Microbiota and metabolome in chronic non-communicable diseases

Nadia Serale (nadia.serale@uniba.it)

Dept. Soil, Plant and Food Sciences, University of Bari Aldo Moro, Italy

Tutor: Prof. Maria De Angelis

Co-Tutor: Prof. Piero Portincasa

This PhD thesis dealt with the study of the gut microbiota and metabolome in association with food and clinical aspects. Specifically, *i*) the relationship between intestinal microbiome and chronic non-communicable diseases (i.e., fructose intolerance, nephropathy, and obesity) were studied; *ii*) the use of probiotics in patients with fructose intolerance and nephropathy (Chronic Kidney Disease, CKD) was evaluated; *iii*) pasta samples contained bioactive waste ingredients were characterized and the antioxidant and microbiological activities were studied, for a possible application as functional food.

Microbiota e metaboloma in patologie croniche non trasmissibili

Questa tesi di dottorato ha riguardato lo studio del microbiota e del metaboloma intestinale in associazione ad aspetti alimentari e clinici. In particolare, *i*) è stata studiata la relazione tra il microbiota intestinale ed alcune patologie croniche non trasmissibili, come intolleranze alimentari, nefropatie e obesità; *ii*) è stato valutato l’impiego di probiotici in soggetti affetti da intolleranza al fruttosio e nefropatia (*Chronic Kidney Disease*, CKD); *iii*) dei campioni di pasta sperimentale contenente ingredienti di scarto bioattivi sono stati caratterizzati e studiati in termini di attività antiossidante e microbiologica, per una possibile applicazione come *functional food*.

**Key words**: intestinal microbiota; intestinal metabolome; chronic non-communicable diseases; functional food.

# **1. Introduction**

In accordance with the PhD thesis project, this oral communication reports the main results of the following five activities directed to:

 A1) Assess *i*) the prevalence of fructose intolerance (FI) in patients with FGIDs and *ii*) the effectiveness of the EQBIOTA probiotic in improving symptoms of FI.

 A2) Explores the effect of the symbiotic NatuREN G on the gut microbiota of stage IIIb-IV CKD patients.

 A3) Provide a comprehensive view of the correlation between the intestinal microbiome and the metabolic alterations in obese patients.

 A4) Investigate the differences of the gut microbiota and metabolome in patients affected by different types of nephropathies.

 A5) Characterize and analyse the microbiological activity of functional pasta samples enriched with different bioactive waste ingredients.

# **2. Materials and Methods**

## **2.1 Assess *i*) the prevalence of FI in patients with FGIDs and *ii*) the effectiveness of the EQBIOTA probiotic in improving symptoms in fructose intolerants.**

The prevalence of FI in a cohort of Romanian adult with Functional Gastrointestinal Disorders (FGIDs) and the effectiveness of treatment with a new probiotic formulation EQBIOTA™ (*Lactiplantibacillus plantarum* CECT 7484 and 7485 and *Pediococcus acidilactici* CECT 7483) were evaluated. FI subjects on fructose-free diet regimen and persistent symptomatology and healthy volunteers (HC) tested the probiotic for 30 days. The gastro-intestinal symptoms (abdominal pain and bloating), bowel habits, and fecal volatile metabolome were evaluated before (T0) and after treatment (T30). The Volatile Organic Compounds (VOCs) were quantified using the gas chromatography coupled by mass spectrometry (GC-MS).

**2.2 Explores the effect of the symbiotic NatuREN G on the gut microbiota of stage IIIb-IV CKD patients.**

The effect of symbiontic (S) NatuREN G® (*Bifidobacterium* *animalis* BLC1, *Lacticaseibacillus* *casei* LC4P1, fructo-oligosaccharides, inulin, quercetin, resveratrol, and proanthocyanidins) on gut microbiota in a single-blind, placebo-controlled, pilot trial, involved both CKD patients at IIIb-IV nephropathy-stage and healthy controls (HC), was evaluated. The placebo (P) used in the present study was based on maltodextrins and aromas. Faecal samples and dietary questionnaires were collected at the beginning of the study (T0), after 60 days of treatment (T60), and after further 30 days of wash out (T90). Fecal samples were analyzed by 16S rDNA metataxonomics and GC-MS to evaluate metabolic profile. Finally, changes in *Lactobacillus* and *Bifidobacterium* genera were also assessed by quantitative PCR.

**2.3 Provide a comprehensive view of the correlation between the intestinal microbiome and the metabolic alterations in obese patients.**

The aim of this study was to characterize gut microbiota and metabolome composition in metabolically healthy (MHO) and unhealthy (OB) obese adult subjects considering their clinical data and dietary conditions. An observational study was conducted on healthy controls (HC) and MHO and OB subjects. Blood samples and 3 days food questionnaires were collected. The gut microbiota was characterized in faecal samples through quantitative PCR with the use of primers for specific bacterial genera and species. GC-MS was performed for the analysis of untargeted metabolites and short-chain fatty acids in faecal samples.

**2.4 Investigate the differences of the gut microbiota and metabolome in patients affected by different types of nephropathies.**

The impaired kidney function in nephropathic patients was linked to an unbalanced microbiota asset. To better detail the reasons of this dysbiosis, an observational study was performed to investigate the gut microbiota and volatile metabolome in four kidney pathologies: Chronic Kidney Disease (CKD), Diabetic Kidney Disease (DKD), Autosomal Dominant Polycystic Kidney Disease (ADPKD), and Immunoglobulin A nephropathy (IgAn). A healthy control group (HC) was added to the study. Blood samples were collected to analyse the biochemical parameters. Moreover, subjects provided faecal samples to investigate the gut microbial population through quantitative PCR and the volatile metabolome through GC-MS.

**2.5 Characterize and analyse the microbiological activity of functional pasta samples enriched with different bioactive waste ingredients.**

Experimental samples of pasta were produced by the Casillo Next Generation Food Group (Corato, Italy). Pasta was enriched with three different ingredients: i) deoleated durum wheat germ; ii) deoleated durum wheat bran; iii) durum wheat oil. By combining the three ingredients, four samples of pasta were formulated: dry pasta with 30% deoleated durum wheat germ (GP); dry pasta with 30% deoleated durum wheat bran (BP); dry pasta with 27% deoleated durum wheat germ and 6% microencapsulated durum wheat oil (GPmO); dry pasta with 27% deoleated durum wheat bran and 6% microencapsulated durum wheat oil (BPmO). Dry pasta obtained from semolina with the addition of integral-like dye was used as control (CP).

*In vitro* tests were performed in order to: i) assess the antioxidant activity and the content of phenols (according to Difonzo et al., 2017 and Limongelli et al., 2023) of pasta extracts, and ii) characterize the microbiological activity of fermented digested pasta samples. Digestion was simulated *in vitro* using enzymes from oral, gastric, and intestinal fluids (De Angelis et al., 2021). Samples of digested pasta were fermented *in vitro*, simulating the colonic fermentation, according to Vacca et al. (2023). For the microbiological activity, some target genera and species were investigated through quantitative PCR and an aliquot of each fermented sample supernatant was analyzed to characterize the Volatile Organic Compound (VOC) through GC-MS.

**3. Results and Discussion**

## **3.1 Assess *i*) the prevalence of FI in patients with FGIDs and *ii*) the effectiveness of the EQBIOTA probiotic in improving symptoms in fructose intolerants.**

The prevalence of fructose intolerants (31.8%) within FGID group was higher than lactose intolerant subjects (6.8%). The final group of enrolled patients consisted of 14 FI and 13 HC subjects. In FI group, the values of gastro-intestinal symptoms (VAS) were significantly higher than HC. Treatment with EQBIOTA caused an overall improvement of symptoms in FI subjects: we observed a significant decrease of bloating (q = 0.0001) and abdominal pain (q = 0.0002).

**Table 1.** Baseline characteristics expressed as mean ± SD and median values of the fructose intolerant and healthy control group enrolled for EQBIOTA treatment.

|  |  |  |
| --- | --- | --- |
|  | **Healthy control** | **Fructose intolerant** |
|  | T0 | T30 | *p*-value | T0 | T30 | *p*-value |
| Bristol Score (BSFS) | 3.3 ± 0.5 | 3.6 ± 0.4 | n.s. | 2.9 ± 1.1 | 3.4 ± 0.5 | n.s. |
| Bloating (VAS, mm) | 6.9 ± 11.8 | 11.2 ± 6.5 | n.s. | 68.6 ± 21.4 | 13.6 ± 17.8 | p = 0.0001 |
| Abdominal pain (VAS, mm) | 2.3 ± 4.4 | 5.9 ± 3.1 | n.s. | 43.6 ± 28.7 | 7.1 ± 13.3 | p = 0.0002 |

118 VOCs were identified and grouped according to chemical classes. The content of the VOCs largely varied within samples, some significant differences were evaluated by comparing HC and FI group at the T0, and at the T30 in FI group. The permutation analyses showed how the VOCs profile of HC-T0, HC-T30, and FI-T30 were grouped in one single cluster, whereas fructose intolerant group at the T0 was un-clustered. In the FI-T30 group, some Medium Chain Fatty Acid (MCFA) resulted higher compared to FI-T0, specifically hexanoic acid and heptanoic acid. Interestingly, the MCFA are resulted discriminant between healthy subjects and patients with gastrointestinal pathologies (De Preter et al., 2015).

A correlation analysis was performed between fecal metabolome and clinical symptoms. The bloating scores in fructose intolerant subjects was negatively correlated with 1-pentanol (r = – 0.60, q = 0.03), hexanoic acid (r = – 0.53, q = 0.04), and carvacrol (r = – 0.59, q = 0.03). The abdominal pain score showed the same trend, in detail showed a negative correlation with 1-pentanol (r = – 0.50, p < 0.05), carvacrol, and beta-bisabolene (r = – 0.50, p < 0.05). Conversely, the ethyl ester of hexadecenoic acid was positive correlated with bloating (r = 0.54, p < 0.05) and abdominal pain (r = 0.60, q = 0.01) scores. This compound belongs to the Fatty Acid Ethyl Esters (FAEE). It has been shown that FAEE are able to induce dysfunctions of the intestinal barrier, with the induction of oxidative stress at the level of the intestinal *epithelium* (Elamin et al., 2013)

The treatment with EQBIOTA determined changes in VOC profile of FI patients with a specific increase of potentially anti-inflammatory and protective compounds. These changes occurred in parallel with a decrease of FAEE, which might have a potential detrimental effect on the intestinal barrier and oxidative stress.

**3.2 Explores the effect of the symbiotic NatuREN G on the gut microbiota of stage IIIb-IV CKD patients.**

The alpha diversity of the GI microbiota reported no differences comparing the run-in (T0) values after the randomization (P *vs* S) of both CKD and HC. NatuREN G® increased the number of identified species in both subgroups (CKD and HC), but the carry-on effect till T90 was found only in CKD. In CKD-S, NatuREN G® significantly shifted the ratio Firmicutes/Bacteroidetes. In detail, Firmicutes were positively affected by the NatuREN G® being found significantly associated to the innovative synbiotic at both follow-ups, whereas Bacteroidetes showed the opposite. At family level, *Coriobacteriaceae* and *Flavobacteriaceae* positively and negatively correlated with the NatuREN G®, respectively, since these tendencies were also detected till the end of the trial (T90). *Blautia* was the only genus that showed a positive correlation with NatuREN G® at both follow-ups. According to 60 days of treatments, only *Selenomonas* significantly differed in CKD patients between subgroups (P *vs* S) in metataxonomic relative abundances. Specifically, the taxa had a higher relative abundance in CKD treated with synbiotic than treated with probiotic too. At T60 and T90 not significantly increased in numbers for *Lactobacillus* were observed in CKD-S, while decreased in CKD-P (p < 0.05). In CKD-S, the abundance of *Bifidobacterium* increased during the study. The metabolic profiles varied between groups after treatment.

The synbiontic NatuREN G® was able to modulate the gut microbiota profiling and the related metabolism in stage IIIb-IV CKD patients. Specifically, NatuREN G® increased the ratio Firmicutes/Bacteroidetes and this was reflected in an increase of the saccharolytic metabolism reducing the proteolytic one, according with an increased concentration of acetic and propanoic acids in faecal samples. Therefore, present work opens the way towards further studies based on nutritional managements and adjuvant therapies based on probiotics and prebiotics administration in diseases, such as the nephropathy.

**3.3 Provide a comprehensive view of the correlation between the intestinal microbiome and the metabolic alterations in obese patients.**

The clinical picture of OB patients differed from HC and MHO ones. Compared to MHO, OB patients showed higher levels of HOMA index (q = 0.01) and Glycate Haemoglobin (q = 0.03), and lower level of Glomerular Filtration Rate (q = 0.03). The DAPC statistical analysis reported the separation of subjects in three groups. Several variables have a greater impact on the subdivision of the groups, including body mass index (BMI), erythrocytes sedimentation rate (ESR), C reactive protein (CRP), and several thyroid hormones.

A target group of intestinal population were investigated (Fig. 1).



**Figure 1.** Statistically significant qPCR tested taxa emerging from pairwise comparison of HC, MHO, and OB cohorts.

The amount of *Clostridium coccoides* was lower in OB and MHO than in HC group (q = 0.0003). Studies reported the same trend of *Clostridium* species comparing obese and control subjects, and *Clostridium coccoides* resulted inversely related to insulin and HOMA index levels (Teixeira et al., 2013). *Lactobacillus* genus (q = 0.02) and *Lactiplantibacillus (Lp.) plantarum* (q = 0.02) were higher in MHO subjects, while *Prevotella* (q = 0.03), *Desulfovibrio* (q = 0.02), and *Lp. plantarum* (q = 0.04) enriched the microbiome of OB patients, compared to HC. *Prevotella* is correlated with the hormone ghrelin, lead to an increment of appetite (Gomes et al., 2018); while higher amounts of *Desulfovibrio*, is correlated with metabolic alteration, as Non Alcoholic Fatty Liver Disease (Lin et al., 2022). Considering volatile metabolome, compared to MHO subjects, the HC group presented higher quantity of tetradecane, 2H-indol-2-one-1,3-dihydro, 2-tridecanone, benzeneacetaldehyde, butanal-3-methyl-2, and gamma-terpinene (p > 0.05). Compared to OB subjects, HC volunteers reported greater amount of nonanoic acid, gamma-terpinene, cyclohexanecarboxylic acid, pentanoic acid, butyl ester, alpha phelladrene, and humulene. On the other hand, OB subjects showed higher levels of 2-undecanone, 2-pentadecanone, and 2-hexadecanone compared to HC subjects, and higher levels of nonadecane, indole, and 1H-pyrrole-2,5-dione, 3-ehyl than MHO group. In addition, a lower presence of butanoic acid was observed in OB patients compared to MHO group.

The preliminary results support the association between metabolic diseases and differences in gut microbiota between healthy and unhealthy obese subjects. Furthermore, such differences in gut microbiota could be used as biomarkers for a less invasive diagnosis of pathological obesity.

**3.4 Investigate the differences of the gut microbiota and metabolome in patients affected by different types of nephropathies.**

Blood biochemical variables highlighted four distinct profiles relative to the four groups of nephropathic patients. A discriminant analysis of principal components separated samples based on the microbial profiles. In detail, HC and ADPKD were separated to CKD, DKD, and IgAn groups, which clustered together (Fig. 2).

****

**Figure 2.** A priori (A) and a posteriori (B) DAPC analysis of qPCR analysis.

The variables that had the main impact on the subdivision in groups include species belonging to *Bifidobacteria*, *Lactobacillus*, *Bacteroides*, and *Clostridium*, and *Bifidobacterium*, *Prevotella*, *Desulfovibrio*, and *Atopobium* at the genus level. The analysis of volatile metabolome led to distinguish three clusters of samples. IgAn was characterized by greater amounts of esters and ketones, ADPK and CKD by aldehydes, terpenes, sulfuric compounds, and fatty acids, while DKD and HC by greater concentration of indoles, hydrocarbons, alcohols, phenols, and carboxylic acids.

The different nephropathies were correlated with altered homeostasis. Our results highlighted the differences of microbial population and intestinal metabolome associated with the different impaired kidney functions. These preliminary results could be useful to understand the role of gut microbiota in nephropathic diseases, and evidence the possibility of identifying some metabolites and taxa as biomarkers useful to predict and stratify patient groups.

**3.5 Characterize and analyse the microbiological activity of functional pasta samples enriched with different bioactive waste ingredients.**

From the analysis of the pasta extracts, the samples subdivision in three groups based on their characteristics (phenols quantity and antioxidant activity) has emerged. In detail, GPmO and GP samples were included in the group with the highest levels of antioxidant activity and phenols quantity; the second group included BPmO and BP samples, characterized by intermediate antioxidant activity and phenolic content; finally, CP sample was excluded from the other groups, with the lowest levels of measured parameters. *In vitro* digestion results highlighted the impact of the pasta tested on the intestinal microbial population (Fig. 3). Coliforms, streptococci, total aerobes microbial count (TAMC), and total anaerobes microbial count (TANMC) were mainly present in GPmO sample. Lactic acid bacteria (LAB) population was significantly underrepresented in GPmO and BPmO samples. GP and BP non-microencapsulated samples showed a greater population of bifidobacteria than the others.



**Figure 3.** Results on cell viability in fermented samples. The first graph (A) shows the arrangement of cases (samples of pasta); the loading plot (B) shows the cell densities of the plated microbial groups.

Based on the analysis of a target group of microbial population, we observed differences between the five samples of pasta, each characterized by a microbial profile. Based on statistically different compounds, a hierarchical clustering analysis was performed. BPmO un-clustered with the other samples. The other two groups included CP and BP samples in the first and GPmO and GP samples in the other one. Finally, a targeted analysis of Short Chain Fatty Acid (SCFA) was conducted. GPmO sample presented the highest quantities of all SCFA and showed statistically significant (p< 0.05) higher amounts of isovaleric acid and isobutyric acid, compared both to CP, GP, and BPmO samples, and higher quantities of butanoic acid and propanoic acid, compared to BPmO and CP samples, respectively. In summary, the analyses carried out reported different antioxidant and microbiological characteristics of the experimental pasta preparations, based on the added ingredients.

# **4. References**

De Angelis M, Siragusa S, Vacca M, Di Cagno R, Cristofori F, Schwarm M, Pelzer S, Flügel M, Speckmann B, Francavilla R, Gobbetti M (2021) Selection of Gut-Resistant Bacteria and Construction of Microbial Consortia for Improving Gluten Digestion under Simulated Gastrointestinal Conditions. *Nutrients*. **13**:992.

De Preter V, Machiels K, Joossens M, Arijs I, Matthys C, Vermeire S, Rutgeerts P, Verbeke K (2015) Faecal metabolite profiling identifies medium-chain fatty acids as discriminating compounds in IBD. *Gut* **64**:447-58.

Difonzo G, Russo A, Trani A, Paradiso VM, Ranieri M, Pasqualone A, Caponio F (2017) Green extracts from Coratina olive cultivar leaves: Antioxidant characterization and biological activity. *Journal of Functional Foods*. **31:**63-70

Elamin E, Masclee A, Juuti-Uusitalo K, van Ijzendoorn S, Troost F, Pieters HJ, Dekker J, Jonkers D (2013) Fatty acid ethyl esters induce intestinal epithelial barrier dysfunction via a reactive oxygen species-dependent mechanism in a three-dimensional cell culture model. *PLoS One* **8**:e58561.

Gomes AC, Hoffmann C, Mota JF (2018). The human gut microbiota: Metabolism and perspective in obesity. *Gut Microbes* **9**:308-325.

Limongelli R, Minervini F, Calasso M (2023) Fermentation of pomegranate matrices with *Hanseniaspora valbyensis* to produce a novel food ingredient. *LWT*. **180**:114687.

Lin YC, Lin HF, Wu CC, Chen CL, Ni YH (2022) Pathogenic effects of *Desulfovibrio* in the gut on fatty liver in diet-induced obese mice and children with obesity. *J Gastroenterol* **57**:913-925.

Teixeira TFS, Grześkowiak LM, Salminen S, Laitinen K, Bressan J, do Carmo Gouveia Peluzio M (2013) Faecal levels of *Bifidobacterium* and *Clostridium coccoides* but not plasma lipopolysaccharide are inversely related to insulin and HOMA index in women. *Clinical Nutrition* **32**:1017-22.

Vacca M, Pinto D, Annunziato A, Ressa A, Calasso M, Pontonio E, Celano G, De Angelis M (2023) Gluten-Free Bread Enriched with Artichoke Leaf Extract In Vitro Exerted Antioxidant and Anti-Inflammatory Properties. *Antioxidants*. **12**:845.