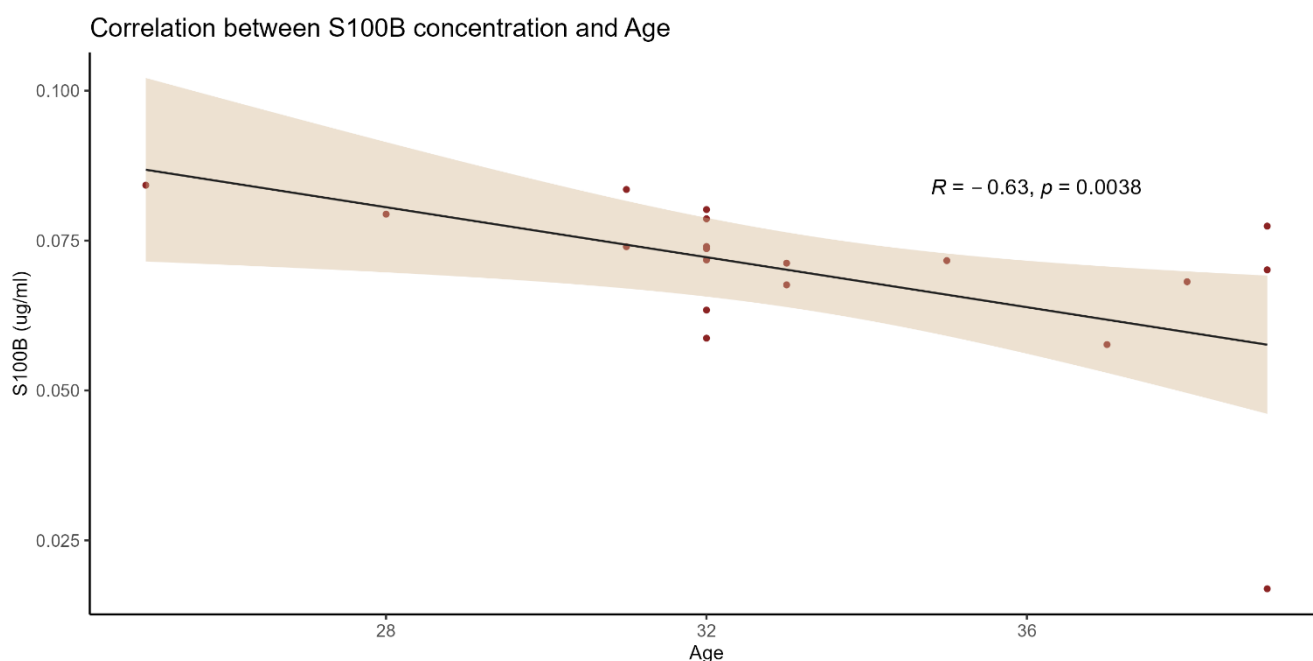


Figure 28: A negative correlation resulted between S100B levels (mean of the kits, absolute values) and age in all the subjects (Spearman's correlation test  $\rho = -0.63$ ,  $p = 0.0038$ ,  $n = 19$ )

to be negatively correlated to age in diet group (Spearman's correlation  $\rho = -$

0.81,  $p = 0.014$ ,  $n = 8$ ) (Figure 29), but not in control group. The negative association in all subjects was attenuated after adjustment for study group, and

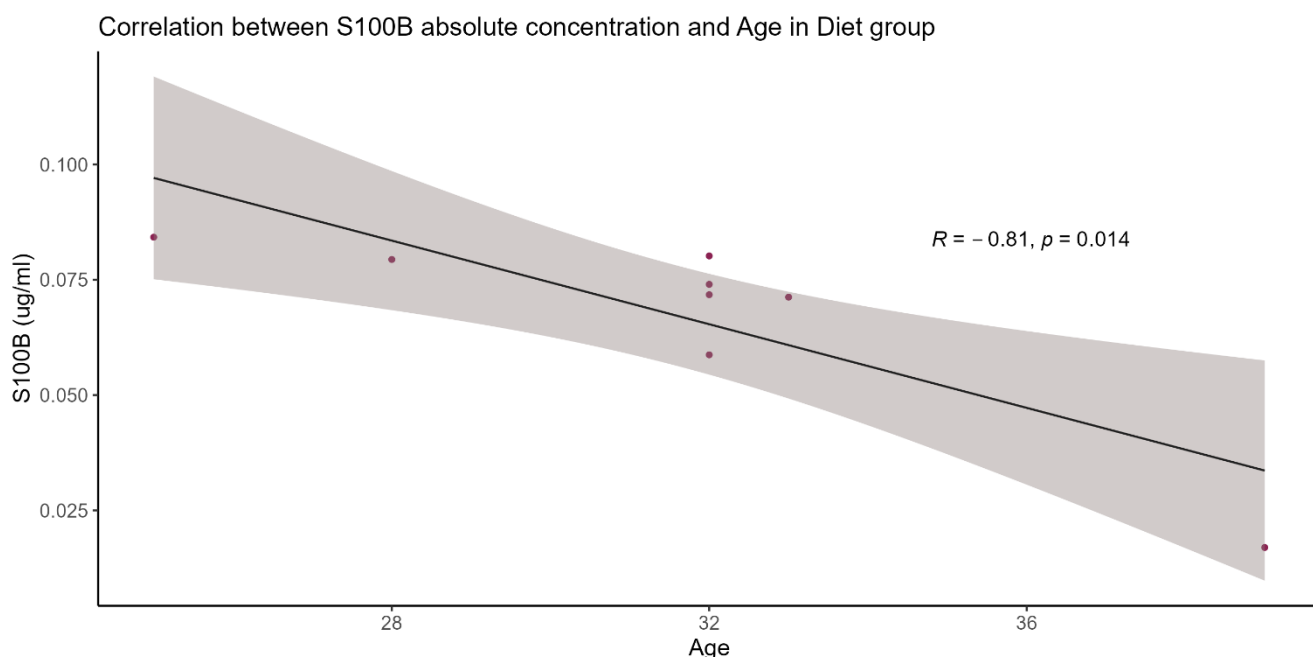


Figure 29: Negative correlation between S100B levels (mean of the kits, absolute values) and age in diet group (Spearman's correlation  $\rho = -0.81$ ,  $p = 0.014$ ,  $n = 8$ )

no significant interaction between age and dietary intervention was detected.

These findings suggest that maternal age may influence S100B concentration, although the evidence remains inconclusive due to the limited sample size.

## 5.4 Results of S100B in diet

### 5.4.1 In silico S100B molecular investigation

The S100B protein motif was expected in silico in different plant species. The root-mean-square deviation (RMSD) serves as a metric to quantify the average spatial separation between the atoms of the examined protein and the reference

S100B structure. A diminished RMSD value correlates with heightened structural similarity, as elucidated in the tabulated results (table 4). Comprehensive protein assessments already yield acceptable outcomes; however, a more refined analysis focusing solely on conserved residues revealed an additional reduction in the RMSD. This observation underscores the notably elevated degree of structural homology within the S100B motif of the investigated proteins.

Table 4 S100B homologs in plants. The table shows the comparison among the aligned proteins having the higher E-value in the alignment result with the reference structure S100B. Root Mean Square Deviation (RMSD full) and TM-align (TM-align score full, version 15/04/2022) were initially calculated with the software TM-align (Y. Zhang & Skolnick, 2005) on the full proteins, with normalization based on the residues of S100B. Solvent Accessible Surface Area (SASA) calculations were performed without normalization, aiming to provide an approximate indicator of the size of the proteins. Afterwards, the conserved N-terminal S100B domain was isolated, and the root mean square deviation (RMSD conserved) and the TM-align score (TM-align score conserved) were calculated for those residues as well.

| Species                    | Accession  | Annotation                  | RMSD | TM-score | SASA (Å <sup>2</sup> ) | RMSD cons. | TM-score cons. |
|----------------------------|------------|-----------------------------|------|----------|------------------------|------------|----------------|
| <b>S100B</b>               | P04271     | S100B ( <i>H. sapiens</i> ) | –    | –        | 6504.42                | –          | –              |
| <i>A. digitata</i>         | Q6EIJ6     | Maturase K                  | 3.67 | 0.4106   | 55361.47               | 2.04       | 0.4728         |
| <i>A. capillus-veneris</i> | A0A9D4UGI1 | EF-hand protein             | 2.46 | 0.5555   | 7227.49                | 2.32       | 0.7455         |
| <i>C. richardii</i>        | A0A8T2T945 | EF-hand protein             | 3.06 | 0.6328   | 12912.80               | 2.36       | 0.7311         |
| <i>C. europaea</i>         | A0A9P0ZHW9 | EF-hand protein             | 3.80 | 0.6159   | 11235.98               | 2.85       | 0.7075         |
| <i>D. zibethinus</i>       | A0A6P6AZ13 | Ca-binding protein          | 2.74 | 0.6694   | 11374.78               | 2.63       | 0.7148         |
| <i>G. klotzschianum</i>    | A0A7J8USE5 | EF-hand protein             | 2.43 | 0.6250   | 11317.84               | 2.41       | 0.7040         |
| <i>H. impetiginosus</i>    | A0A2G9G7M9 | EF-hand protein             | 3.07 | 0.6160   | 15050.24               | 2.19       | 0.7337         |
| <i>H. syriacus</i>         | A0A6A3BQP9 | PfkB-like kinase            | 2.82 | 0.6195   | 10389.12               | 2.13       | 0.7627         |
| <i>M. domestica</i>        | A0A498KP84 | EF-hand protein             | 3.50 | 0.6018   | 37161.19               | 2.81       | 0.6325         |
| <i>M. acuminata</i>        | A0A804HRU8 | Hypothetical protein        | 2.53 | 0.6163   | 5706.66                | 2.03       | 0.7767         |
| <i>O. europaea</i>         | A0A8S0U3M0 | Ca-binding CML18            | 2.51 | 0.6307   | 12185.49               | 2.38       | 0.7467         |

However, from a purely phylogenetic point of view, the S100B protein alone is not informative enough to clearly define the evolutionary history of green plants, but it can be noticeably regarded as an interesting biomarker. Afterwards, the conserved domain demonstrating significant structural homology with the benchmark protein S100B was identified and isolated. The obtained structures were aligned with the benchmark protein structure, and RMSDs and TM-align were computed to evaluate the precision of the structural similarity between the analysed peptides and the reference protein (D. Zhang et al., 2007). The RMSD and TM-align calculations were normalized with respect to the reference structure of S100B. Finally, a satisfying alignment of both peptides analysed with S100B was clearly observed. Notably, the comprehensive analysis of the entire protein structures had already revealed substantial similarities. However, a more discerning examination, isolating and comparing the S100B motif, elucidated an augmented level of similarity when contrasted with the reference protein. The results of the comparative structural analysis indicated that the three-dimensional structural similarity is significantly improved when only the conserved domain is considered. This observation supports the hypothesis that proteins have retained this biological function throughout evolution in the plant kingdom. These findings support the presence of proteins with a conserved S100B motif in different plants belonging to the plantae kingdom. Proteomic sequences from species belonging

to the *Viridiplantae* clade with the highest homology with the human S100B motif were selected to study the eventual presence of the latter among green plants. The top 100 organisms with the closest similarity to the human S100B protein were retained. The individual proteomes were used to infer a phylogenetic tree, aiming to obtain an evolutionary history close to current taxonomic beliefs. Despite the limited size of the S100B protein, the analysis yielded a satisfactory phylogeny. Species with a close common ancestor were correctly clustered in the same groups, e.g., *Musa balbisiana* and *Ensete ventricosum*, the *Gossypium* genus, *Hibiscus syriacus*, and *Durio zibethinus*, the *Brassica* genus, *Eutrema salsugineum*, and *Raphanus sativus*.

#### **5.4.2 S100B-like immunomaterial in plant extract**

In the same species where S100B motif was identified *in silico*, S100B-like immunoreactive material was also detected in plant samples. A total of 50% of tested samples were positive (n = 11/22) (Table 5). Among the powder samples, 50% were positive (n = 5/10), and 33.3% (n = 3/9) of fresh samples were positive. Regarding fruits, 77.8% of them were positive (n = 7/9), but all the tested vegetables were negative. Interestingly, aromatic plants (sage and laurel) tested positive. Only açai samples were positive among berries. Notably, two members of the same family (*Malvaceae*), durian and baobab fruit, were positive for S100B-like immunoreactive material among the samples that resulted positive. The S100B epitopes immunodetection in different plants supports the

*in silico* findings, further confirming the presence of this molecular structure in selected plants.

Table 5 Presence or absence of S100B-like immunoreactive material detected through ELISA assay in samples of plant tissue or derivatives. The source, plant's scientific name and author of the name and the plant family are indicated.

| Sample                   | Scientific notation                                                          | S100B presence |
|--------------------------|------------------------------------------------------------------------------|----------------|
| Banana (powder)          | <i>Musa acuminata</i> , Colla, <i>Musaceae</i>                               | -              |
| Banana (fresh)           | <i>Musa acuminata</i> , Colla, <i>Musaceae</i>                               | -              |
| Cocoa (powder)           | <i>Theobroma cacao</i> , Linneo, <i>Malvaceae</i>                            | -              |
| Spinach (powder)         | <i>Spinacia oleracea</i> , Linnaeus, <i>Amaranthaceae</i>                    | -              |
| Spinach (fresh)          | <i>Spinacia oleracea</i> , Linnaeus, <i>Amaranthaceae</i>                    | -              |
| Sunflower (powder)       | <i>Helianthus annuus</i> , Linnaeus, <i>Asteraceae</i>                       | -              |
| Açaí (powder)            | <i>Euterpe oleracea</i> , Von Martius, <i>Arecaceae</i>                      | +              |
| Kombucha(powder)         | <i>Camellia sinensis</i> , Linnaeus, <i>Theaceae</i>                         | +              |
| Reishi Mushroom (powder) | <i>Ganoderma lucidum</i> , (Curtis)<br>P. Karst. <i>Ganodermataceae</i>      | -              |
| Mela Annurca (cps)       | <i>Malus domestica</i> , Borkhausen, <i>Rosaceae</i>                         | +              |
| Durian (fresh)           | <i>Durio zibethinus</i> , Linnaeus, <i>Malvaceae</i>                         | +              |
| Durian (lyophilized)     | <i>Durio zibethinus</i> , Linnaeus, <i>Malvaceae</i>                         | +              |
| Durian (powder)          | <i>Durio zibethinus</i> , Linnaeus, <i>Malvaceae</i>                         | -              |
| Baobab (powder)          | <i>Adansonia</i> , Linnaeus, <i>Malvaceae</i>                                | +              |
| Jack fruit (lyophilized) | <i>Artocarpus heterophyllus</i> , Lamarck, <i>Moraceae</i>                   | +              |
| Graviola (powder)        | <i>Annona muricata</i> , Linnaeus, <i>Annonaceae</i>                         | +              |
| Broccoli (fresh)         | <i>Brassica oleracea</i> var. <i>italica</i> , Linnaeus, <i>Brassicaceae</i> | -              |
| Kiwi (fresh)             | <i>Actinidia chinensis</i> , Planch, <i>Actinidiaceae</i>                    | -              |
| Cabbage (fresh)          | <i>Brassica oleracea</i> var. <i>capitata</i> , <i>Brassicaceae</i>          | -              |
| Salad (fresh)            | <i>Lactuca sativa</i> , Linnaeus, <i>Asteraceae</i>                          | -              |
| Laurel (fresh)           | <i>Laurus nobilis</i> , Linnaeus, <i>Lauraceae</i>                           | +              |
| Sage (fresh)             | <i>Salvia officinalis</i> , Linnaeus, <i>Lamiaceae</i>                       | +              |

## 5.5 Results of S100B as microbiota-modulator: application in vivo

### 5.5.1 Storage method influences microbiota composition

The 16S rRNA V1-V2 region sequencing determined the gut microbiota mice identification. The alpha-diversity indices didn't show any significant difference over time (Figure 30).

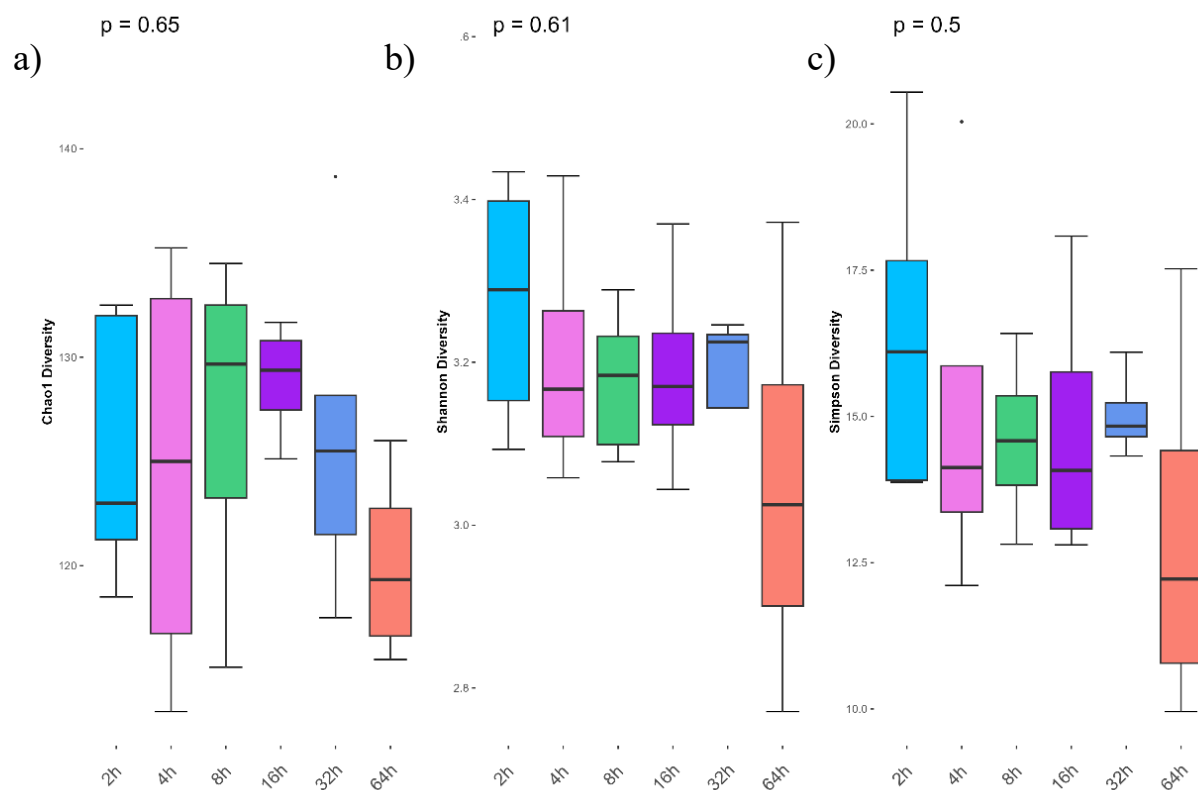


Figure 30: Three boxplot graphs showing mean of Alpha diversity indexes changes over time: (a) mean Chao1 richness index over time. (b) mean Shannon diversity index over time. (c) mean Simpson Diversity index over time. All three graphs report a non-unique trend and, on average, show a stable richness between 4 and 16 hours, while arrange on a lowering of microbiota richness 64 hours after the solution administration.

However, the Shannon and Simpson index were significant different when comparing the two different faecal pellet storage methods (respectively

Wilcoxon test  $W = 35$ ,  $p = 0.0096$  and Wilcoxon test  $W = 46$ ,  $p = 0.041$ ) (Figure 31).

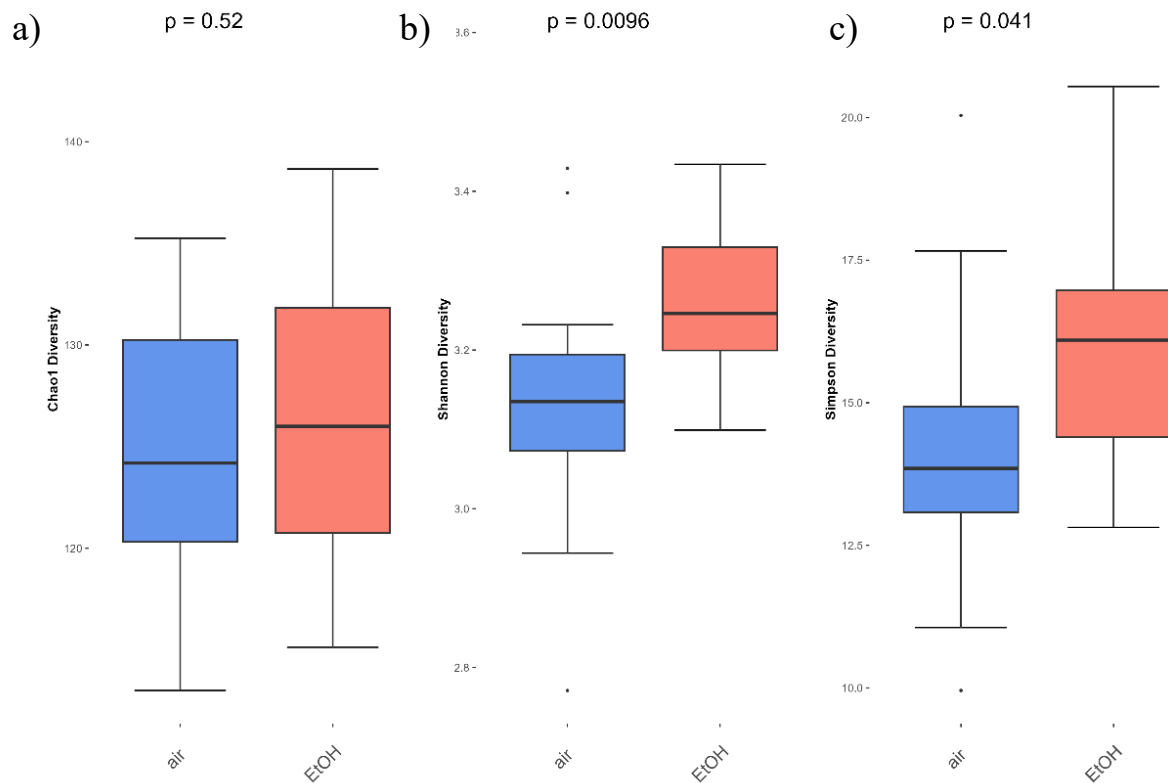


Figure 31: Comparison of alpha diversity indexes between storage methods: (a) the Chao1 richness index didn't show a significant difference between air and ethanol. (b) Shannon diversity index showed statistically significant differences the two storage methods (Wilcoxon test  $W = 35$ ,  $p = 0.0096$ ). (c) Simpson diversity index also showed significant difference between air and ethanol methods (Wilcoxon test  $W = 46$ ,  $p = 0.041$ ). These differences could indicate a presence of rarer and no prevalent species in samples stored in ethanol

These differences remained significant when running a linear mixed-effects model on storage, timepoint and their interaction: with faecal pellet storage method and timepoint as fixed effects and mouse identity as a random effect, the storage influenced significantly the Shannon index ( $F(1, 19.59) = 6.48$ ,  $p = 0.019$ ) and the Simpson index ( $F(1, 14.15) = 4.7$ ,  $p = 0.047$ ). No significant interaction between storage and timepoint was detected neither with Shannon ( $F(5, 16.70) = 1.33$ ,  $p = 0.300$ ) nor with Simpson ( $F(5, 11.16) = 0.63$ ,  $p = 0.67$ ). Beta

diversity was demonstrated as a Principal Component Analysis (PCA) on Aitchison distance matrix. No evident clustering has been observed, apart from

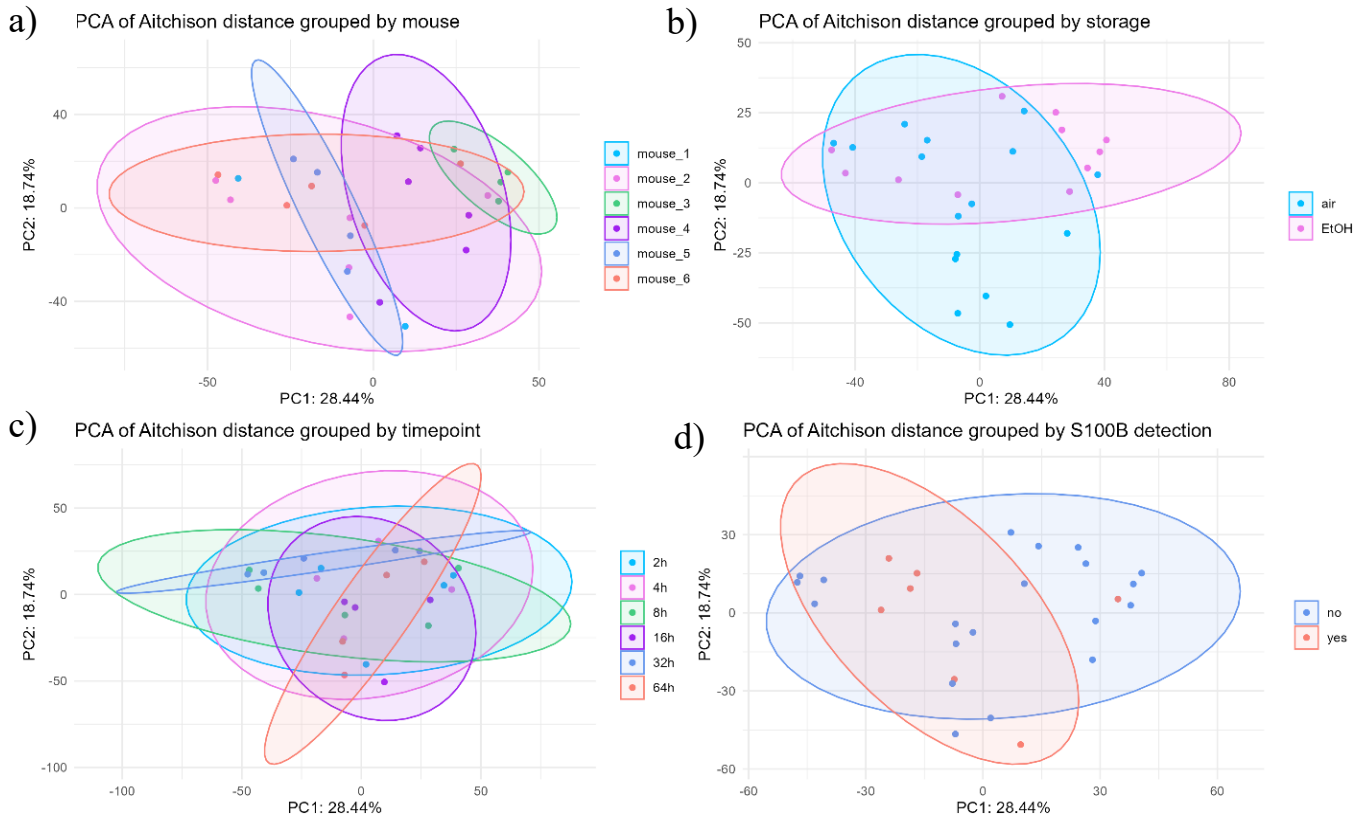


Figure 32: Principal Component Analysis of microbiota composition based on Euclidean distance between CLR transformed sample abundances showing: the beta diversity by mouse (a), beta diversity by faecal pellet storage method (b), beta diversity by timepoint (c), and beta diversity by S100B detection (d). The PC1 and PC2 explain respectively the 28.44% and 18.74% of the total variance.

mouse 1, 3 and 4 whose corresponding ellipsoids appear separate and slightly

overlapping, indicating that individual microbiome is an important source of variability (Figure 32a).

Beta diversity differed significantly according to storage method when checking for the three variables (timepoint, storage and S100B detection), on Aitchison-distanced data stratified by mouse (PERMANOVA,  $F(1,25) = 1.59$ ,  $R^2 = 0.06$ ,  $p = 0.038$ ). The storage method persisted to have a significant effect on

microbiota composition after adjusting for timepoint (PERMANOVA,  $F(6,20) = 1.18$ ,  $R^2 = 0.26$ ,  $p = 0.031$ ). Differential abundance analysis using linear mixed-effects models on CLR-transformed abundances (mouse as random effect) identified time-dependent changes in individual taxa, in particular in *Dysosmobacter* (late decrease), *Faecalibaculum* (later increase), *Laedolimicola* (decrease), *Pseudobutyricicoccus* (decrease), UBA2658 (later decrease) and WRAH01 (decrease). This last one also showed a significant monotonic decrease in relative abundance over time (FDR-adjusted  $p = 0.047$ ). Lastly, a pairwise comparison between individual timepoints restricted within mouse didn't show any significant differences on microbiota composition: this result is in accordance with the previous non-significant effect of time and could be also explained by the limited number of mice and restricted permutation. Nevertheless, effect sizes ( $R^2$  up to 0.30) suggest progressive temporal divergence of community structure, particularly between later timepoints (16h vs 32h).

### **5.5.2 S100B kinetic**

Lastly, the protein quantification in urine samples showed a peak of protein excretion at 4h followed by a decrease over time with almost complete clearance within 64h (Figure 33). This trend is consistent with the renal kinetics expected for a protein of approximately 21 kDa (Mishra et al., 2005).

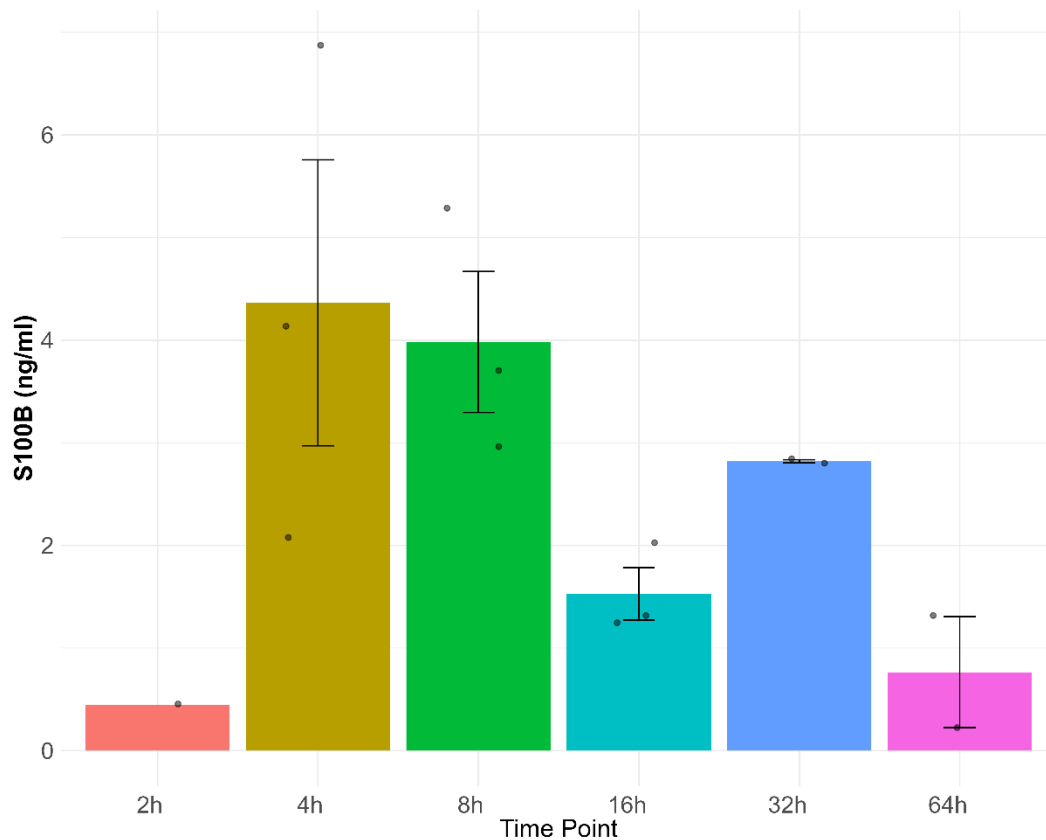


Figure 33 S100B urine levels (ng/ml) over time. A peak of excretion is detected at 4 hours after the one administration of the protein solution. Bars represent the mean  $\pm$  standard error of the mean (SEM), while dots indicate individual measurements.

## 6. Discussion

The number of patients with inflammatory bowel diseases, including Crohn's disease and ulcerative colitis, is rising globally (Alatab et al., 2020). Given the chronic-relapsing course that characterises the pathology, and the lack of resolute treatment, it is worth emphasizing elements that could improve patients' quality of life. Among these, there are diet and physical activity, equally able to have an influence on gut microbiota composition, a key factor in the IBD aetiology (Bertin et al., 2026; Clauss et al., 2021; Wegierska et al., 2022).

Moreover, both can and should be personalized to each subject, as future medicine aims to, in order to give the maximum improvements. In fact, this work showed, through the literature review, that physical activity can, most of the time, represent an efficacious preventive tool. In the management of the pathology, it also gives improvements in preventing relapses and above all quality of life, but it doesn't have to be excessive and strenuous. In line with this, the IBD cohort reported a negative correlation between dysbiosis marker indican and physical activity, especially in patients, even though an opposite trend of association between microbiota biodiversity and physical activity was found between healthy subjects and patients. In mother's cohort the physical exercise reported an effect in increasing the S100B quantity in breast milk. In IBD patients the adherence to Mediterranean diet was lower than in healthy controls. As a confirmation of this, indican and microbiota diversity were also lower in patients than controls. Additional support for the beneficial effect of the MD was provided by the similarly positive trends observed between Faith's PD and age, and between MD adherence and age. Among mothers, enriched diet vs standard MD resulted in significant difference between microbiota composition of the groups and different microbiota composition between mothers and newborns. An additional information was also provided by the negative correlation between S100B concentration in breast milk and age. Regarding about S100B, the experimental data confirmed the *in silico*

predictions of its immunomaterial presence in vegetables such as *Malus domestica* and *Durio zibethinus*. The pilot study of S100B as modulator of microbiota revealed the methodological importance of storing the samples in the best way and implies a change in biodiversity that could be more evident with repeated doses and larger number of subjects. Therefore, this present work can contribute to a multi-approach on IBD illness supplying a more in-depth knowledge of healthy and personalized lifestyle attitude to possibly prevent or help in pathology management.

IBDs pose a major public health concern due to increasing prevalence, deaths and disability-modified life and, thus, require specific preventive and management policies (R. Wang et al., 2023). PA positively influences all physiologic systems and it is regarded as a “medicine” towards several conditions and diseases (E. Anderson & Durstine, 2019). The first step in this work established a systematic review to examine the possible beneficial role of PA in counteracting the development of IBDs and/or managing these diseases after their occurrence. The first emerging fact from this systematic analysis is that the preventive role of PA towards IBD is less considered than the therapeutic one. In fact, only six articles out of 29 evaluate PA for this purpose. In this respect, it is well demonstrated that regular PA is a protective factor for several noncommunicable diseases (NCDs) such as cardiovascular diseases, diabetes and several cancers (E. Anderson & Durstine, 2019). Indeed, it has been

proved that the lack of PA can cause an imbalance in the Th1/Th2 lymphocyte ratio. This imbalance in turn leads to preponderant inflammation status, at the base of the dysregulated immune responses developed by the patients (Huang & Chen, 2016; Steensberg et al., 2001). This mechanism is supported by the evidence that the incidence of other autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis or psoriasis is higher in participants less engaged in PA (Lautenschlager et al., 2023; Sharif et al., 2018). As for the role of PA on IBDs management, seven studies reported positive effects of PA/exercise on disease symptoms and activity, while three did not observe significant improvements with respect to controls and one showed negative effects. These contrasting results can be due to so many non-harmonized factors like different type and intensity of PA, characteristics of the population, stage of the disease etc. Talking about the IBD complications it is important to mention the preventive role of PA towards mainly bone loss, osteoporosis and metabolic bone diseases (Robinson et al., 1998). The effect of PA on bone health can be explained considering that exercise increases muscle mass and this can determine osteogenic effects through muscle pull on the bones; whereas aerobic activities at high-impact such as running or step aerobics can produce positive changes in bone strength and can reduce fracture risk (N. Lee et al., 2005). Furthermore, except for the study by Lamers et al., all the studies included in this review reported positive effects of PA on IBD comorbidities, mental health and quality

of life. This result is in line with previous literature data reporting that PA can contribute to decreasing the frequency of mental disorders, in particular depression and anxiety, by reducing health disparities and mental health symptoms (Schuch & Vancampfort, 2021). However, the question of whether these patients are able to engage in PA must be considered. In fact, although data support the assumption that PA is feasible in IBDs patients, this may not always be easy to practice due to abdominal pain (Coates et al., 2023). De Filippis et al. found that nearly 40% of the participants in their study felt that IBD limited their exercise for various reasons, most commonly fatigue, joint pain, embarrassment, weakness and abdominal pain, while some reported a subjective increase in symptoms immediately following exercise (DeFilippis et al., 2016). These limitations may result in lower PA levels after the diagnosis of IBDs than before. There is, indeed, a reduction in the percentage of patients participating in sports activities after diagnosis (Gatt et al., 2019; Van Langenberg & Gibson, 2010) including and most notably in relation to the disease stage (Mack et al., 2011; Tew et al., 2016; Wiestler et al., 2019). Lastly, some limitations should be considered when interpreting these results. First, the examined studies differed in design, type and level of PA/exercise, outcomes investigated and assessment methods, and this did not allow a meta-analysis to be performed, nor to obtain more consistent results. Moreover, it should be noted that the results were not controlled for possible confounding

factors in all the selected studies, which contributed to their generally low quality.

The IBD cohort study reported, regarding diet, a positive trend between age and adherence to MD. This finding may reflect generational differences in dietary habits: older adults are more likely to maintain traditional Mediterranean eating patterns, whereas younger individuals may be more prone to adopt westernized dietary habits. This is confirmed by various studies reporting a lower adherence to Mediterranean diet among youngest compared to older population (García-Arenzana et al., 2012; Veronese et al., 2020), even though, lately, the observance among the latter was also diminishing (Foscolou et al., 2018). Older adults who follow the Mediterranean diet principles end up with a better health status and more balanced gut microbiota composition compared with non-adherent subjects (Merra et al., 2020). This is here confirmed by a positive trend that resulted between biodiversity alpha index Faith's PD and age. The three main results of this cohort about diet and microbiota, particularly lower MD adherence and consecutive lower microbiota biodiversity and higher indican in IBD patients compared to healthy controls, summarise and confirm the concept afore mentioned. The MD is characterized by high consumption of cereals, vegetables, fruit, olive oil, small portions of dairy products, pastry, sugar, meat products and a moderate intake of fish and wine (Ratajczak et al., 2023). The benefits of such diet are widely known,

comprising, for example, reducing biomarkers of inflammation and oxidative stress, protection against the onset of many chronic and degenerative diseases, cardiovascular disease prevention, decreased risk of chronic kidney disease and lower risk in cognitive decline in older adults without dementia (Aleksandrova et al., 2021; Coelho-Júnior et al., 2021; Hansrivijit et al., 2020; Serra-Majem et al., 2019; Sofi et al., 2014). Keeping this in mind, the lower adherence of IBD patients to the Mediterranean diet cannot simply be ascribed to personal choice. In fact, adherence to MD can be significantly influenced by disease activity and previous surgery. The stage of the illness determines the reduction in high-fibre food consumption; indeed, during flare-ups this results in a detrimental effect. Actually, even patients in clinical remission showed lower MD adherence compared to general Italian population, suggesting a symptoms-linked fear that persists after the acute phase (Fiorindi et al., 2021). The same study by Fiorindi et al. found also an interesting association between previous surgery and MD observance, but just for CD: patients who had undergone a single abdominal operation showed significantly higher MD adherence than patients who underwent surgery more than twice. It has been hypothesized that various surgeries can negatively influence patient's nutritional behaviour, leading to a restricted diet, usually based on personal beliefs and not supported by clinical evidence. With respect to the results regarding intestinal permeability, microbiota diversity and sex differences, the biological differentiation between

males and females, including hormonal regulation, immune responses and gut barrier function, can contribute to these distinct associations (S. Ahmed & Spence, 2021; Santos-Marcos et al., 2023). Indeed, there are several studies showing how different types of diet can affect the gut microbiota in relation to sex differences in diverse animal models, but also in humans (Valeri & Endres, 2021). This very intriguing topic is extensive and merits dedicated attention. Moreover, it has to be considered that women have higher adherence to MD and lower dropout rates than men (De Santi et al., 2020; Rose et al., 2024). Regarding physical exercise, higher intensity was matched by lower microbial diversity in patients, while the opposite happened for healthy controls. This result may seem controversial but is actually in line with existing data; in fact, mouse models have demonstrated that exhaustive exercise promotes intestinal inflammation and increases the growth of *Ruminococcus gnavus*, *Butyrivibrio* spp., *Oscillospira* spp., and *Coprococcus* spp., with a concomitant decrease in *Turicibacter* spp. (Clark & Mach, 2016). Similarly in humans, intense (>70% VO<sub>2</sub> max) exercise sessions seem to cause more significant disturbances to the body's homeostasis than moderate exercise (Clauss et al., 2021). Following an acute bout of exercise, elite athletes have been shown to experience high levels of inflammation (Castell et al., 1996; Suzuki et al., 2000), as attested by an increase in blood and urine markers of inflammation (Suzuki et al., 2002). This side effect also covers endurance exercise. A study observed the consequences of a long-

distance triathlon and concluded that bacterial lipopolysaccharides (LPS) do enter the circulation after ultraendurance exercise. Also, mild endotoxemia (ranging from 5 to 15 pg/ml) was evident in 68% of the athletes and a 27-fold increase in IL-6 production was observed immediately after the race (Jeukendrup et al., 2000). Even if there was no significant correlation between LPS and IL-6 concentrations, these results indicate that increased intestinal permeability could occur simultaneously with an increased cytokine response and thus could contribute to an increased inflammatory response after exercise. It is worth noting that these changes, although negatively impacting host health, are just temporary. This means that it is pivotal to consider the background of the subject practicing excessive physical activity: in a subject affected by IBD, excessive physical activity might worsen an already existing dysbiosis status contributing to inflammation. In addition, the worsening of inflammation could be reflected by the accentuated abdominal pain and fatigue that is perceived by patients engaging physical exercise and who for the same reason, tend to have less tolerance to exercise, creating a self-perpetuating cycle of physical inactivity, declining fitness, and worsening exhaustion (Gatt et al., 2019; Mack et al., 2011; Zaltman et al., 2014). Physical exercise also resulted in a negative correlation with indican levels in all subjects but mainly for IBD patients. Despite the limited sample size, this trend suggests that increased physical activity may be associated with lower intestinal protein fermentation.

This concept falls within the point that moderate physical exercise leads to positive effects on gut microbial diversity, increasing butyrate-producing bacteria belonging to the *Firmicutes* phylum (*Clostridiales*, *Roseburia*, *Lachnospiraceae*, and *Erysipelotrichaceae*) at the expense of the organic acid-producing bacteria (Estaki et al., 2016). Butyrate, together with acetic acid, and propionic acid are the most abundant short-chain fatty acids (SCFAs) produced by gut microbiota and they are regarded to be used as energy sources by host cells and the intestinal microbiota itself (Aziz et al., 2013). They can also contribute to shaping the gut environment, participating in different host-signalling mechanisms, as well as possessing some anti-inflammatory effects (Hsu et al., 2015).

The study on mother's cohort pointed out the importance of nutritional counselling, especially while pregnant, and how diet shapes microbiota composition despite individual differences. In fact, the composition of mother's microbiota was statistically different between the two observed groups. This change was driven by 23 taxa, of these, six, *Dysgonomona*, *Clostridium\_P*, *Veillonella\_A*, *Staphylococcus*, *Klebsiella\_724518* and *Faecalimonas* are incremented in diet group. *Veillonella* is not considered a characteristic marker of the Mediterranean diet and previous studies have reported inconsistent findings in this context, yet has been associated with a fibre-rich dietary pattern (Filippis et al., 2016; Meslier et al., 2020). Regarding *Faecalimonas*, it has been characterized

just recently and associated with metabolically healthy gut ecosystems. It may represent a fibre-responsive taxon, but additional data is currently scarce (Parks et al., 2020). As for *Dysgonomonas*, members of this genus are known for producing short-chain fatty acids such as acetate, lactate and propionate, but clinically they have been recognized as opportunistic pathogens, particularly in immunocompromised individuals. To date, there are no studies linking this genus to a Mediterranean diet pattern, so this would be an exploratory result. As well it is for *Staphylococcus* that acts as an opportunistic pathogen in the gut and *Clostridium* that is mainly associated with pathogen strains (Barber et al., 2023; Khavandegar et al., 2024). These singular results must be considered in a context of limited number of subjects and a treatment that was not a proper intervention leaving space for personal application.

Despite the statistical difference between mothers and infant's microbiota composition, it is worth noting that half taxa were in common between the top 10 genus describing the mothers and newborns separately. Specifically, these 5 taxa were *Bacteroides\_H*, *Dysgonomonas*, *Escherichia\_710834*, *Faecalibacterium* and *Phocaeicola\_A\_858004*. This result doesn't surprise, as it is generally assumed that the first major bacterial newborn encounters are of maternal origin and occur during and directly after birth (de Goffau et al., 2019). Vertical mother-to-infant transmission of microbiota has been studied by several research groups. These studies mainly focused on the infant gut microbiota and possible

maternal source among which there are the vagina, breastmilk, and maternal gut microbiota (Bogaert et al., 2023). Birth mode i.e., vaginal birth compared with birth by caesarean section (CS), has been found to be an important determinant of infant gut microbiota (Bäckhed et al., 2015; Bokulich et al., 2016). For example, it was shown that CS is associated with a perturbed transmission of health-associated bacteria like *Bifidobacterium* and *Bacteroides* strains (Shao et al., 2019). Similar to the gut, the microbiota of other niches correlates with health outcomes. For example, respiratory-tract bacterial communities have been related to immune education, respiratory-tract-infection susceptibility, and development of asthma (Tang et al., 2021; Teo et al., 2018). Particularly, in IBD context, clinical investigations demonstrated a strong association between maternal illness and newborn health outcomes. A study demonstrated that children born to mothers with IBD exhibit higher faecal calprotectin levels from 2 weeks to 36 months of age compared with those born to healthy mothers (E. S. Kim et al., 2021). In addition, prenatal exposure to parental CD has been associated with impaired gut barrier, increased risk of CD in long-term, and alterations in specific microbial communities in their babies (S.-H. Lee et al., 2025). Therefore, it is pivotal to care for a healthy microbiota composition in women who will become mothers and influence, in part, the future composition and health status of their offsprings. It is also curious to note that, comparing the taxa composition among groups (diet and control) separately on mothers

and infants, *Streptococcus* and *Bifidobacterium\_388775* decreased and *Veillonella\_A* increased in diet group both in mothers and newborns. In previous studies *Streptococcus* have been associated with a lower adherence to MD, higher BMI and a high-fat diet pattern (Filippis et al., 2016; Garcia-Mantrana et al., 2018; Zeng et al., 2016). Thus, its decrease is in line with a Mediterranean diet base enriched with other vegetables, whereas the *Bifidobacterium\_388775* decrease is in contrast with previous studies. In point of fact, this genus has been shown to be significantly and meaningfully increased in diets high in fibre (Inoue et al., 2025; So et al., 2018). Talking about the *Veillonella* increment, data literature support its higher presence in Mediterranean diet, particularly in an energy-restricted model (McLeod et al., 2020) and Italian Mediterranean non-Organic Diet (Di Renzo et al., 2025). An interesting meta-analysis conducted by Illeascas et. al compared subjects following MD or other diets and patients with colorectal cancer (CRC) and IBD. Their results revealed that *Veillonellaceae* family was increased in MD, CRC, and IBD compared to healthy controls and precisely, the increase in IBD group was mainly driven by the genus *Veillonella*. Physical exercise in mothers brought an intersecting result that deserves further investigation: sufficiently active mothers had higher levels of S100B in their breast milk compared to non-sufficiently active ones. As mentioned before, there are still no studies analysing the amount of S100B in milk in relation to physical activity, nevertheless its incremented presence is in line with a review

reporting that moderate-intensity exercise (30–45 min per day) does not adversely affect milk volume, nutrient composition, or infant growth. Instead, it may enhance antioxidant levels in breast milk, improve lipid and hormonal profiles (Calcaterra et al., 2025). The S100B concentrations also had a negative correlation with mother's age. To the present knowledge in literature there is still no work displaying a relation between breast milk levels and age. Nevertheless this result reflects an already known negative correlation between S100B and age, even though found in a different biological fluid, the serum (Portela et al., 2002).

On the subjects of S100B, the study investigating its implication in diet showed experimentally that the same plant species which were observed to display the S100 motif *in silico*, actually contain S100B-like immunoreactive material. The calcium signalling system is quite relevant and diffused over animal and vegetal kingdoms. The detection in plants of a protein domain containing a calcium-binding site, such as S100B protein, is hence not surprising. In fact, in addition to calmodulin, which is probably the more known example of the EF-hand  $\text{Ca}^{2+}$ -binding proteins, calcineurin and other novel calcium-dependent protein kinases have also been detected in plants (Luan & Wang, 2021). Interestingly, the observation that the three-dimensional structural similarity is significantly improved when only conserved domains are considered, might underline the hypothesis that proteins have retained their biological functions

throughout evolution in plant kingdom. Of course, it should be noted that these findings do not make it possible to determine that these plants contain molecules that functionally overlap with the corresponding animal S100B protein. Moreover, a larger number of different antibodies would significantly strengthen the observed data. In addition, antibodies specifically binding vegetable S100B are not available, as this study is the first to definitively address this topic. Thus, we have to define the molecule(s) reacting with the antibodies currently available as “S100B-like” immunoreactive material. However, the experimental data together with the in silico finding of a conserved S100B protein domain in plants, allow to infer that such molecule(s) might exhibit structural and probably functional characteristics that reasonably do not diverge significantly from those considered in animal tissues. Remembering that in mammals S100B protein exerts a trophic beneficial effect at low (nanomolar) concentration and a pathogenic effect at a high (micromolar) concentration, it can be hypothesized that the same occurs in plants. This may in turn suggest for the protein both a role as a phyto-therapeutic molecule and as a possible therapeutic target (Michetti et al., 2019, 2021, 2023). S100B-like immunoreactive material was detected in different vegetables and fruits, such as aromatic sage and laurel, but also açai berries, durian, baobab and apples fruits from the *Malvaceae* and *Rosaceae* families. Consuming these fruits and vegetables has been shown to have a significant positive impact on the health

of the gastrointestinal tract and the overall well-being of human subjects (Maleš, Dragović-Uzelac, et al., 2022). Indeed, medicinal herbs, such as aromatic plants, are rich in phytochemicals and their extracts are already added to functional food products as diet supplements, making them desirable ingredients also for functional beverages to improve gut microbiota and overall health (Kompoura et al., 2024; Maleš, Pedisić, et al., 2022). Brazilian native fruits, such as açai (*Euterpe oleracea* Mart.), contain bioactive compounds investigated for their ability to modulate intestinal microbiota (Lima et al., 2023). Specifically, they seem to lower the concentration of hostile microorganism to intestinal microbiota as some genera of *Clostridium* (Alqurashi et al., 2017). In addition, studies have shown that Baobab fruits (*Adansonia digitata*) have several phytochemical and biological activities. In particular, the pulp powder has been found to stimulate the growth and/or activity of certain bacteria, increasing the biodiversity indices (Bester et al., 2023). Recently, a study showed that *Durio zibethinus* was correlated with a reduction in pathogenic bacteria such as *Desulfovibrio* and an increase in the relative abundance of beneficial bacteria such as *Lachnospiraceae* in animal models (Jiang et al., 2020). Some molecules from *Artocarpus heterophyllus* Lam. (jackfruit) exhibited immune-stimulating activity in a mouse model, as well as changes in gut microbiota (Ma et al., 2021; Tan et al., 2013). In conclusion, the beneficial effects of these vegetable-derived bioactive compounds are not yet fully explained (Zhumaliyeva et al., 2023).

Considering the role proposed for the S100B as a neural trophic factor in milk (Gazzolo et al., 2003, 2009) and as an modulator of microbiota biodiversity (Orsini et al., 2020; Romano Spica et al., 2023) it can participate in the explanation of these unclear phenomena, although, additional data is required. The last study aimed to integrate the previous pilot study by Romano Spica et al. (Romano Spica et al., 2023) confirming an effect of S100B on animal's gut microbiota and additional data on kinetics and best way of storing faeces. The non-significant effect of time reinforces the concept of consistency in treatment, especially when it comes to microbiome shaping: In fact, to have a noticeable changing in mice gut microbiota composition a repeated treatment ranging from 2 to 6 weeks is required (Gryaznova et al., 2022; M. T. Khan et al., 2014). Going into more detail, three out of the six strains detected having a relevant increase/decrease in single timepoints have been already documented in mice and seem to be associated with positive health outcomes (exception made for *Laedolomicola*). *Dysosmobacter welbionis* was first found in the human gut and was also identified in the Human Microbiome Project (Le Roy et al., 2020). *Dysosmobacter welbionis* strain J115 is strictly anaerobic, it doesn't produce spore and was found to protect mice from brown adipose tissue (BAT) inflammation in association with increased mitochondria number and non-shivering thermogenesis (Le Roy et al., 2022). This genus is a butyrate producer whose presence in stool is correlated with improved metabolic health (C.-H. Lee et al.,

2026). Treatment with strain J115T showed improvement in glycaemia and hepatic steatosis in high-fat diet fed mice (Moens de Hase et al., 2024). *Faecalibaculum* genus is also part of the SCFA producing bacteria and has been positively associated with improvement of induced ulcerative colitis in mice (Song et al., 2025). Some strains of the genus *Faecalibaculum* have been also associated with an anti-tumorigenic function (Zagato et al., 2020). Lastly, *Laedolimicola* genus has been documented at relatively elevated levels in Streptozotocin (STZ)/High fat diet (HFD) mice but the effect of this genus on host is rarely reported (Si et al., 2024). Concluding, with regard to storage method, it is generally known that ethanol has been widely used as bactericidal and virucidal solution for disinfection (Sauerbrei, 2020). The possible bactericidal deletion of prevalent species could explain the bigger alpha diversity indices (Shannon and Simpson) observed in ethanol-preserved samples compared to air-preserved samples. It is therefore preferable to store the samples in tubes with only air or at least avoiding ethanol.

## 7. Conclusions

Inflammatory bowel diseases continue to represent a growing public health burden worldwide, and their chronic, relapsing nature calls for management strategies that go beyond pharmacological treatment alone. This work approached the question from different complementary angles — physical activity, diet, and the emerging role of S100B protein — with the overarching aim of contributing evidence toward a more personalized, lifestyle-integrated approach to IBD prevention and care. Research efforts are moving towards a better understanding of the relationship between IBD and the microbiota. Diet and physical activity confirm themselves as optimal instruments, particularly when applying the Mediterranean model and moderate intensity level, respectively. The good premises of a phyto-therapeutic role for S100B-like molecules, easily applicable through diet enrichment, completes the picture of the study aim.

However, some limitations must be acknowledged: the sample size was relatively small across all cohorts (mostly for mothers and mice), the imbalanced age of controls vs IBD patients and the constraint of using only two antibodies for the S100B-like material in plants. Beyond their scientific value, these findings carry practical implications for patients and the medical community. Regarding patients, this work reinforces the message that diet and

physical activity are not merely adjunctive lifestyle advice, but modifiable factors directly linked to gut microbiota composition and, potentially, to disease course. The observation that even a short nutritional counselling intervention measurably shifted Mediterranean diet adherence and microbiota composition in the mother's cohort suggests that relatively low-cost, low-risk interventions could be integrated into perinatal care. As for IBD patients, the MD adherence followed by a healthier gut microbiota encourages patients to take an active role in managing their condition. Similarly, the cautionary finding on physical activity intensity offers practical guidance: moderate exercise appears beneficial, while excessive/strenuous activity may worsen symptoms, a nuance that is often missing from generic "stay active" recommendations. This could help medical professionals to personalize exercise prescriptions for IBD patients, particularly during periods of active disease. Moreover, the S100B findings, both in maternal milk and in the newly explored plant sources, open a novel research stream: if confirmed in larger and mechanistic studies, S100B-like molecules could represent an additional step toward phyto-derived therapeutic or nutraceutical strategies.

Taken together, translating these exploratory findings into actionable clinical guidance will require larger, longitudinal and, where feasible, interventional studies capable of establishing causality, particularly regarding physical activity intensity thresholds in IBD patients, together with repeated-dose in

vivo protocols and biochemical characterization of the plant-derived S100B-like material. Multidisciplinary collaboration between gastroenterologists, dietitians, exercise physiologists and researchers will be essential to move from the hypothesis-generating evidence presented here toward interventions that are both scientifically validated and genuinely usable in everyday clinical and patient-facing settings.

## **8. Topic-related publications during my three-years PhD. Program (2023 – 2026)**

### **The Estimated Intake of S100B Relates to Microbiota Biodiversity in Different Diets**

Ghaffar T, **Volpini V**, Platania S, Vassioukovitch O, Valle A, Valeriani F,  
Michetti F, Romano Spica V.

*Biomolecules*. 2025; 15(7):1047. <https://doi.org/10.3390/biom15071047>

### **Regulation of the gut microbiota through meditation and exercise: Potential for enhancing physical well-being across all ages in the twenty-first century**

**Volpini V**, Bricca S, Ubaldi F, Ghaffar T, Platania S, Valeriani F, Romano Spica V.

*Sports Med Health Sci.* 2025;8(3):350-362. Published 2025 Jul 28.  
doi:10.1016/j.smhs.2025.07.007

**The Role of Gut Microbiota in Different Types of Physical Activity and Their Intensity: Systematic Review and Meta-Analysis**

Ghaffar T, Ubaldi F, Volpini V, Valeriani F, Romano Spica V.

*Sports.* 2024; 12(8):221. <https://doi.org/10.3390/sports12080221>

**Meditation in Motion: Sport Type and Meditation Level Shape Gut Microbiota Profiles in Aikido and Tai Chi Practitioners**

Ghaffar T, Volpini V, Ubaldi F, Romano Spica V, Valeriani F.

*Microorganisms.* 2026; 14(2):275.  
<https://doi.org/10.3390/microorganisms14020275>

# Systematic Review and Meta-Analysis of Dietary Interventions and Microbiome in Phenylketonuria

Ubaldi F, Frangella C, **Volpini V**, Fortugno P, Valeriani F, Romano Spica V.

*International Journal of Molecular Sciences.* 2023; 24(24):17428.

<https://doi.org/10.3390/ijms242417428>

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