UNIVERSITÀ POLITECNICA DELLE MARCHE

DIPARTIMENTO SCIENZE DELLA VITA E DELL'AMBIENTE

Corso di Laurea Magistrale

BIOLOGIA MOLECOLARE E APPLICATA

Disordini metabolici causati dall'esposizione di zebrafish a BPA: riduzione degli effetti tossici mediante trattamento con probiotici.

Probiotic modulation of metabolic disorders caused by BPA exposure in zebrafish

Tesi di Laurea Magistrale di:

Giommi Christian

Relatore

Chiar.ma Prof.ssa.
Carnevali Oliana

Correlatori:

Dott.ssa.
Maradonna Francesca

Prof.
Habibi Hamid R.

Sessione Luglio 2020

Anno Accademico 2019/2020

INDEX

ABSTRACT

Il plastificante Bisfenolo A (BPA), usato estensivamente nella produzione di resina epossidica e plastica in policarbonato, ha dimostrato di possedere capacità di distruttore endocrino a concentrazioni ambientali, producendo preoccupazione a causa della sua ampia distribuzione nell'ambiente. Numerosi studi documentano la compromissione metabolica causata da questo contaminante a livello epatico, dove stimola l'accumulazione lipidica promuovendo quindi lo sviluppo della epato steatosi e della sindrome metabolica. Al contrario invece, ceppi e formulazioni probiotiche si sono dimostrati in grado di ridurre il contenuto lipidico epatico, i livelli sanguigni di glucosio e l'appetito. Per queste ragioni, i probiotici sono ampiamente usati e raccomandati come supplementi nutritivi per il trattamento di malattie metaboliche, suggerendo quindi il loro uso nel contrastare gli effetti negativi dei contaminanti a livello metabolico. In questo studio vengono investigati gli effetti modulatori della formulazione probiotica SLAB51 sulla esposizione cronica da BPA. L'esperimento ha avuto una durata di 28 giorni e gli zebrafish adulti, femmine e maschi, sono stati divisi in 4 gruppi: 1. Gruppo di Controllo (C) nutrito con un mangime commerciale, 2. (BPA) nutrito con mangime commerciale ed esposto ad una concentrazione di 10µg/L di BPA tramite

acqua, 3. (P) nutrito con mangime commerciale arricchito con 10^9 CFU della formulazione probiotica SLAB51, 4. (BPA+P) nutrito con mangime commerciale arricchito con 10^9 CFU della formulazione probiotica SLAB51 ed esposto ad una concentrazione di BPA di 10µg/L dall'ottavo giorno del trattamento. Alla fine dell'esperimento, i pesci sono stati uccisi con un eccesso di anestetico e i campioni di fegato sono stati prelevati. I metaboliti estratti dal fegato sono stati analizzati tramite un UHPLC-ESI-MS così da identificare il profilo metabolico epatico dei diversi trattamenti. Le analisi preliminari, PCA, OPLS-DA e Analisi Gerarchica, mostrano che il gruppo BPA+P clusterizza insieme e sovrapposto al gruppo P invece che con il gruppo BPA. Successivamente sono state condotte analisi statistiche quali ANOVA, t-test e Pathway Analysis. Il BPA ha indotto una up-regolazione dei livelli dei metaboliti riscontrati in questo gruppo, tra cui l'Acido Acetoacetico, coinvolto nella degradazione degli amminoacidi e nella sintesi dei corpi chetonici, la Creatina, nel metabolismo dell'arginina e glicina, la O-Fosfoetanolammina, nel metabolismo degli sfingolipidi, l'Acid Docosaexaenoico, nella biosintesi degli acidi grassi insaturi e l'Alanina prodotta dall'enzima alanina transaminasi. Questi metaboliti e i loro pathway supportano studi pre-esistenti sulla stimolazione dell'accumulo lipidico epatico e delle capacità da distruttore endocrino da parte del BPA. Il trattamento con probiotico ha portato ad una up-

regolazione dei livelli di Anserina e Acido Oftalmico con concomitante diminuzione dei livelli di Cistationina. Ciò è coinvolto nel miglioramento del potere antiossidante della cellula. I livelli di O-Fosfoetanolammina sono stati ridotti dall'uso del probiotico, permettendo di ipotizzare un possibile effetto di questa formulazione nel ridurre il contenuto lipidico epatico. Il gruppo BPA+P presenta come unico punto di somiglianza con il gruppo BPA la up-regolazione dell'Acido Acetoacetico. Presenta invece, una up-regolazione dell'Anserina e una down-regolazione della Cistationina e O-Fosfoetanolammina come il gruppo P. In questo trattamento è anche possibile notare, comparato con il gruppo BPA, una diminuzione dei livelli di Acido Retinoico che è necessario alla eliminazione del BPA. Ciò conferma i dati preliminari che suggeriscono un possibile meccanismo di azione del probiotico nella modulazione degli effetti del contaminante, incluso il miglioramento del potere antiossidante e l'aumentata eliminazione del contaminante.

L'mRNA totale estratto dai fegati è stato analizzato tramite Real Time-PCR per determinare l'espressione genica di geni coinvolti nell'infiammazione, nel metabolismo dei lipidi e del glucosio e nell'appetito. I risultati mostrano una up-regolazione dell'espressione di *ptgs2a* nel gruppo BPA maschi, mentre nel gruppo BPA+P maschi è presente una down-regolazione. Nei maschi, l'esposizione al BPA ha indotto una up-regolazione dei geni *gpr55bp* e *pparα*.

Nelle femmine è presente una up-regolazione del gene *cebpα* rispetto al gruppo BPA+P e nei maschi questo gene è up-regolato sia nel gruppo BPA che P. Il gene *gcga* è up-regolato nel gruppo P femmine e nel gruppo BPA maschi. Il BPA causa una up-regolazione del gene *mboat4* nei maschi mentre il gruppo P presenta una down-regolazione di questo gene. Nessun cambiamento è poi presente nei livelli di questo gene tra BPA+P e C. Nelle femmine, *mboat4* presenta una down-regolazione in tutti i gruppi rispetto a C. Il probiotico produce una up-regolazione del gene *nucb2a* nelle femmine. Presi insime, questi risultati mostrano come, a livello trascrizionale, il BPA abbia un maggiore impatto sui maschi che sulle femmine e questo si traduce nell'aumento dell'espressione dei geni *ptgs2a*, *gpr55bp*, *pparα*, *cebpα*, *gcga* e *mboat4* mentre il probiotico produce una riduzione dello stato infiammatorio, tramite down-regolazione di *ptgs2a* nel gruppo BPA+P, e diminuzione dell'appetito come dimostrato dall'espressione dei geni *mboat4* and *nucb2a*.

La colonizzazione batterica intestinale è stata determinata con un approccio di Metagenomica e mostra come la formulazione SLAB51 induca una colonizzazione di batteri produttori di antibiotici, amminoacidi e vitamine del gruppo B, esercitando un controllo della composizione microbica intestinale e rendendolo efficace come supplemento nel trattamento delle disbiosi e delle carenze da vitamine del gruppo B. L'esposizione al BPA ha invece causato una

colonizzazione di batteri che usano questo composto come nutriente producendone quindi la degradazione.

INTRODUCTION

1.1 Focus on EDC: The Plasticizer Bisphenol A

Endocrine disrupting compounds (EDCs) are exogenous substances that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine functions". They interfere with hormonal signaling and balance, thus causing different biological effects on the organism's physiology. These compounds include a wide range of natural, i.e. phytoestrogens or anthropogenic chemicals present in air pollution, industrial discharge, pesticides, herbicides, plastic additives. Among plastic additives, noteworthy is Bisphenol A (BPA), which has been used to make certain plastics and resins since the 1960s. BPA is an organic compound formed by two phenolic vicinal groups and its chemical name is 2,2-bis-(4-hydroxyphenyl)-propane. It is used as plasticizer in the synthesis of plastic materials and additives, with applications in the synthesis of polystyrene, polysulfonate, polyether ether ketone (PEEK), antioxidant in some plasticizer and as polymerization inhibitor of PVC. In 2003 BPA production was about 2-3 million tons and in 2015 it has achieved 5,4 million tons. Being a key polymer in polycarbonate production, a transparent and very hard and resistant material, it can be found in a lot of common household objects, as baby bottle and toys, and also medical and

dental objects. It is also relevant that BPA is used in the synthesis of epoxy resins which are used to coat the internal surfaces of plastic and aluminum food containers (Allard, 2014). Recycling codes 03 (PVC) and 07 (other plastic), contain BPA, which is present to increase flexibility in polycarbonate and epoxy resins, while in PVC plastic, is found as antioxidant (National Institute of Environmental Health Science, NIH).

BPA binding to polymers is not covalent and this leads to release of this compound in packaged foods and liquids, and also in the environment (Cousins et al., 2002). This allows the BPA uptake by the organism, thus acting as Endocrine Disrupting Chemical (EDC). In humans exposed to this contaminant, blood concentrations reach 0.3-4.4 ng/mL (1-19.4 nM) (Vandenberg et al., 2007) while urine ones are 0.4-149 µg/L (1.7-653 nM) (Calafat et al., 2008). The estimated contamination of packaged foods and liquids by BPA is about 1-5 µg/Kg/D (European Commission, 2002; Thomson et al., 2003), significantly increases in fat foods, and accumulates in body fat and liver (Nunez et al., 2001; Geens et al., 2012).

With a similar structure to that of estradiol, BPA behaves as an estrogen-like compound by binding to estrogen receptors and thus activating the same signaling pathways (Allard, 2014). BPA has also an activity on the Androgen Receptor (AR) causing an antagonistic effect on 5α-Dihydrotestosterone

(DHT)-induced AR transcriptional activity and also binding to the AR ligand binding domain, abolishing the DHT-dependent dissociation of AR from its chaperone and blocking the DHT-induced AR nuclear translocation (Huang et al., 2019)

For this main reason, BPA has been linked to health problems including kidney and liver pathologies, infertility, breast cancer, brain and behavioral disorders, weakening of immune system, diabetes and obesity. There are evidences that liver damage and obesogenic properties of this compound are due to the activation of the endocannabinoid system (Martella et al., 2016), involved in energy balance, food assumption and lipid metabolism. These authors demonstrate that adult zebrafish developed hepatic steatosis which was associated with an increase of the obesogenic endocannabinoids 2-arachidonoylglycerol and anandamide levels, and a concomitant decrease in palmitoylethanolamide. These changes were associated with variations in the expression of key endocannabinoid catabolic and metabolic enzymes and an increase in the expression of the endocannabinoid receptor *cnr1*. Acute and chronic in vitro treatments with nano- and micromolar BPA doses showed increased anandamide levels in line with decreased activity of fatty acid amide hydrolase, the main anandamide hydrolytic enzyme, and induced triglyceride

accumulation in HHL-5 cells in a CB1-dependent manner (Martella et al., 2016)

In another study exposing zebrafish to BPA via the water, a chronic exposure to 3 different concentrations (5, 10 and 20 µg/L) causes lipid vacuoles accumulation in the liver triggering the onset of hepatosteatosis (Forner Piquer et al., 2018) by interfering with the expression of genes involved in the metabolism of 2-AG and Anandamide endocannabinoids that control metabolism at hepatic and brain levels. The lowest dose increased the triacylglycerides storage promoting fatty acids synthesis, the highest concentration promoted de novo lipogenesis and cholesterologenesis (Forner Piquer et al., 2018). Similarly, seabream exposed to BPA presented an overexpression of genes involved in lipid metabolism and transport including *pparα*, *β* and *γ*, *fas* and *lpl* and a downregulation of *hsl* gene expression (Maradonna et al, 2015). The enhancement of fatty acids biosynthesis and the impairment of lipid transport are the major causes of hepatic lipid accumulation and steatosis. In marine medaka, BPA stimulate the fatty acids metabolism by upregulating *apo A-IV*, *apo A-I*, *Acsl1*, *Elovl* and *fabp* (Kim et al, 2018). Recently, the impact of EDCs on lipid metabolism has been reviewed (Maradonna and Carnevali, 2018). A central role has been represented by *ppars* genes, which in turn activate expression of genes involved in lipid homeostasis

and fatty acid metabolism. Also, at hepatic level, *c/ebps* family of transcription factors promotes downstream adipocyte-related genes. SREBPs activate the complete program of hepatic cholesterol and fatty acids metabolism. In general term, BPA and other xenobiotics alter hepatic lipid metabolism by upregulating the expression of these genes thus leading to the liver lipid accumulation in vacuoles.

For all these reasons, in 2011, European union banned the use of BPA in baby bottles (Europe Commission, 2011) and the use of this compound is limited, by 2 January 2020, to doses lower than 0.02% /weight in the production of thermal paper (Europe Commission, 2016). However, it is still present in other commonly used products, including food containers and bottles.

1.2 The use of probiotic to improve organism's health

The term Probiotic derives from the two Latin words Pro, which means in favor of, and Bios, which means life. Probiotics are live non-pathogenic microorganisms that benefit the guest's health if administered in adequate quantities (Hamilton-Miller et al., 2003). Lactic acid bacteria are usually part of probiotics and in this category lactobacilli and bifidobacteria are the most common. Other bacilli and yeast can be included in the list of probiotics.

The requirements to be defined as probiotics are (Morelli and Salari, 2007):

- Safety for Human consumption and therefore there must be no acquired and/or transmissible antibiotic resistance.
- They must be active and viable in the intestine in quantities sufficient to justify any beneficial effects observed in efficacy studies.
- They must persist and multiply in Human intestine.
- They must confer a physiological benefit to the organism and each effect should be attributed only to the single strain or formulation tested, without generalizing.

Regarding their beneficial effects, probiotics can stimulate the intestinal mucosal immune system through the improvement of phagocytosis, by increasing the number of T lymphocytes, natural killer cells and immunoglobulin-producing plasmacytes. In addition to this effect, they exercise a protective function from pathogenic microorganisms by inibiting their growth and competing for resources. A study in which the *Lactobacillus rhamnosus* strain was administered to Zebrafish, showed the beneficial effects of this strain administration on the gut immune system and also an improvement in the hepatic oxidative stress response (Gioacchini et al. 2014).

Numerous studies have shown that metabolic diseases, including obesity and diabetes, are caused by gut dysbiosis, that is an alteration of composition and

quantity of the intestinal microbial flora. The use of probiotic strains and formulations was therefore introduced to heal this type of diseases. The administration of *Lactobacillus rhamnosus* to Zebrafish larvae, induced a reduction of fat levels acting on the microbiota and on the transcription of genes involved in lipid metabolism including *fit2*, *agpat4*, *dgat2*, *mgll*, *hnf4a*, *scap*, and *cck* that are involved in cholesterol and triglycerides metabolism and which resulted downregulated; moreover effects on appetite and the glucose levels were observed (Falcinelli et al., 2015; Falcinelli et al., 2016). The expression of *nucb2a*, *gcga* and *insulin* was upregulated in the probiotic treatment while *mboat4* was downregulated. This produced a whole total body glucose level reduction in larvae. *Leptin* and *mc4r* gene expression were upregulated while *cb1* and *npy* were downregulated and food intake was decreased in probiotic fed group, suggesting a possible role for *L. rhamnosus* in the treatment of impaired glucose tolerance and food intake disorders by gut microbiota manipulation (Falcinelli et al., 2016). In zebrafish, VSL#3 formulation of probiotics can also activate the endocannabinoid system (ECS), a novel biomarker to study the immune system, with the induction of Tlr signaling which in turn can modulate the immune system (Gioacchini et al., 2017). Even if the mechanism of action is not yet full known, preclinical studies have shown that this formula can modulate and enhance the host's immune system in mice,

through the DNA released by these bacteria (Chapman et al., 2006). Also, a lot of double-blind, randomized and placebo-control clinical trials confirm those observation and numerous evidences confirm that this formulation can be used to treat a wide spectrum of inflammatory diseases (Chapman et al., 2006).

Lactobacillus plantarum ST-III treatment in zebrafish showed protective effects against Triclosan exposure (Zang et al 2019) bringing back to the control level the expression of immune-related genes *NF-κB*, *IL-1β* and *TNF-α* that were upregulated in Triclosan exposed fish. The *lysozyme* level, that is a biomarker of inflammation aggravation, also decreased in fish co-exposed to probiotic and Triclosan (Zhang et al., 2019). In rats exposed to BPA and receiving *Bifidobacterium breve* and *Lactobacillus casei*, an increased fecal elimination of BPA and a concomitant decreased of its blood level were observed. This was possibly caused by the protective effects of probiotics that reduced the intestinal absorption of this contaminant (Oishi et al., 2008).

1.2.1 A case study: the probiotic mixture SLAB51

Considering the beneficial effects of probiotic administration, in this study a commercial probiotic mixture was chosen to investigate on its eventual capacity to counteract/mitigate the negative effects induced by BPA.

This probiotic is a high-concentrated multi-strain probiotic formulation containing eight freeze-dried different strains of live lactic bacteria and bifidobacterium which are normal components of the gastrointestinal microbial flora. This formulation contains five strains of lactobacilli, *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus helveticus* and *Lactobacillus brevis*, two strains of *Bifidobacterium lactis* and the *Streptococcus thermophilus*.

Veterinary trials demonstrated that this formulation can improve the prognosis of chronic constipation and idiopathic megacolon in cats treated with SLAB51 (Rossi et al., 2018) suggesting an anti-inflammatory effect of this formulation, on the gut mucosa. SLAB51 treatment also induces region-specific changes in morphology and carbohydrate composition of mucins secreted in the intestinal tract of pigs (Desantis et al., 2019) ameliorating the health status of animals and improving their growth. This formulation can also reduce signs of colony dysmotility in dogs through microbiota modulation and epithelial cell receptor-mediated signaling in intestinal mucosa (Rossi et al., 2020) by increasing cannabinoid receptor type 1- and 2-positive epithelial cells number and decreasing the number of mast cells. In an Alzheimer's disease mouse model, this formulation reduces oxidative stress in brain by activating SIRT1-dependent mechanisms through gut microbiota modulation exerting an

antioxidant and neuroprotective effects (Bonfili et al., 2018) and slowing down the Alzheimer's disease progression in mice (Bonfili et al., 2017).

1.3 "omics" technologies to assess EDC toxicity

In recent years, "omic" approaches (transcriptomics or gene expression profiling, proteomics, metabolomics, and so on) are proving to be powerful tools in advancing knowledge regarding toxicological effects and risk assessments. Due to their high sensitivity, "omic" methods are advantageous for studying effects occurring at doses which are relevant for environmental exposure either to a single chemical or a chemical mixture. Since a molecular signature does not always produce an adverse outcome at the physiological level, proteomic data, although very precise, need to be carefully interpreted (Beyer et al., 2014). Nonetheless, omics are a valuable tool for toxicity analysis of mixtures as well as for single compounds because mechanistic insight regarding the MOA and upcoming affected pathways can be evaluated and better characterized. Furthermore, in conjunction with standard *in vitro* methods and computational tools, "omic" technologies have the potential to offer valuable insight concerning MOA obtained in animal models (Brockmeier et al., 2017). Omic methodologies assess if a certain compound induces changes that could develop into adverse effects and helps identify

MOAs. The resulting omic data provide class comparisons (which gene/protein best discriminate the studied groups), class predictions (the pattern of gene/protein expression induced by the test compound to predict the MOA and its effects) and class discovery (the case when unpredicted, but biologically relevant patterns are generated by omics data) (Sauer et al., 2017).

1.3.1 Metabolomics as Tool to Assess Toxicity

To better study the complexity of biological responses, including toxicological responses after exposure to a contaminant or a response to a drug treatment, an "omic" approach (genomics, transcriptomic, proteomic and metabolomic) is necessary, and it is a great challenge for systematic biology to integrate all omics fields to have a complete overview of living organisms physiology. Metabolomic analysis show us what happened and what is happening in the samples that we are analyzing. This occurs because is a technique that analyzes the "chemical fingerprints" produced by cellular processes. Doing this, metabolomic provides a snapshot of the cellular physiology and gives a direct functional interpretation of the physiological state of the organism. Metabolomic, indeed, studies the metabolome, the complex and complete set of metabolites contained in a cell, organ or organism. In the metabolomic field, the term metabolite refers to substrates, metabolic intermediates, hormone,

signaling molecules and secondary products of the metabolism, which have a molecular weight lower than 1,5 kDa. Metabolome forms a big network of metabolic reactions, where the output of an enzymatic reaction becomes the input of another reaction in a system called hypercycle. Like transcriptome and proteome, metabolome is dynamic and dependent on the physiological and pathological state as well as on external stimuli.

In 2007, scientists from University of Alberta and University of Calgary completed the first draft of the human metabolome, the Human Metabolome Data Base (HMDB), the largest of today with 40.000 entries which comprises 2.500 metabolites, 1.200 drugs and 3.500 food components find in human. This project is still far to be completed because of the complexity of the metabolome and because every type of cells and tissues has its own metabolic fingerprint which is dependent on many factors (Wishart et al., 2007; Wishart et al., 2009*).*

The flowchart of the metabolomic analysis consists of (Dettmer et al., 2007):

- Sampling: the sample can consist of a biological fluid, a tissue, an organ or a whole organism.
- Extraction of metabolites: usually with the inclusion of an internal standard and derivatization.
- Sample analysis: identification and quantification of the metabolites.
- Raw data collection and statistical analysis.

The most common used detection method to analyze the samples is the Mass Spectrometry (MS), an analytical technique used to generate a mass spectrum, a plot of signal intensity of detected ions as a function of the mass-to-charge ratio (m/z) of the ions. To do this, ions in the sample are subjected to an electric or magnetic field to accelerate the motion of ions produced by an analyte of interest. Ions with the same m/z ratio will have the same amount of deflection and will be detected by an electron multiplier. Analyte identity in the sample are evaluated by correlating known masses to the measured masses or using a characteristic fragmentation pattern (Ho et al., 2003). The MS is usually preceded by a separation method used to simplify, separate, ionize and fragment the original mixture of chemicals. To do this Ultra High Performance Liquid Chromatography (UHPLC) has emerged as the most common separation technique for metabolomic analysis and it's possible to couple this technique with the MS by the Electrospray Ionization (ESI) methods. The UHPLC is used to separate the mixture of analytes in the liquid phase (Xin Lu et al., 2007) and then the ESI vaporizes this liquid using a high voltage and producing an aerosol that is then introduced in the MS for the detection. During the vaporization, molecules into the sample can assume a charge or break into ions, the result is a mixture of ions that in the negative mode of ESI are negative charged (Ho et al., 2003).

UHPLC-ESI-MS is the technique used in this study, is one of the most powerful, sensitive and specific technique and has one of the highest resolutions between chromatographic techniques. The other advantages of this method are the low sample volume (about 10-100 µL), the detection of most of the organic molecule and some inorganic molecules, it has a very good sensitivity, is a very flexible technology and is compatible with solid and liquid samples. The disadvantages are the destruction of the sample that cannot be run for a second time, the fact that usually require sample separation, it is slow, with about 15-40 minutes for each sample, is less quantitative than other techniques and is not compatible with gases.

The detection process produces a metabolite data matrix, where all the metabolites identified are plotted with the samples. This table shows for every sample the concentration of all the metabolites. After the table generation, statistical analyses are needed to identify changes and pattern in the sample classes.

In a lot of studies, like those that evaluate the toxicity of a compound, the metabolites of interest are not known a priori. It is possible then, to use unsupervised methods, those that does not have prior assumptions on the class membership. The most common of these methods is the Principal Component Analysis (PCA), that is an unsupervised data reduction technique. PCA can

reduce efficiently the dimension of dataset to few dimensions that still have the greatest variation. The purpose of PCA is to replace all the correlated variables with a minor number of non-correlated variables, called principal components (PC), that still retain most of the information of the original dataset. In this lower-dimension space, clustering of the samples with similar metabolic fingerprints can be detected, patterns can be elucidated and we can determine biomarkers for disease or metabolites that correlate most with a class.

Partial Least Square Discriminant Analysis (PLS-DA) and its Orthogonal version (OPLS-DA) are usually used to find the fundamental relations between two matrixes. They are supervised methods that explain and predict the subdivision of observations in classes using quantitative and qualitative variables. They not only divide the samples in groups but also gives information on how those groups differ between them and how much variability there is between the single group (Lu et al., 2007).

To analyze metabolomic data, also univariate methods can be used where the variables are analyzed one by one using classical statistics tools like T-Test and Anova where only variables with sufficient low p-value (< 0,05) are considered relevant. When multiple comparison is conducted, the use of those classic tools needs correction strategies, like the use of False Discovery Rate (FDR), to reduce false discoveries.

Another important metabolomic tool is the Pathway Analysis. It is used to understand and interpret the omics data using prior knowledge organized in pathways diagrams. With this tool we can find distinct cellular processes, disease or signaling pathways that changes between the experimental groups and we can determine the metabolic fingerprint of a treatment easily allowing us to compare it with that of other groups.

Therefore, metabolomics analysis is useful for the toxicity assessment and toxicology because provides a metabolic profile with which is possible to detect the physiological changes caused by the toxic compound. Usually the observed changes can be related to specific metabolic syndromes and liver lesions.

1.3.2 Transcriptomic as tool to assess toxicity

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is used to quantify the expression profile of target genes by quantitatively measuring the levels of mRNA that they have produced. After total cellular RNA extraction, using a thermostable viral RNA dependent DNA polymerase, complementary DNA (cDNA) molecules are produced. PCR allow to produce an exponential number of copies of this cDNA. Coupling PCR with Ethidium Bromide, that intercalates in the DNA double helix, is possible to quantify the number of

copies produced by measuring the emitted fluorescence of Ethidium Bromide that, when exposed to UV rays, increases about 20 times. Comparing the data from an inducible gene with that of a housekeeping gene allow to standardize the gene expression. After this, gene expression of treated groups can be compared to that of control establishing the statistically significant changes induced by the treatments. So, this technique allows to investigate the expression of a wide range of genes involved in different cellular, tissue and organ functions, e.g. inflammation, oxidative stress, growth and appetite and lipid, amino acids and energy metabolisms.

1.3.3 Metagenomic as tool to assess gut microbial colonization

The development of advance genomic tools, in the last two decades, leads to the possibility of microbial community study directly in their environment without the need of microbial cultivation. So, metagenomics is used to determine the microbial composition in environmental and organism samples and determine the presence of uncultivable microorganisms. The modification of gut microbiota can be associated to the development of inflammatory and metabolic diseases, and growing evidences suggest a role of EDCs in this. Probiotics instead, can positively modulate the gut microbiota and restore the normal colonization. The study of intestine microbiota is then important to

assess the changes that contaminants, like BPA, induce and to investigate if the probiotics manage to mitigate these effects and restore the normal situation. The metagenomic technique makes use of 16S rRNA V1-V2/V3-V4 variable regions amplification with PCR after the total DNA extraction from the samples. Sequencing of all the DNA amplified is perform with Ion Torrent technology on a chip and then data are processed with bioinformatics tools to remove noise and low-quality reads. Databases are used to identify the phylogeny of the microorganisms and then statistical analysis are used to assess differences between treatments. This allow the study of the gut microbial composition after the administration of probiotics and the contaminant exposure.

1.4 Aim of the study

Previous studies using zebrafish demonstrated that the administration of different probiotic strains potentiates the organisms' stress and immune response (Gioacchini et al., 2014; Chapman et al., 2006; Gioacchini et al., 2017) improve lipid and glucose metabolism (Falcinelli et al., 2016; 2017, 2018) and exerts a beneficial effect on reproduction (Gioacchini et al 2010; 2012 . A positive effect of its administration was also demonstrated in case of intestinal pathologies (Chapman et al., 2006). BPA, as endocrine disruptor, can

modify the classic hormonal cascade inducing deleterious cellular effects including the activation of the inflammatory response and increasing oxidative stress, thus affecting the organism's health, which results more prone to develop metabolic diseases, e.g. liver steatosis, obesity and diabetes (Forner-Piquer et al., 2018; Legeay and Faure, 2017; Hwang et al., 2018 . Starting from these evidences, the aim of this study was to verify if the probiotic mixture, Slab51, Mendes S.A. (Lugano, Switzerland) can counteract the toxic effects of BPA and improve the health of fish exposed to this contaminant. To verify this hypothesis, an experimental trial was set up, in addition to Control fish, receiving a standard diet, one group was exposed to BPA via water and fed a standard diet, another one was fed a diet enriched with probiotic and in the last group, to verify the role of probiotic in mitigating BPA toxicity, fish were fed probiotic for four weeks and, starting by the 8^{th} day of the trial, exposed for three weeks to an environmental dose of BPA.

This last group features will help in verifying the leading hypothesis of the study.

Using an "omic" approach, expression of genes involved in metabolism and main metabolite changes were analyzed, providing evidences of main changes occurring at the cellular level following the exposure to BPA and/or probiotics. In addition, in the last years, great interest arose concerning the role of Gut

Microbiota as Second Brain. Microbiota alterations are linked to obesity, metabolic and central nervous system disorders. Recent studies on the microbiome show that its composition is highly heterogeneous and that rapid changes in the relative abundances in the main microbial populations caused by stress, smoking, or vitamin D deficiency and diet, induce severe health problems. On this regard, considering the large number of pathologies and comorbidities associated to BPA exposure, it could be hypothesized that an alteration of fish microbiome, contributing to the onset of these pathologies occurs and probiotics could counteract its toxic effect, thus influencing the intestinal and brain network and in turn positively acting on hepatic metabolism.

1.5 Zebrafish as Experimental Model and Synteny with Humans.

The experimental model of this study is zebrafish, *Danio rerio*, a small fish recognizable by its blue and silver side stripes. It originates from tropical and subtropical areas of the world, living in stagnant freshwater. They belong to the class of *Actinopterygii*, order of *Cypriniforms*, family *Cyprinidae*, subfamily *Danionidae*, genus *Danio* and species *Danio rerio* (Spence et al., 2007). An experimental model should offer practical and technical advantages to study the main biological processes, effects and mechanisms and should be

representative of a large group of organisms. Among its attributes, zebrafish is small and robust, so large number of specimens can be easily and cheaply maintained under controlled laboratory conditions. Females spawn every day and they can produce up to 100-200 eggs per week. Male is necessary for both deposition and fertilization of the eggs. They can breed all year round and they have external fertilization with the production of a transparent embryo that hatches after three days post fertilization. This allows an easy follow up and assessment all the embryonic developmental stages. At five days post fertilization they start swimming and feeding independently. They reach reproductive maturity in about 3-4 months, and they live for a maximum of 2-3 years in laboratory conditions. All these characteristics make this fish and excellent experimental model (Spence et al., 2007). Second, the whole genome has been sequenced, leading to the development of bioinformatic tools, dedicated database, that simplifies the study of this organism. Several zebrafish mutants have been so far realized, allowing the study of a particular phenotype or genetic disorders. In addition to this, a screening of 26.000 protein coding genes revealed that at least 70% of Human genes has an orthologous in Zebrafish's genes (Howe et al., 2013)

For all the features mentioned, Zebrafish is considered an excellent experimental model for developmental, reproductive, genetics and toxicology

studies. As embryonic, larval or adult stage, can be easily used in screening trials, to establish the effects and mechanisms of action of Endocrine Disruptor Chemicals (EDC) (Segner, 2009).

MATERIALS AND METHODS

2.1 Fish Maintenance

The experiments were carried out on adult zebrafish (Danio rerio, wild-type strain). The week before starting the treatments, a number of fish of similar sizes and no observable abnormalities were randomly selected from the facility. Fish were maintained in 100-L aquaria (static system) with oxygenated water under controlled conditions (28.0 ± 0.5 °C under a 14/10 h of light/dark period). Chemico-physical water parameters were constantly monitored. A total of 200 fish were equally distributed into 8 aquaria (25 fish per group) and fed as follows:

- (C), a control group which was fed with commercial food and *Artemia salina*,
- (P), a probiotic-treated group (SLAB51), that received a commercial diet containing the lyophilized probiotic at a final concentration of 10^9 CFU/g for 28 days. The probiotic formulation utilized was SLAB51 multi-strain probiotic mixture containing live bacteria (*Streptococcus thermophilus* DSM 32245®, *Bifidobacterium lactis* DSM 32246®, *Bifidobacterium lactis* DSM 32247®, *Lactobacillus helveticus* DSM

32242®, *Lactobacillus acidophilus* DSM 32241®, *Lactobacillus plantarum* DSM 32244®, *Lactobacillus paracasei* DSM 32243®, *Lactobacillus brevis* DSM 27961®). The probiotic strain was mixed into the diet prior to feeding.

- (BPA) fed commercial diet and *Artemia salina* and exposed to 10 µg/l BPA (98% analytical purity, Sigma-Aldrich, Milano, Italy) via the water.
- (BPA+P) fed for 4 weeks on a commercial diet containing the lyophilized probiotic at a final concentration of 10^9 CFU/g and exposed to 10 µg/l BPA starting from 8^{th} day of the trial.

All fish were served with a quantity of food ranging from 2.5% to 3% of their bodyweight per day. The experiment was conducted in duplicate

All the procedures involving animals were conducted according to University of Calgary animal care protocol for care and use of experimental animals). All efforts were made to minimize suffering. After 28 days of treatment, fish were euthanized using MS-222 (3-aminobenzoic acid ethyl ester (Sigma Aldrich) buffered to pH 7.4. according to University of Calgary animal care protocol for care and use of experimental animals) and tissue were sampled and stored at -80°C per molecular biology and metabolomic assays.

2.2 Metabolites Extraction and LC-MS Analysis

For metabolite extraction, the samples were taken form the -80 °C freezer and always kept on ice. 50 mg of liver sample were weighted and placed in a 2 mL test tube without letting the tissue thaw completely. The test tubes were incubated in the -20 °C freezer for 10 minutes because samples have to be frozen when homogenized. Then, 500 µL of cold (-20 °C) 50% methanol solution (50% Me-OH and 50% ultra-pure water) were added. One stainless steel bead was added in each tube and all the tubes were placed in a TissueLyser II (Quiagen, Hilden Germany) for 1 minute at a frequency of 30 shakes/s. The tubes were putted back into ice for 1 minutes and after that the samples were homogenized for 1 minute at a frequency of 30 shakes/s. 500 µL of cold (-20 °C) 50% methanol solution (50% Me-OH and 50% ultra-pure water) were added and then the beads were removed. The tubes were vortexed for 10 seconds, incubated at -20 °C for 5 minutes and then vortexed again for 10 seconds. The samples were then centrifuged for 20 minutes at 14.000 rpm at 4 °C and then centrifuged for 2 minutes at 21.000 g at room temperature. 500 µL of supernatant were collected and stored at -80 °C.

Extracted samples were thawed and centrifuged for 10 min at 14.000 rpm at 4 °C and then 200 µL of each sample have been used to prepare a 96-well plate. A quality control was also included formed by 4 extracted livers mixed

together. All the plate preparation was made in ice to prevent metabolites degradation. The plate was then covered with a film that allow the LC-MS needle to remove all the sample volume from the well.

Samples were analyzed with Ultra-High Performance Liquid Chromatography (UHPLC) coupled with Mass Spectrometry (MS). To separate the metabolites in the liquid phase a Hydrophilic Interaction Liquid Chromatography Column (Syncronis HILIC, Thermo Fisher, stationary phase) was used and high-resolution full scan MS data were acquired on a Thermo Fisher Scientific Q-Exactive HF Mass Spectrometer using negative-mode electrospray ionization. MAVEN freeware was used for the targeted profiling of the resulting MS spectra and the metabolites identification using the retention times of standards.

2.3 RNA extraction and cDNA synthesis

For Real Time PCR analysis, total RNA was extracted from 24 female fish livers (5 Control; 7 BPA; 6 Probiotic; 6 BPA + Probiotic) and 25 male fish livers (4 Control; 6 BPA; 7 Probiotic; 8 BPA + Probiotic) using RNAeasy® Minikit (Qiagen, Milano, Italy), following the manufacturer's instructions, and eluted in 50 µl of RNAse-free water. The final RNA concentration was determined using the Nanophotometer^{TM}P-Class (Implem GmbH, Munich,

Germany); RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at -80 °C until use. Total RNA was treated with DNAse (10 IU at 37 °C for 10 min, MBI Fermentas, Milano, Italy). A total amount of 1 µg RNA was used for cDNA synthesis with iScript cDNA Synthesis Kit (Bio-Rad, Milano, Italy).

2.4 Real-time PCR

PCRs were performed with SYBR green in an iQ5 iCycler thermal cycler (Bio-Rad) in triplicate as described previously (Maradonna et al., 2014). 60S ribosomal protein L13 (rplp13) and 60S acidic ribosomal protein P0 (rplp0) were the housekeeping genes. For primer sequences, accession number and annealing temperature see **Table 1**.

Table 1. Genes Name, Primers and Annealing Temperature.

Gene	ZFIN Name	Forward Primer 5'-3'	Reverse Primer 3'-5'	Accession Number	Ta (°C)
rplp13	60S ribosomal protein L13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	NM_212784	59
rplp0	*60S acidic ribosomal protein P0*	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	NM_131580.2	60
ptgs2a	*prostaglandin-endoperoxide synthase 2a*	ATCCAGGATGAAGTCTACAAT	GCTGTTGACGCCATAATC	NM_001025504.2	57
cebpa	*CCAAT enhancer binding protein alpha*	TTACAACAGGCCAGGTTTCC	CTCTGGGATGGATCGATTGT	NM_131837.1	57
gpr55bp	*G protein-coupled receptor 55b, processed pseudogene*	AACTGAAGGTGTGGATAC	ACGCCATAATGTTTAACG	XM_005163567.2	53
pparα	*Peroxisome proliferator-activated receptor alpha*	TCTTCAGGAGAACCATTC	ATCGGCAGTATTGACATT	NM_001161333	59
gcga	*glucagon a*	CAGAGGGAACTTTCTCCAACGA	AGTTCATACCCATTTCTCTTAGCGT	NM_001008595.3	60
mboat4	*membrane bound O-acyltransferase domain containing 4*	GCACACTGGAAGATGAGCGA	AACCGAAGGACGACTGGATG	NM_001122944.1	58
nucb2A	*nucleobindin*	AGGAGCGGCATGAAGAATTT	GATGGTTGACTTTGGGGTGA	NM_201493.1	60

2.5 DNA extraction and PCR

Zebrafish were surface sterilized with 0.1% NaOCl for 30 s and then washed 3 times with Tris-EDTA buffer. DNA was extracted from intestine following (Standen et al., 2015). PCR amplification of the 16S rRNA V1-V2 regions was conducted as described by (Roeselers et al., 2011). One μL of DNA template was used in the PCR reactions. The PCRs was performed in a TC-512 thermal cycler (Techne, Staffordshire, UK) under the following conditions: initial denaturation at 94 °C for 7 min, then 10 cycles at 94 °C for 30 s, followed by a touchdown of 1 °C per cycle from 62 − 53 °C for 30 s and 72 °C for 30 s. A further 20 cycles were performed at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s and a final extension 72 °C for 7 min.

2.6 High-throughput sequence analysis

PCR products from zebrafish intestine were sequenced using a 318^{TM} chip (LifeTechnologies^{TM}) on an Ion Torrent Personal Genome Machine (LifeTechnologies^{TM}) at the Systems Biology Centre in Plymouth University (UK) as described by (Standen et al., 2015). Sequences were binned by sample and filtered within the PGM software to remove low quality reads. Data were then exported as FastQ files. Taxonomic analyses of sequence reads were performed after the removal of low-quality scores (Q score < 20 at 80%

probability) with FASTX-Toolkit (Hannon Lab, USA). Sequences were concatenated and sorted by sequence similarity into a single fasta file. Sequences were denoised and analyzed with QIIME (Caporaso et al., 2010). Briefly, OTU mapping was performed using the USEARH quality filter pipeline (Edgar 2010), to remove putatively erroneous reads (chimeras), then OTU picking was achieved with a minimum pairwise identity of 97%. The most abundant sequence in each OTU were selected to assign a taxonomic classification based on the Greengenes database (DeSantis et al., 2006) using the RDP classifier (Wang Q. et al., 2007), clustering the sequences at 97% similarity with a 0.80 confidence threshold. PyNast was used to create a multiple alignment of the representative sequences for each OUT (Caporaso et al., 2010) with minimum sequence length threshold of 150 bp and 95% identification. Sequences were filtered to remove outliers, filter positions with gaps (0.95) and singletons. Highest homologous species were identified at 97% and minimum of 150 bp using nucleotide collection database at BLAST-NCBI. Alpha diversity metrics were calculated on rarefied OTU tables with QIIME to assess sampling depth coverage using observed species, phylogenetic diversity, Chao1, Shannon's diversity index and Good's coverage. QIIME was also used to calculate Beta diversity metrics among samples using weighted Unifrac distances (Abràmoff et al., 2004) and Bray-Curtis similarity (Bray and Curtis,

1957) The distance matrixes were represented by a two-dimensional Principal Coordinates Analysis (PCoA) plot.

2.7 Statistical analysis

For metabolomics analysis the SIMCA (Umetrics, Umeå Sweden) software was used to generate the PCA, PLS-DA, OPLS-DA and Hierarchical Analysis models and to assess their quality with the R^2, Q^2 and $p_{CV\text{-}ANOVA}$ parameters. Concerning R^2 and Q^2, a significant threshold of > 0.5 was used and a statistical significance for $p_{CV\text{-}ANOVA}$ was set at $p < 0.05$. The MetaboAnalyst online tools (University of Alberta, Alberta Canada) were used for the T-Test, ANOVA, Volcano Plot and Heatmap analysis using a Log Transformation normalization and a significant threshold of $p < 0.05$ and FDR correction set at < 0.05 were used. for the Pathway Analysis was used the MetaboAnalyst online tools using a Log Transformation coupled with Pareto Scaling normalization and setting the significant threshold at $p < 0.05$ and FDR correction at < 0.05.

The transcript abundance results were analyzed with the one-way ANOVA analyses of variance followed by Tukey multiple comparison tests. Significance between groups was set at $p < 0.05$. Results are presented as mean ± standard deviation. All statistical procedures were run using GraphPad Prism 6.

STAMP, Venny diagram, ape and vegan packages of R were used to analyze the high-throughput sequencing data. P-values < 0.05 were considered significant.

RESULTS

3.1 Metabolomic Analysis

The application of metabolomic study to analyze changes of the hepatic metabolism among different experimental zebrafish groups, starts from their separation according to the metabolic characteristics they possess. Therefore, analyzes like Principal Component Analysis (PCA), Orthogonal Partial Least Square Regression – Discriminant Analysis (OPLS-DA) and Hierarchical Analysis are initially used for this purpose. This allows to view at glance correlations and differences among the treatments, with groups that present similar features clustering together, differently from those which do not share common aspects. Statistical analysis using ANOVA and Heatmap, will determine differences among experimental groups and determine which analytes vary significantly among them. At this point, comparing each group with the C, metabolites that changes significantly and that characterize each treatment can be identified. Then, with the Pathway Analysis, metabolites are identified inside the metabolic pathways allowing to determine the metabolic phenotypes of each specific treatment respect to C one. This allows to make hypothesis on how treatments influenced and impacted the metabolism and

analyzing the related pathways is possible to verify eventual changes in the healthy state of the organism.

3.1.1 PCA, OPLS-DA and Hierarchical Analysis

The first step of the analysis consists in the elaboration of a PCA, including all experimental groups and a quality control group. As quality control, 5 extracted goldfish liver samples were mixed together to make a homogenous solution of metabolites and then aliquoted in 5 wells of the 96-well plate where the samples were present. This analysis plotted the two first principal components which best contain the information regarding the samples and is done primarily to see how the quality control cluster together. This internal control is used to determine how precisely the UHPLC-ESI-MS worked. So, the closer the quality controls are to each other, the more the machinery is reliable and precise in the analysis. In the present study, quality controls cluster very well together forming a well-cohesive group. This provides evidence that the samples have been correctly analyzed and provides biologically validity to the analysis conducted starting from the data generated. In addition to this, from PCA is possible to see that BPA+P and P groups clusters close and are overlapped. The C group is near and get in touch with them, while the BPA group has a very

wide distribution that encloses all the other three groups and the quality controls in it. The PCA analysis is shown in the **Figure 1**.

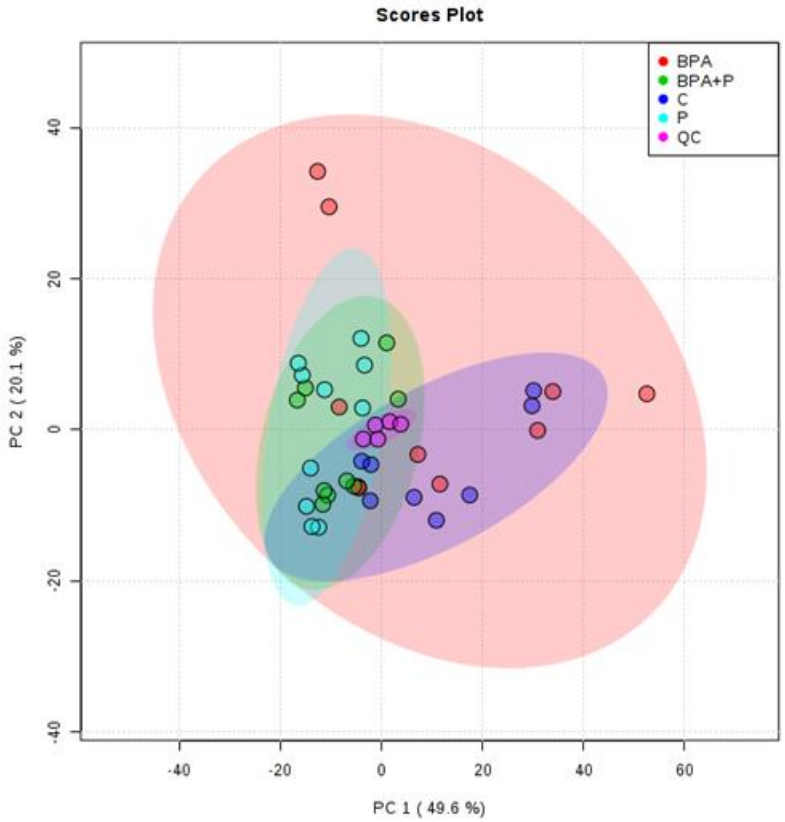

Figure 1. PCA Analysis of all groups.
Quality Control Samples (pink) cluster very close to each other. C (blue), P (light blue) and BPA+P (green) groups cluster close to each other and show an overlapped region. P and BPA+P groups show a big overlap. BPA (red) has a wide distributrion that contain all the other groups.

The resolution of this analysis is not very high and does not allow to appreciate the separation among groups and the eventual correlation among them but provides important information regarding quality controls. Further analysis is

therefore necessary. The OPLS-DA Analysis provides a more intense separation of groups and not only shows differences among groups but also gives information on differences within each group. In the present study, the OPLS-DA analysis confirmed that BPA+P group clusters together with P group, instead of BPA group, and that they are overlapped. This result suggests that probiotic administration mitigates BPA effects and similarities in the hepatic metabolism between BPA+P and P groups are hypothesized. The OPLS-DA result is also confirmed by the Hierarchical Analysis that shows a closer correlation of the BPA+P group with the P group respect to the BPA group. Further insights on this regard will be provided by subsequent metabolomic analysis and transcripts analysis. In **Figure 2** OPLS-DA analysis is shown and **Figure 3** Hierarchical Analysis is presented.

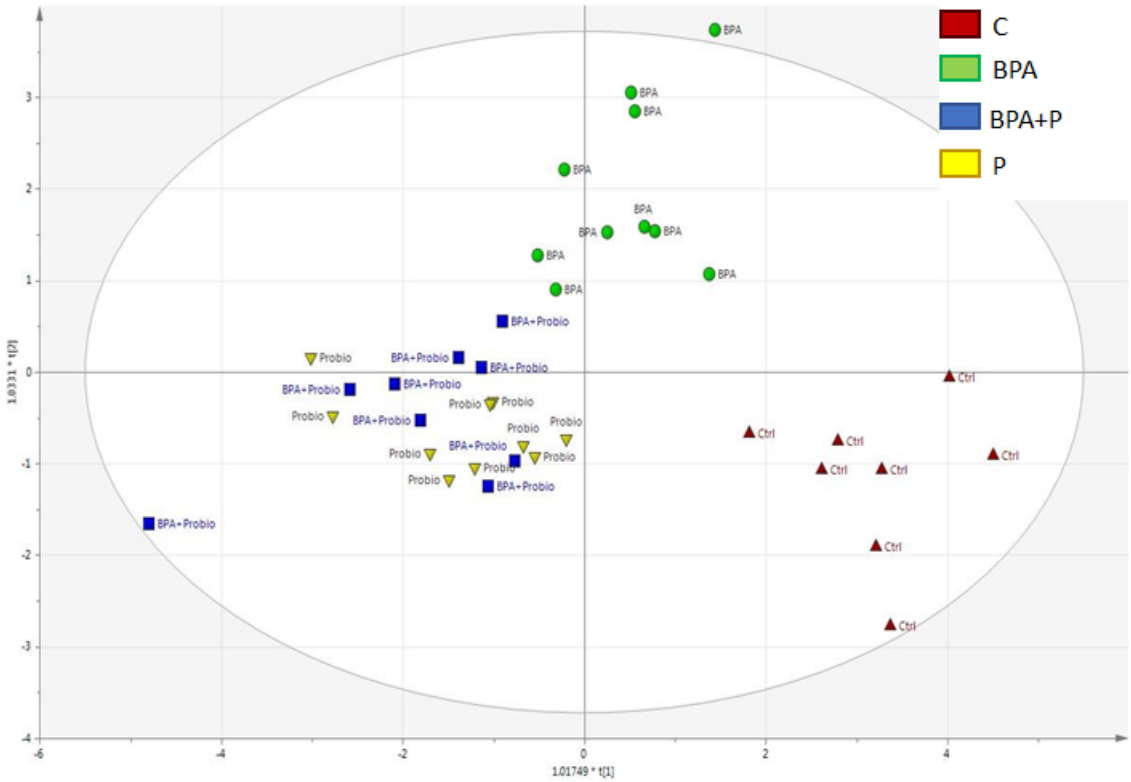

Figure 2. OPLS-DA Analysis of all groups.
P (yellow) and BPA+P (blue) cluser together and overlapped. The quality parameters value of this analysis are: $R^2 = 0.628$; $Q^2 = 0.262$; p-value CV ANOVA = 0.145.

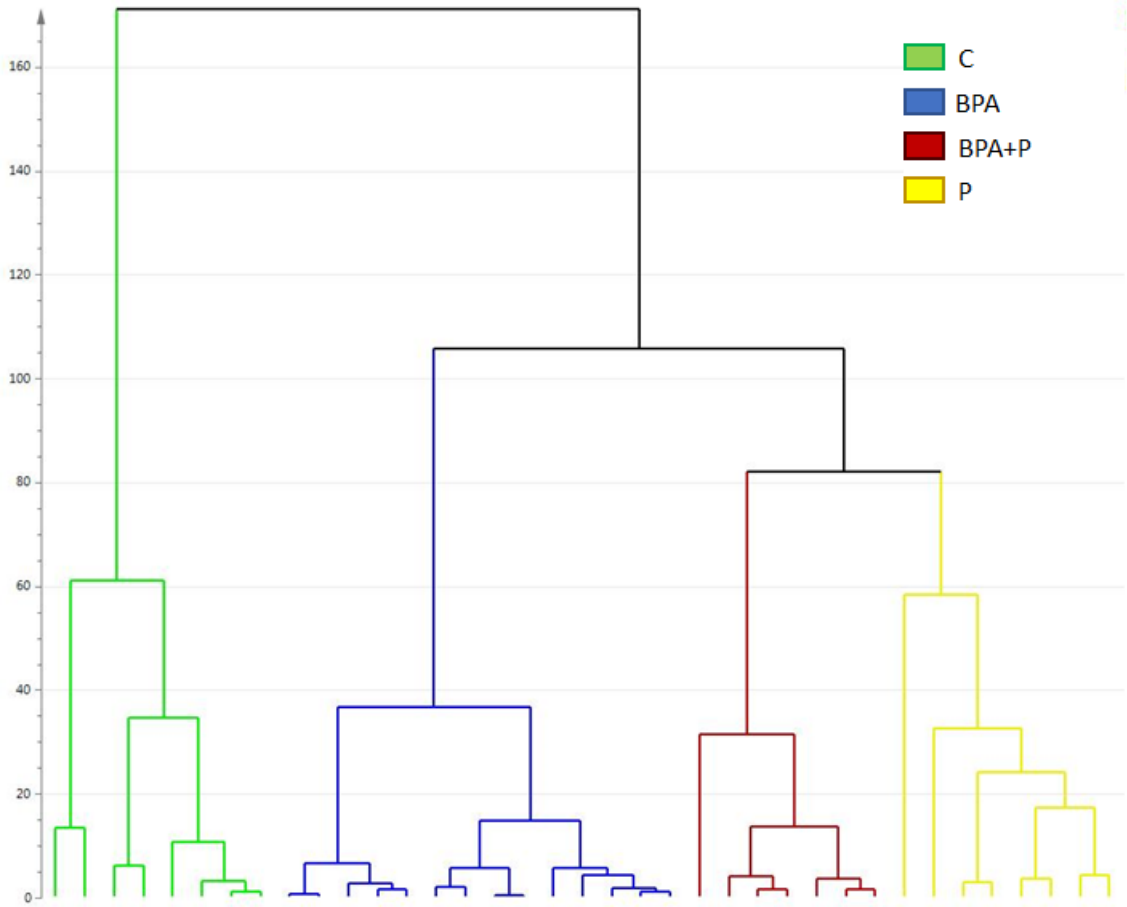

Figure 3. Hierarchical Analysis of all groups.
The metabolic profile of BPA+P (red) is more similar to that of P (yellow) than that of BPA (blue).

3.1.2 Heatmap and One-Way ANOVA Analysis

Once the interactions among groups have been established, a statistical analysis through ANOVA has been conducted and a Heatmap is produced to determine the changes among treated groups; the analyzes are shown in the **Figures 4** and **5**.

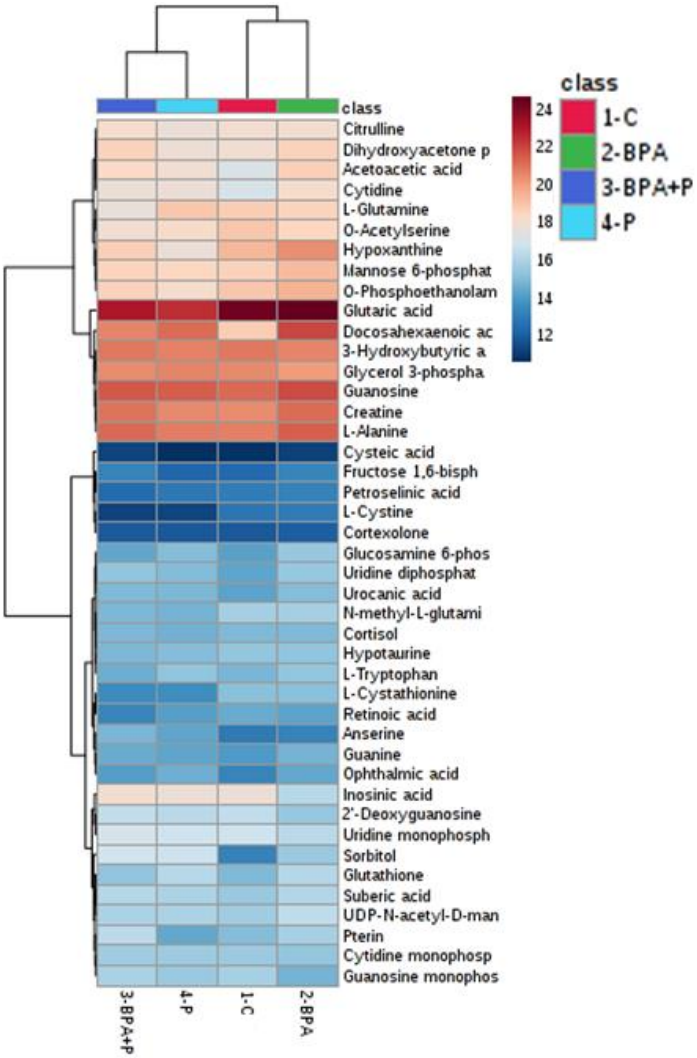

Figure 4. Heatmap Analysis of all groups.

By ANOVA analysis, statistically significant changes (p-value and FDR < 0.05) were found for 13 metabolites; metabolite list is shown in **Table 2**.

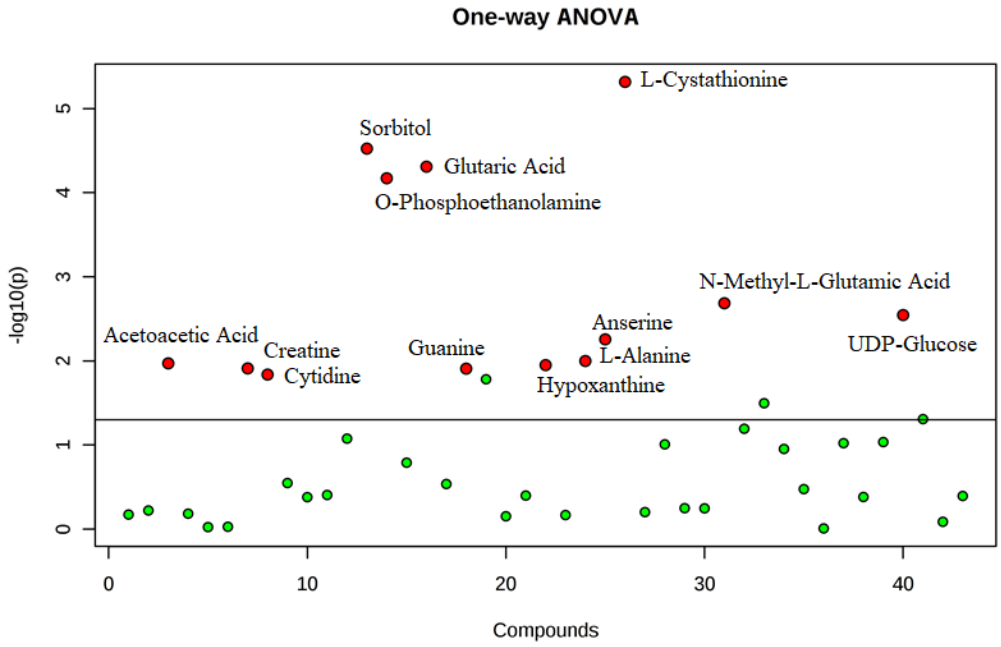

Figure 5. One-Way ANOVA Analysis of all groups.
Metabolites with p-value and FDR < 0.05 are shown with red dots.

Table 2. ANOVA Metabolites.		
Metabolites	**p.value**	**FDR**
L-Cystathionine	4,81E-03	2.07E-4
Sorbitol	2,99E-01	6,43E+00
Glutaric acid	4,91E-01	7,03E+00
O-Phosphoethanolamine	6,73E-01	7,24E-01
N-methyl-L-glutamic Acid	0.0020612	0.017726
Uridine diphosphate glucose	0.0028482	0.020412
Anserine	0.0055399	0.034031
L-Alanine	0.010017	0.044262
Acetoacetic acid	0.010688	0.044262
Hypoxanthine	0.011208	0.044262
Creatine	0.012266	0.044262
Guanine	0.012352	0.044262
Cytidine	0.014525	0.048045

A decrease of Cystathionine, Glutaric Acid and N-Methyl-L-Glutamic Acid levels were measured in the P and BPA+P groups respect to C, while the levels of these metabolites did not vary in the BPA group. Sorbitol, UDP-Glucose and Cytidine levels were significantly increased in all the treated groups respect to C. The O-Phosphoethanolamine metabolite levels increased in the group BPA compared to C, while they decreased in the P group. Levels of this metabolite remained unchanged in BPA+P group respect to C and P. Levels of Anserine metabolite raised in the P and BPA+P groups compared to C and BPA groups.

Levels of the amino acid Alanine, the metabolites Creatine and Guanine significantly increased in BPA and BPA+P groups compared to C, while the P group did not show any significant change compared to C and BPA+P groups. BPA and BPA+P groups show a significant increase in Acetoacetic Acid levels

compared to C, not significant in the P group which did not significantly vary from BPA and BPA+P groups. There is also a significant change in Hypoxanthine levels that are decreased in BPA+P and P groups compared to BPA group, while in BPA the level does not change compared to C. The Hypoxanthine concentration does not change significantly in the P and BPA+P groups compared to C. Metabolite concentration changes are shown in **Figures 6-a and b**.

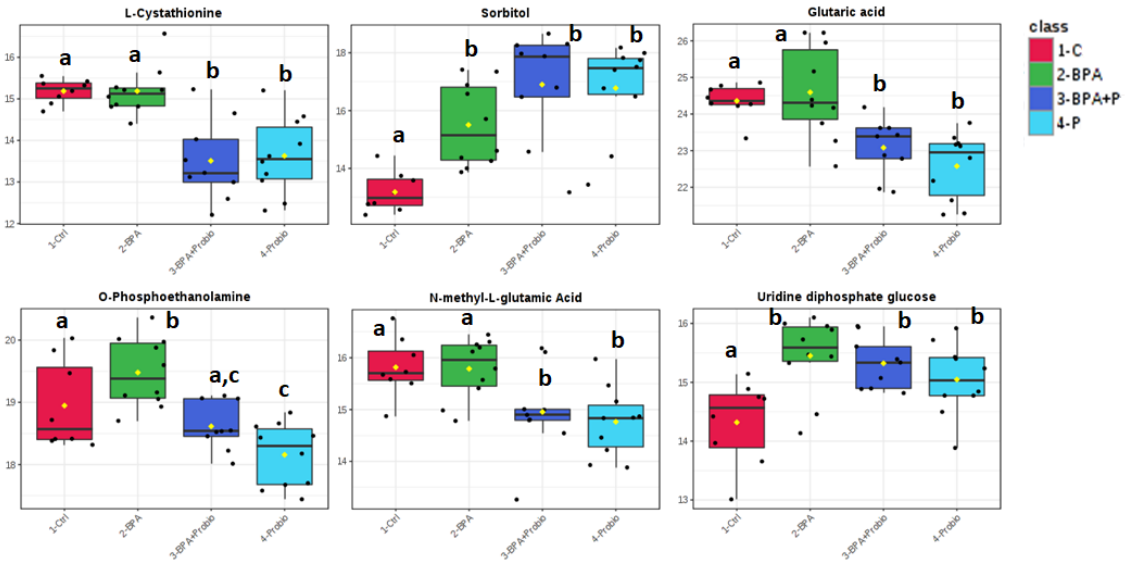

Figure 6-a. One-Way ANOVA Comparison of all groups for each metabolite.
Different letters means a Statistically Significant Difference (p-value and FDR < 0.05) between the groups

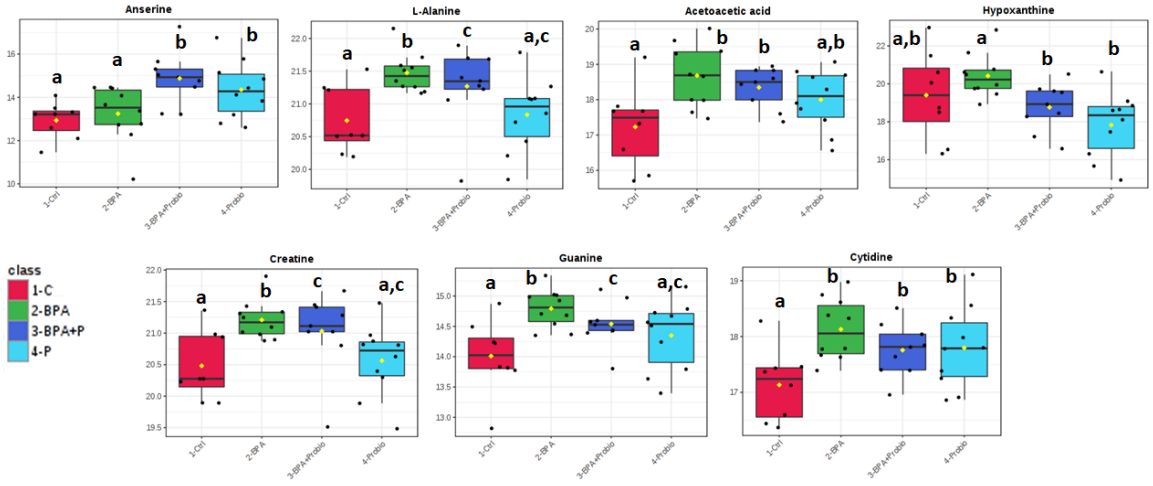

Figure 6-b. One-Way ANOVA Comparison of all groups for each metabolite.
Different letters means a Statistically Significant Difference (p-value and FDR < 0.05) between the groups

All the ANOVA determined metabolic changes mentioned above will be considered and analyzed in the Discussion, with data from the comparison of the three treated groups with C, in the determination of metabolic profiles of these groups.

3.1.3 Metabolomic Evaluation of BPA Metabolism

To analyze the metabolic profile of BPA group, first OPLS-DA analysis was conducted to determine how the samples are distributed in the BPA group compared to Control and to verify the presence of separation between these two groups. The OPLS-DA analysis is shown in **Figure 7**.

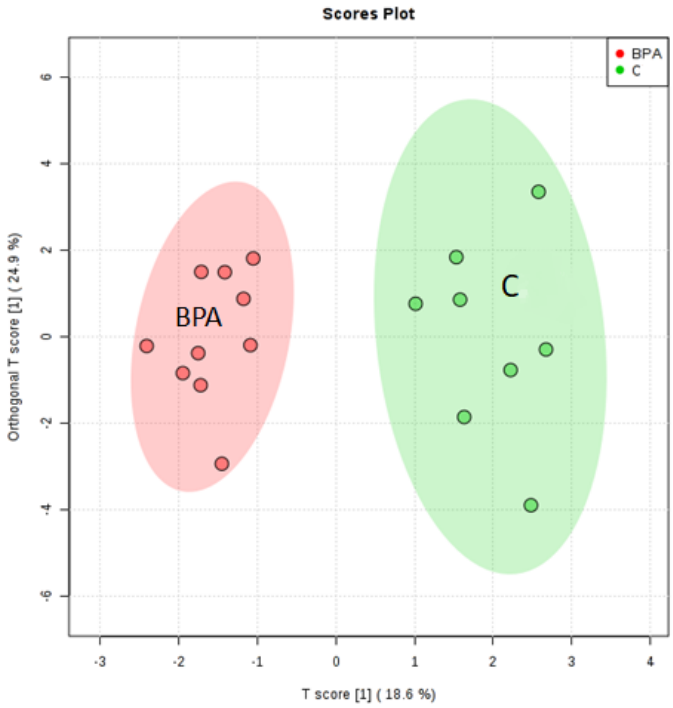

Figure 7. OPLS-DA of C vs BPA.
The quality parameters values of this analysis are: $R^2 = 0.987$; $Q^2 = 0.847$; p-value CV ANOVA = 0.018

After this, a T-TEST was performed and a Heatmap was realized to see the statistically significant changes of metabolites in the BPA group compared to C group. A statistically significant increase was found in 11 metabolites levels respect to C; the metabolites are shown together with their p-value and FDR in **Table 3**. The Heatmap is shown in **Figure 8** and the T-TEST is shown in **Figure 9**.

Table 3. T-TEST Metabolites of C vs BPA.

Name	p.value	FDR
Sorbitol	7,23E+00	0.014613
Guanosine	8,19E+00	0.014613
Suberic acid	0.0010195	0.014613
L-Alanine	0.0016031	0.017233
Creatine	0.0025268	0.017498
Cytidine	0.0026761	0.017498
Guanine	0.0029121	0.017498
Uridine diphosphate glucose	0.0032555	0.017498
Docosahexaenoic acid	0.005008	0.023927
UDP-N-acetyl-D-mannosamine	0.0071402	0.030703
Acetoacetic acid	0.0080048	0.031292

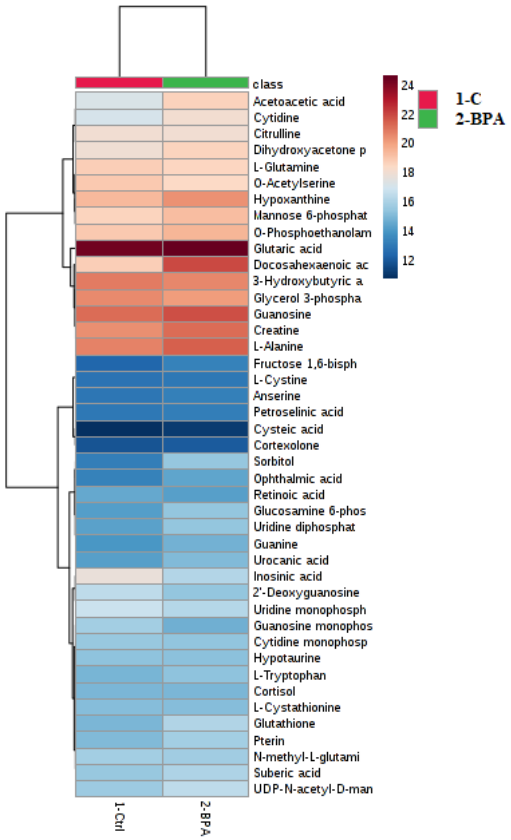

Figure 8. Heatmap of C vs BPA.

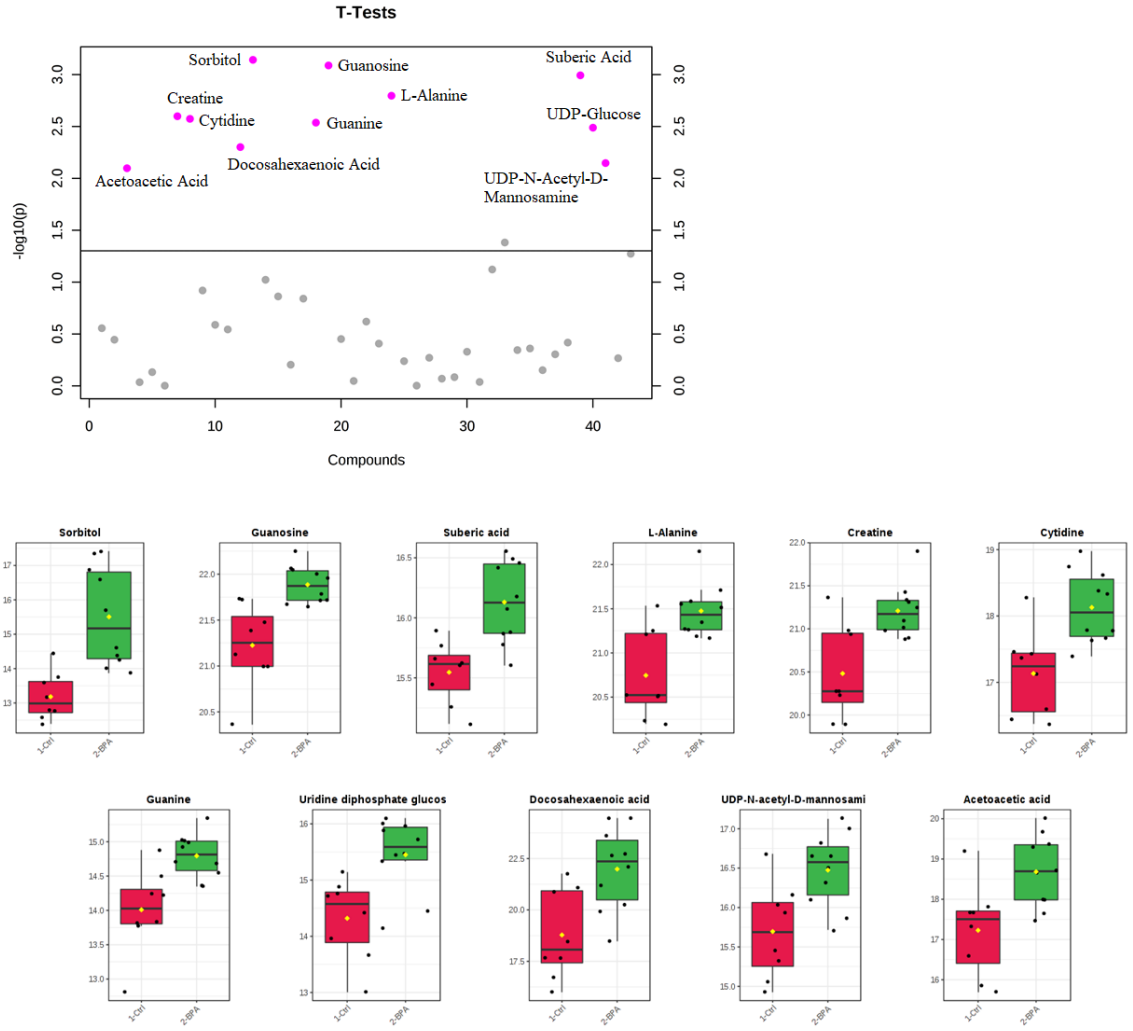

Figure 9. T-TEST of C vs BPA.
The upper figure show the metabolites that present a statistically significant difference, p-value and FDR < 0.05, compared to Control with pink dots. In the figures below, the changes are visible for all the metabolites.

In addition to these analyzes, a Pathway Analysis was conducted and the results are visible in **Table 4**, while **Figure 10** shows two graphs that for all the matched pathways plot the impact values from the pathway topology analysis and the p-values from the pathway enrichment analysis.

Table 4. Pathway Analysis of C vs BPA				
Pathway Name	**p**	**FDR**	**Impact**	**Metabolites Involved**
Galactose metabolism	3,25E-01	0.0010724	0,00228	Sorbitol; UDP-Glucose
Selenocompound metabolism	0.0016031	0.017905	0	Alanine
Arginine and proline metabolism	0.0025268	0.017905	0,01206	Creatine
Pentose and glucuronate interconversions	0.0032555	0.017905	0,07792	UDP-Glucose
Starch and sucrose metabolism	0.0032555	0.017905	0,011	UDP-Glucose
Ascorbate and aldarate metabolism	0.0032555	0.017905	0	UDP-Glucose
Fructose and mannose metabolism	0.0038853	0.018317	0,19359	Sorbitol; Mannose-6-Phosphate; Glicerone Phosphate
Biosynthesis of unsaturated fatty acids	0.005008	0.020658	0	Docosahexaenoic Acid
Glycine, serine and threonine metabolism	0.0076061	0.024015	0	Creatine; Cystathionine
Valine, leucine and isoleucine degradation	0.0080048	0.024015	0	Acetoacetic Acid
Tyrosine metabolism	0.0080048	0.024015	0	Acetoacetic Acid
Synthesis and degradation of ketone bodies	0.010045	0.025499	0,6	Acetoacetic Acid; 3-Hydroxybutanoate
Butanoate metabolism	0.010045	0.025499	0,11111	Acetoacetic Acid; 3-Hydroxybutanoate
Amino sugar and nucleotide sugar metabolism	0.015882	0.037435	0,1819	UDP-Glucose; Mannose-6-Phosphate; Glucosamine-6-Phosphate

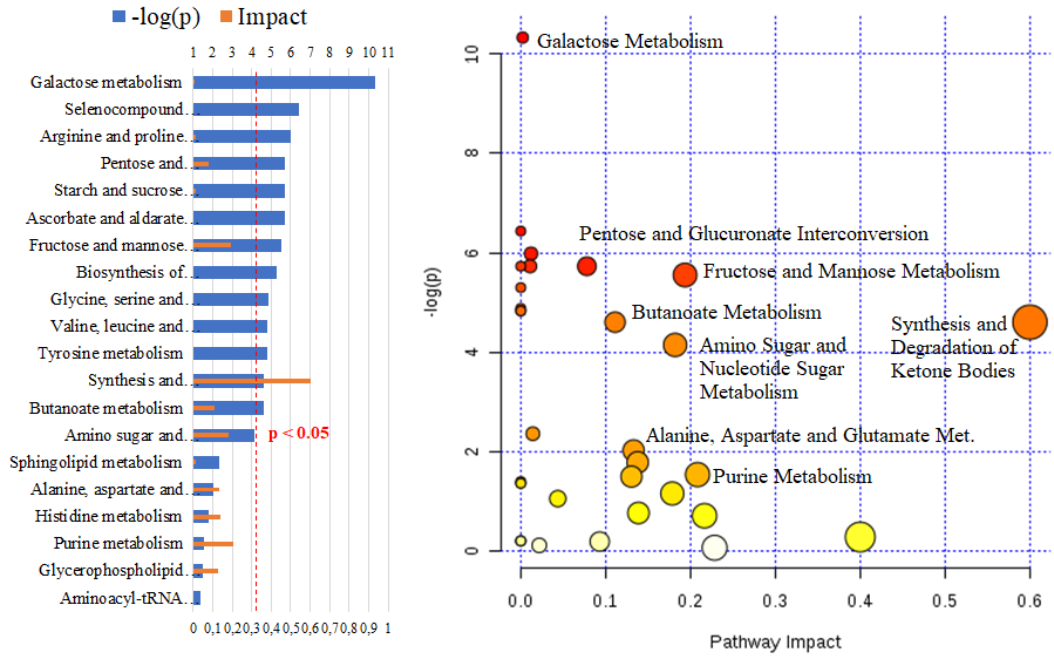

Figure 10. Pathway Analysis of C vs BPA.
These graphics plotted the impact of the metabolites found in the pathway analized with the logaritm of the p-value. In the lesft graph, the dotted red line represents the cut-off of the p-value. In the right graph the color of the dots represents the statistically significance, where red is the most significant and the white is the least significant; the diameter of the dots represents the impact of the metabolites, where the largest has the biggest impact and the smallest has the least impact.

Pathways analyzed in the BPA group are shown in **Figures 11 – 16**. These pathways are described in the discussion.

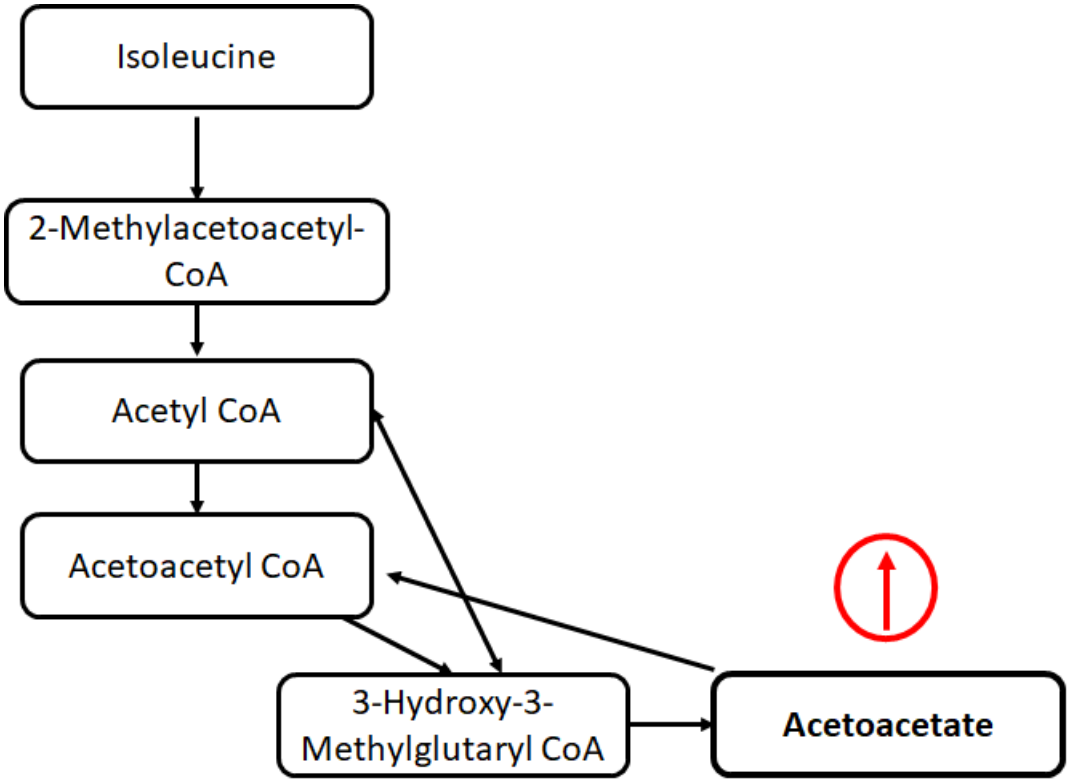

Figure 11. Valine, Leucine and Isoleucine Metabolism.
In this figure only the Isoleucine degradation pathway is shown. The increased Acetoacetic Acid level (p-value and FDR < 0.05; red arrow pointing up) is shown.

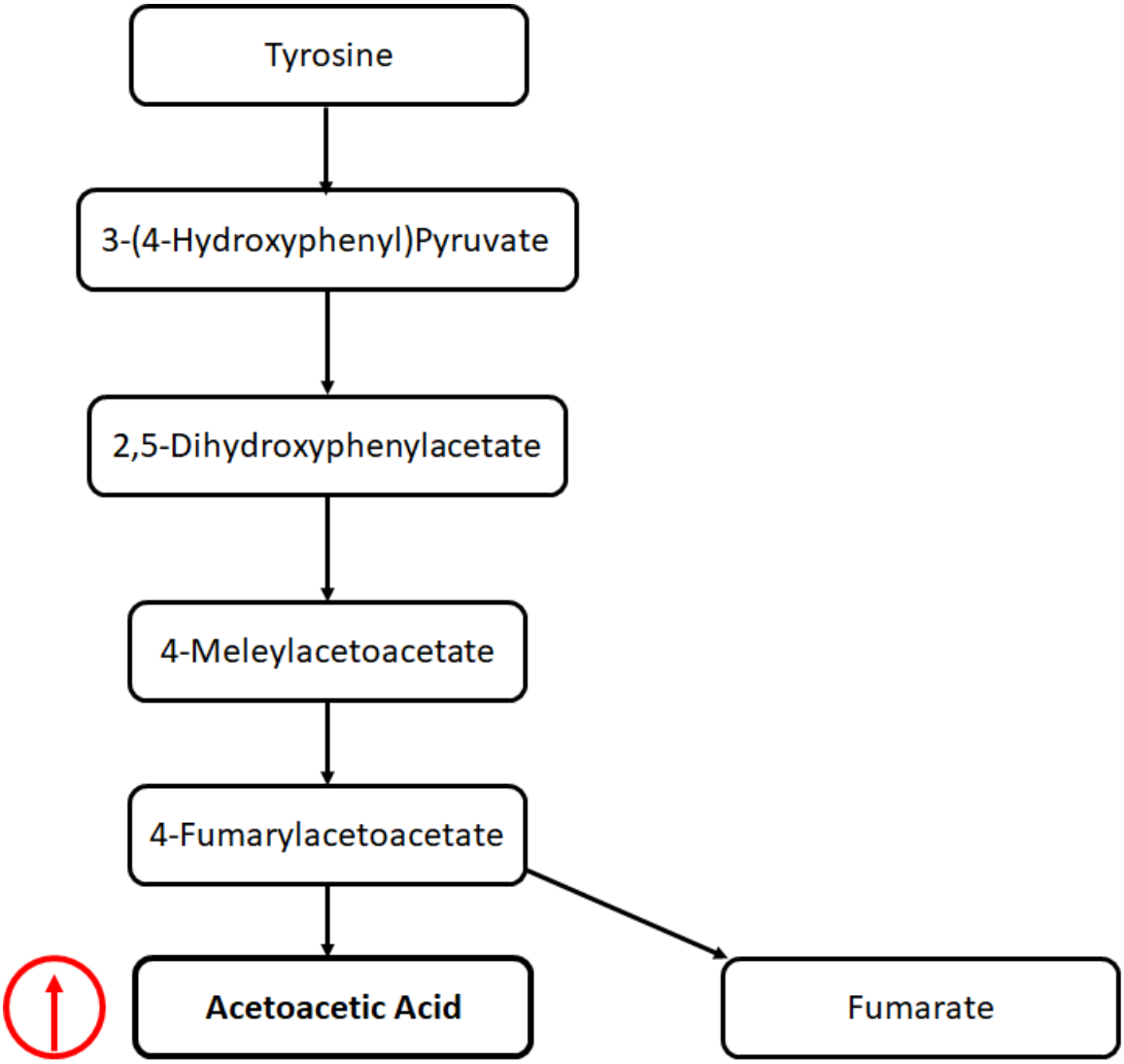

Figure 12 . Tyrosine Metabolism.
In this pathway there is a significant increase (p-value and FDR < 0.05; red arrow pointing up) in the Acetoacetic Acid level.

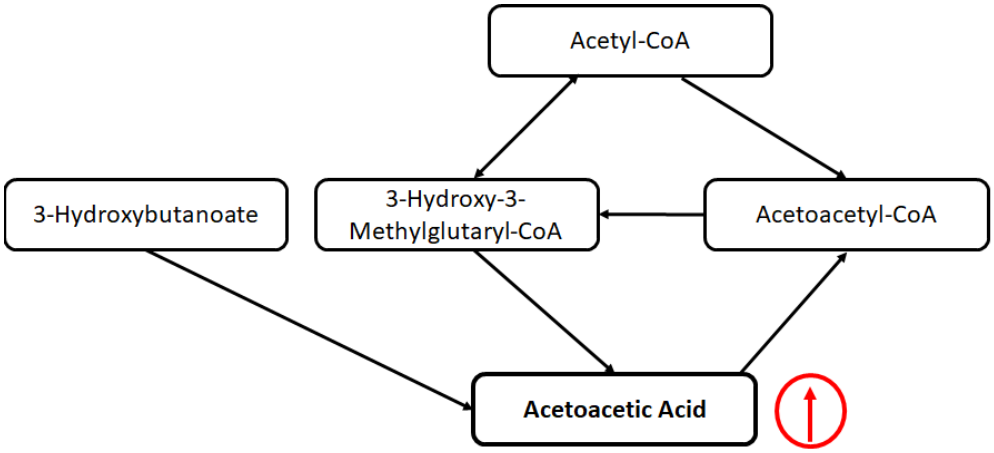

Figure 13. Synthesis and Degradation of Ketone Bodies.
In this pathway there is a significant increase (p-value and FDR < 0.05; red arrow pointing up) in the Acetoacetic Acid level.

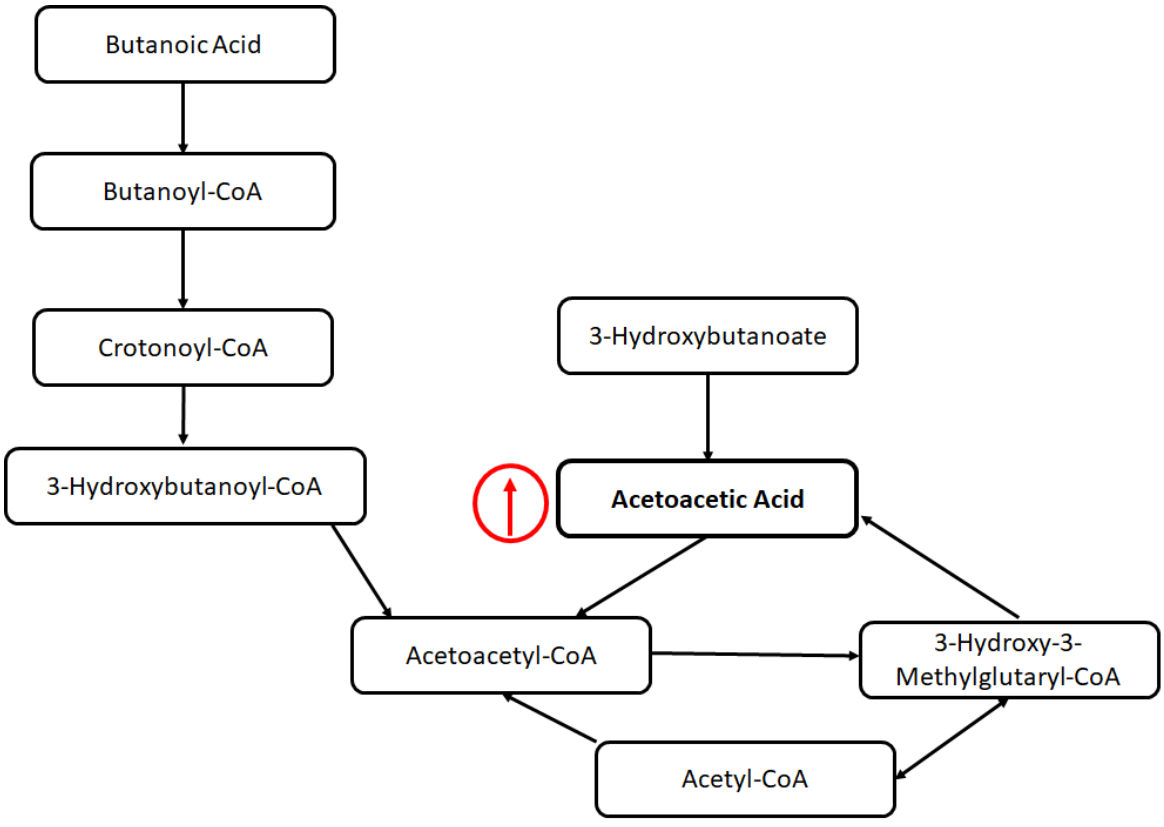

Figure 14 . Butanoate Metabolism.
In this pathway there is a significant increase (p-value and FDR < 0.05; red arrow pointing up) in the Acetoacetic Acid level.

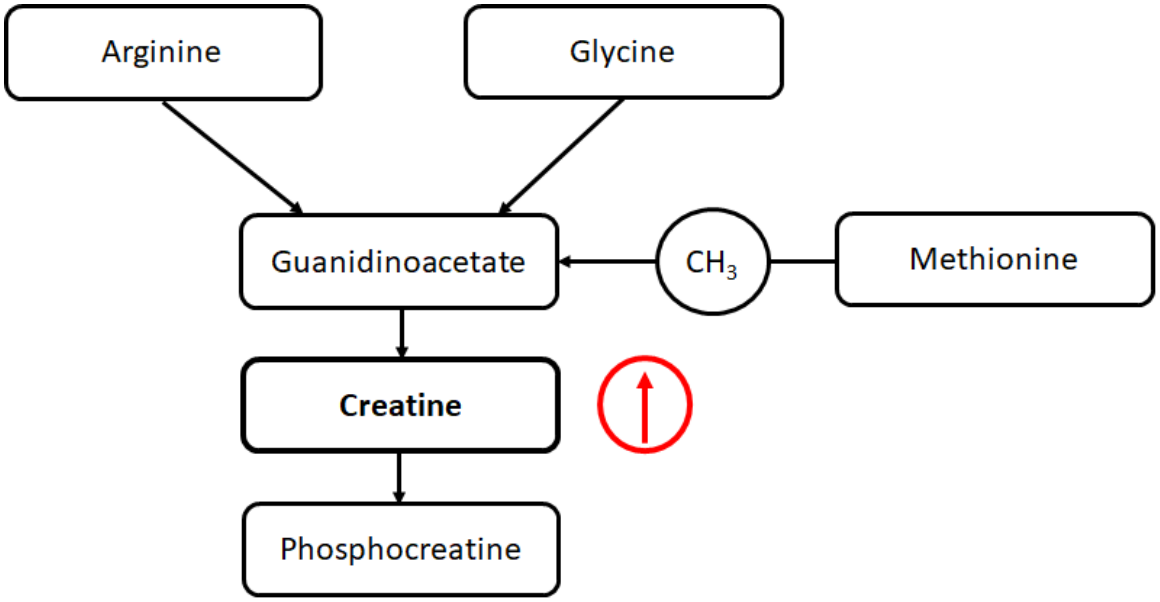

Figure 15. Creatine Biosynthesis.
Guanidinoacetate is synthetized in the kidney from Arginine, Glycine and the methyl group of Methionine, and then converted in Creatine in the liver. In this pathway there is a significant increase (p-value and FDR < 0.05; red arrow pointing up) in the Creatine level.

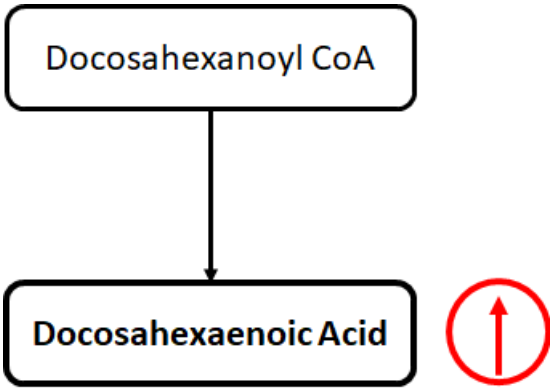

Figure 16. Biosynthesis of Unsatureted Fatty Acids.
In this pathway there is a significant increase (p-value and FDR < 0.05; red arrow pointing up) of the Docosahexaenoic Acid level.

3.1.4 Metabolomic Evaluation of Probiotic Metabolism

An OPLS-DA analysis was performed and a separation between the P and C groups was found. Despite a clear separation is visible, a portion of the area covered by the group overlaps (see in the OPLS-DA graph shown in the **Figure 17**).

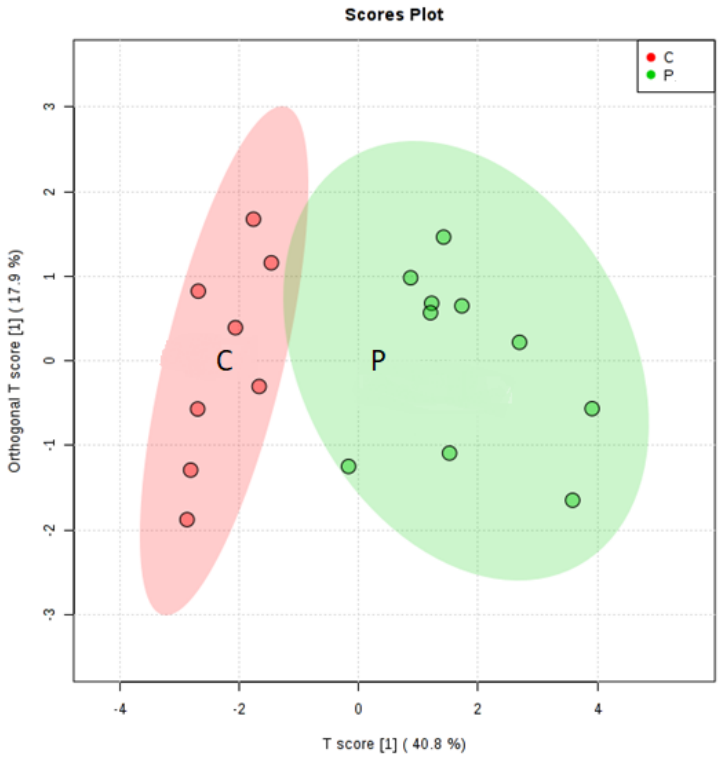

Figure 17. OPLS-DA of C vs P
The quality parameters values of this analysis are: $R^2 = 0.998$; $Q^2 = 0.92$; p-value CV ANOVA = 0.005.

The T-TEST analysis identified a statistically significant changes (p-value and FDR < 0.05) for 4 metabolites; Sorbitol showed increased level while Glutaric

Acid, Cystathionine and N-Methyl-L-Glutamic Acid showed decreased levels, compared to C. Other 4 metabolites shown a p-value < 0.05 but an FDR > 0.05; O-Phosphoethanolamine showed decreased level while Anserine, Ophthalmic Acid and UDP-Glucose showed increased levels, compared to C. Some of these last metabolites are included in the succeeding analyzes because of their presence in the ANOVA test or because they are found in the Pathway Analysis. The metabolites are visible in the **Table 5**. The Heatmap is shown in the **Figure 18** while the T-TEST graphs are visible in the **Figure 19**.

Table 5. T-TEST Metabolites of C vs P

Metabolites	p.value	FDR
Sorbitol	2,31E-01	9,94E+00
Glutaric acid	1,38E+00	0.0029632
L-Cystathionine	3,26E+00	0.0046692
N-methyl-L-glutamic Acid	0.0027502	0.029564
O-Phosphoethanolamine	0.014856	0.12777
Anserine	0.018731	0.13424
Ophthalmic acid	0.023932	0.14701
Uridine diphosphate glucose	0.034033	0.18293

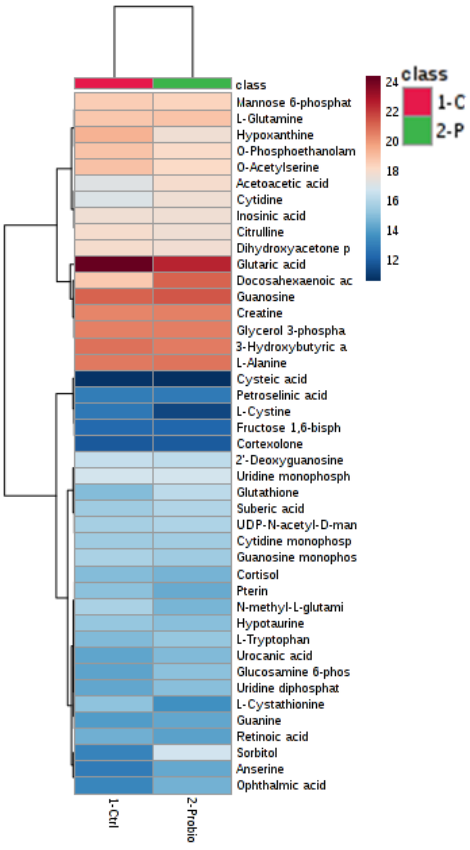

Figure 18 . Heatmap of C vs P

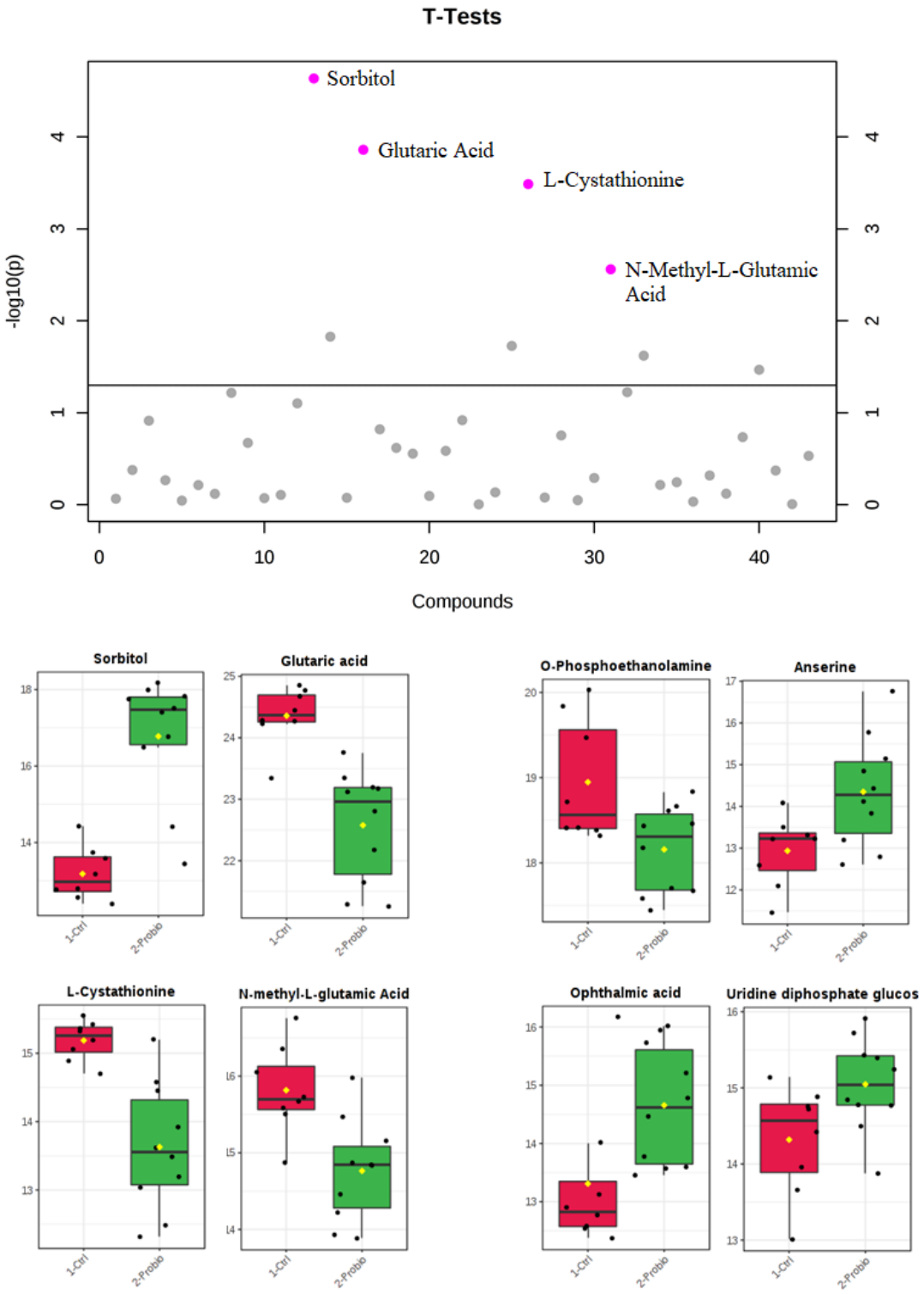

Figure 19 . T-TEST of C vs P
In the upper figure the pink dots show the statistically significant changes (p-value and FDR < 0.05). In the figures below the metabolites changes are visible. The left figure show the metabolites with p-value and FDR < 0.05; in the right figure the metabolites with p-value < 0.05 but FDR > 0.05 are shown.

A Pathway Analysis has been conducted. The pathways involved in this treatment are shown in the **Table 6**, while graphs are reported in the **Figure 20**.

Table 6. Pathway Analysis of C vs P

Pathway Name	p	FDR	Impact	Metabolites Involved
Galactose metabolism	3,39E-01	0.0011183	0,00228	Sorbitol; UDP-Glucose
Fructose and mannose metabolism	3,02E+00	0.0049894	0,19359	Sorbitol; Mannose-6-Phosphate; Glycerone Phosphate
Glycine, serine and threonine metabolism	5,21E+00	0.0057349	0	Creatine; Cystathionine
Cysteine and methionine metabolism	0.0077859	0.064234	0,17854	Cysteate; Cysteine; Cystathionine; Ophtalmate
Sphingolipid metabolism	0.014856	0.098053	0,0142	O-Phosphoethanolamine
Histidine metabolism	0.032125	0.12479	0,13776	Anserine; Urocanate
Pentose and glucuronate interconversions	0.034033	0.12479	0,07792	UDP-Glucose
Starch and sucrose metabolism	0.034033	0.12479	0,011	UDP-Glucose
Ascorbate and aldarate metabolism	0.034033	0.12479	0	UDP-Glucose

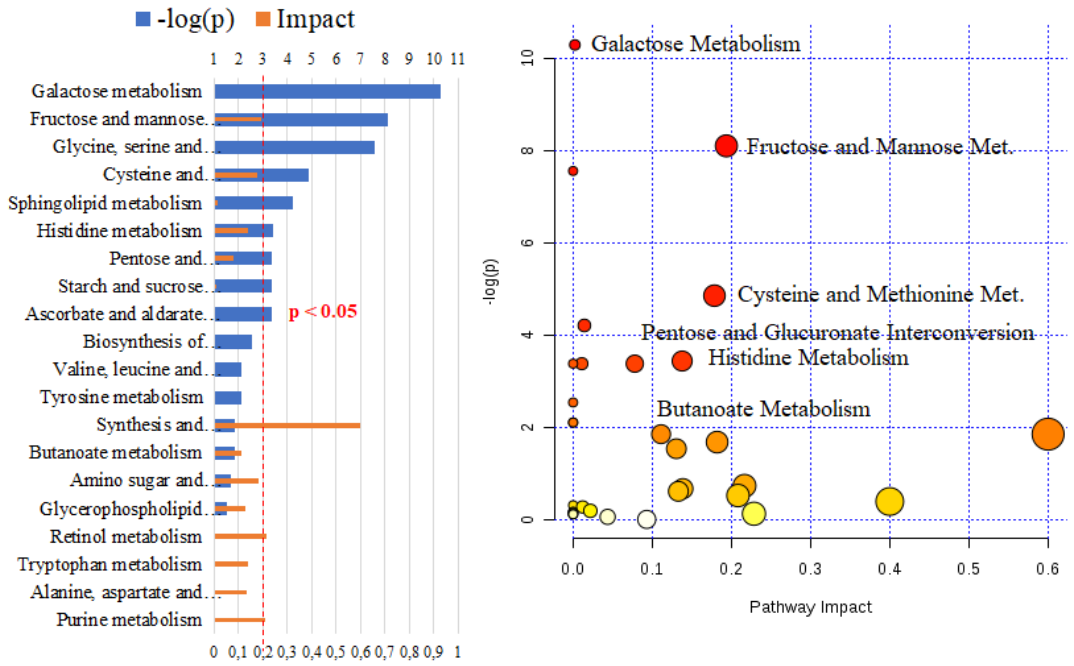

Figure 20. Pathway Analysis of C vs P
These graphics plotted the impact of the metabolites found in the pathway analized with the logaritm of the p-value. In the lesft graph, the dotted red line represents the cut-off of the p-value. In the right graph the color of the dots represents the statistically significance, where red is the most significant and the white is the least significant; the diameter of the dots represents the impact of the metabolites, where the largest has the biggest impact and the smallest has the least impact.

Pathways analyzed in P group are shown in **Figures 21 – 23** then described in the discussion.

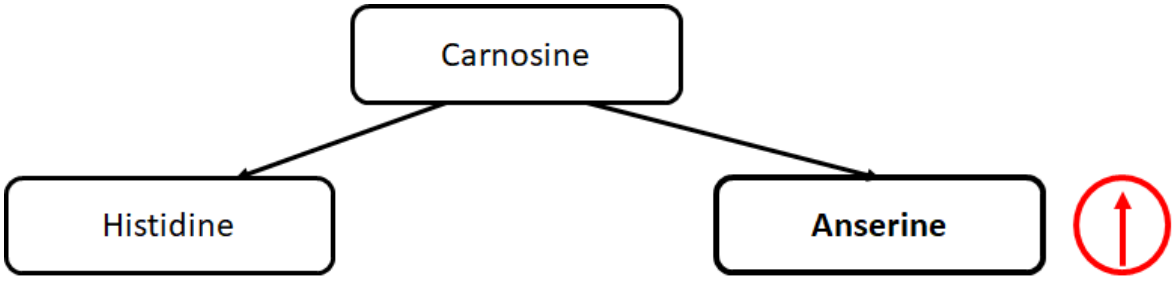

Figure 21. Histidine Metabolism.
In this pathway there is a significant increase (p-value < 0.05; red arrow pointing up) of Anserine level.

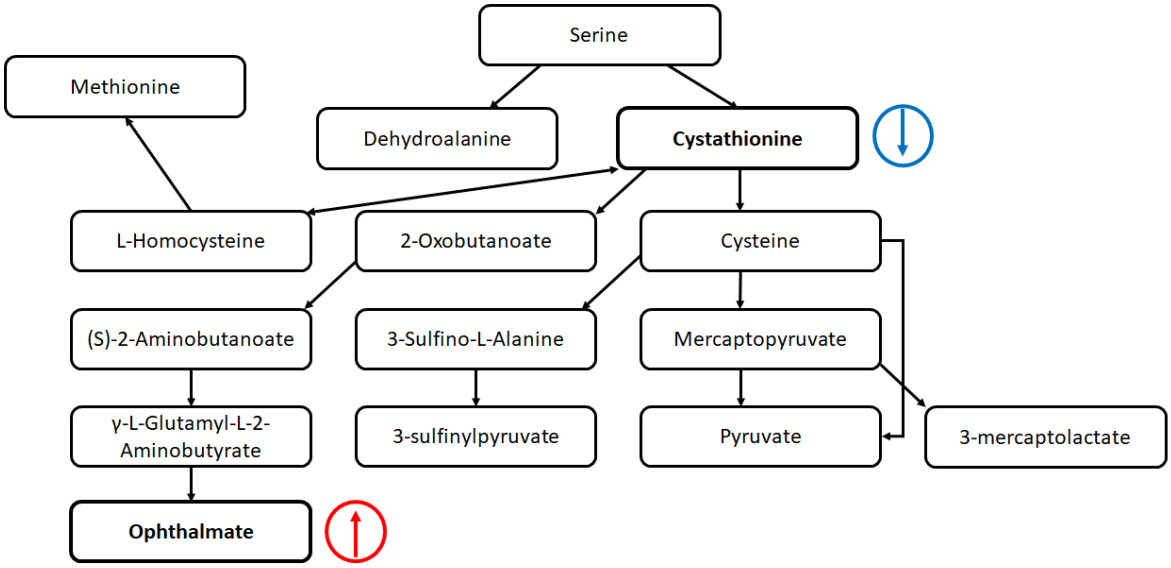

Figure 22. Cysteine and Methionine Metabolism.
In this pathway there is a significant decrease of Cystathionine level (p-value and FDR < 0.05; blue arrow pointing down) and a increase in the Ophthalmic Acid level (p-value < 0.05; red arrow pointing up).

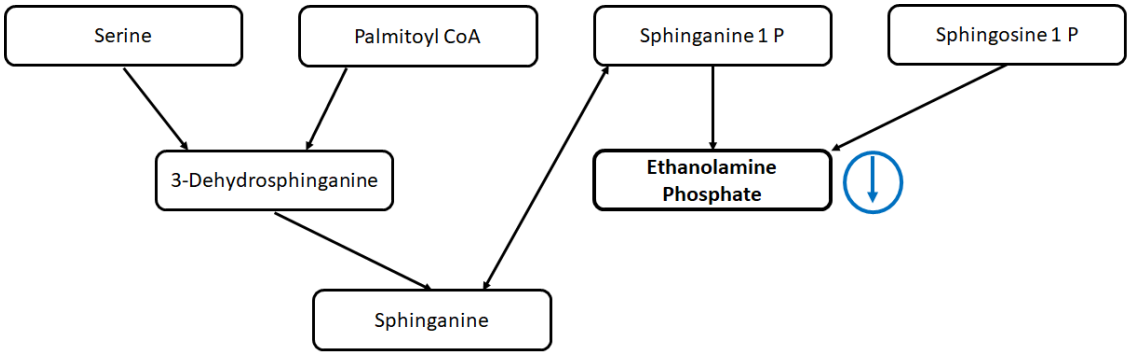

Figure 23 . Sphingolipid Metabolism.
In this pathway there is a decrease in the O-Phosphoethanolamine level (p-value < 0.05; blue arrow pointing down).

3.1.5 Metabolomic Evaluation of BPA+Probiotic Metabolism

The OPLS-DA analysis show a neat separation of the group BPA+P respect to C group. The **Figure 24** show the OPLS-DA graph.

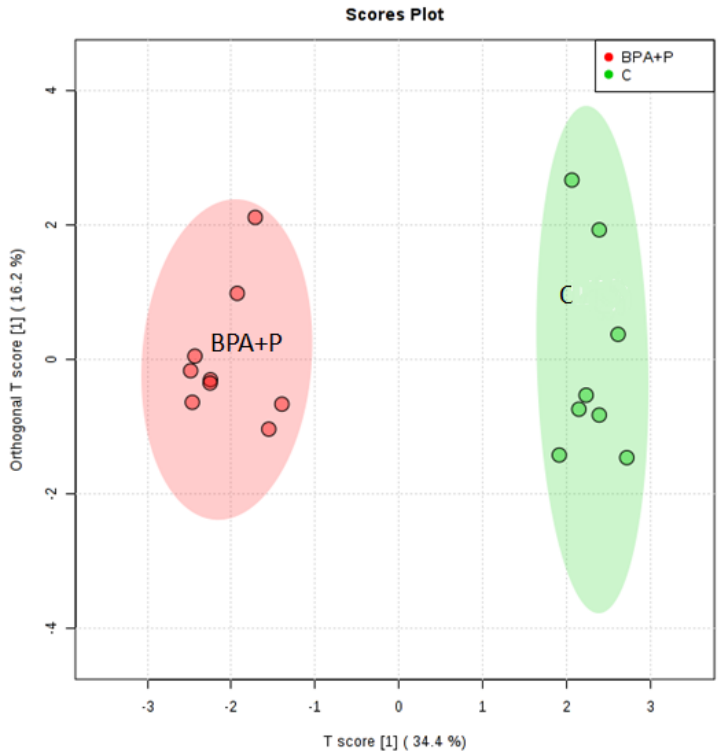

Figure 24. OPLS-DA of C vs BPA+P
The quality parameters values of this analysis are: $R^2 = 0.997$; $Q^2 = 0.899$; p-value CV ANOVA = 0.067.

The Heatmap and T-TEST analysis found a metabolic significant change (p-value and FDR < 0.05) in 6 metabolites; Cystathionine and Glutaric Acid show decreased levels while Sorbitol, Anserine, UDP-Glucose and Suberic Acid show increased levels, compared to C. Other 6 metabolites present a metabolic change with p-value < 0.05 but FDR > 0.05; O-Acetylserine, Retinoic Acid and N-Methyl-L-Glutamic Acid showed decreased levels while Acetoacetic Acid,

Cytidine and Guanosine showed increased levels, compared to C. Metabolites and their p-value and FDR are shown in the **Table 7** while the Heatmap is shown in the **Figure 25** and the T-TEST visible in the **Figure 26**.

Table 7. T-TEST Metabolites of C vs BPA+P

Metabolites	p.value	FDR
Sorbitol	9,75E-01	0.0041943
L-Cystathionine	2,94E+00	0.0063131
Glutaric acid	0.0011595	0.01662
Anserine	0.0019978	0.021476
Uridine diphosphate glucose	0.0031668	0.027234
Suberic acid	0.0061847	0.044324
O-Acetylserine	0.010672	0.065556
Retinoic acid	0.01835	0.097134
Acetoacetic acid	0.02033	0.097134
N-methyl-L-glutamic Acid	0.030271	0.13016
Cytidine	0.036981	0.14456
Guanine	0.045475	0.16295

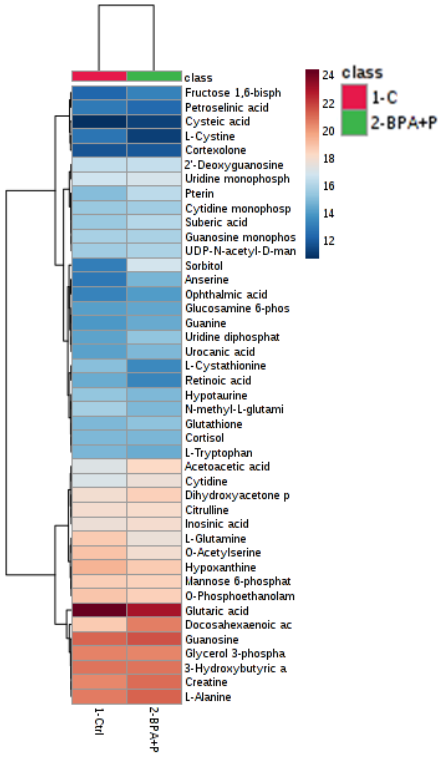

Figure 25. Heatmap of C vs BPA+P

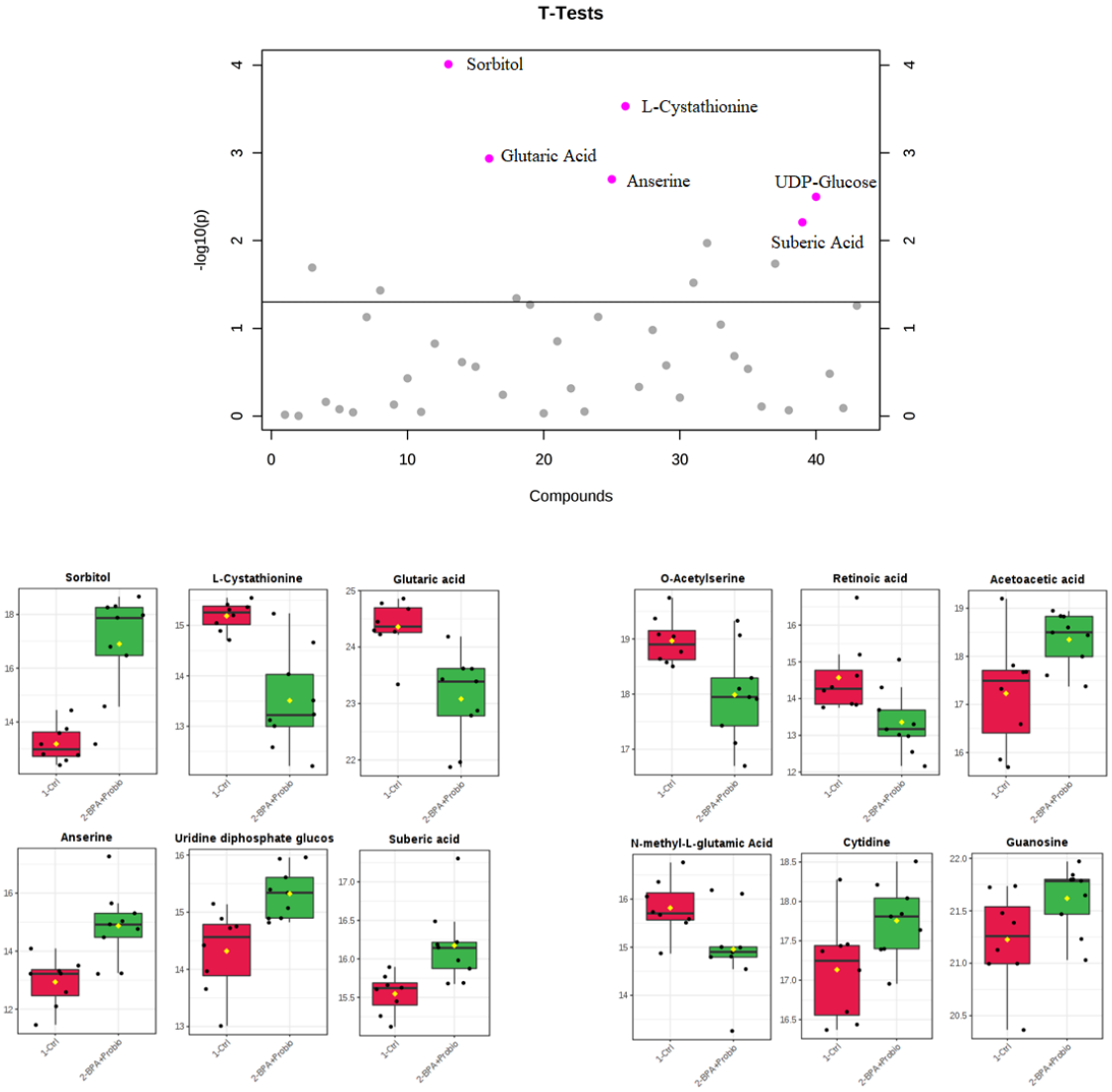

Figure 26. T-TEST of C vs BPA+P
In the upper figure the pink dots show the statistically significant changes (p-value and FDR < 0.05). In the figures below the metabolites changes are visible. The left figure show the metabolites with p-value and FDR < 0.05; in the right figure the metabolites with p-value < 0.05 but FDR > 0.05 are shown.

A Pathway Analysis has been conducted and the pathways found are shown in the **Table 8** while in the **Figure 27** the Pathway Analysis is visible.

Table 8. Pathway Analysis of C vs BPA+P				
Pathway Name	**p**	**FDR**	**Impact**	**Metabolites Involved**
Galactose metabolism	1,87E-01	6,19E+00	0,00228	Sorbitol; UDP-Glucose
Glycine, serine and threonine metabolism	1,44E+00	0.0023775	0	Creatine; Cystathionine
Fructose and mannose metabolism	8,93E+00	0.0087836	0,19359	Sorbitol; Mannose-6-Phosphate; Glycerone Phosphate
Histidine metabolism	0.0010647	0.0087836	0,13776	Anserine; Urocanate
Pentose and glucuronate interconversions	0.0031668	0.014929	0,07792	UDP-Glucose
Starch and sucrose metabolism	0.0031668	0.014929	0,011	UDP-Glucose
Ascorbate and aldarate metabolism	0.0031668	0.014929	0	UDP-Glucose
Cysteine and methionine metabolism	0.0037225	0.015355	0,17854	Cysteate; Cysteine; Cystathionine; Ophtalmate
Retinol metabolism	0.01835	0.060991	0,21649	Retinoic Acid
Valine, leucine and isoleucine degradation	0.02033	0.060991	0	Acetoacetic Acid
Tyrosine metabolism	0.02033	0.060991	0	Acetoacetic Acid
Synthesis and degradation of ketone bodies	0.029918	0.075947	0,6	Acetoacetic Acid; 3-Hydroxybutirrate
Butanoate metabolism	0.029918	0.075947	0,11111	Acetoacetic Acid; 3-Hydroxybutirrate

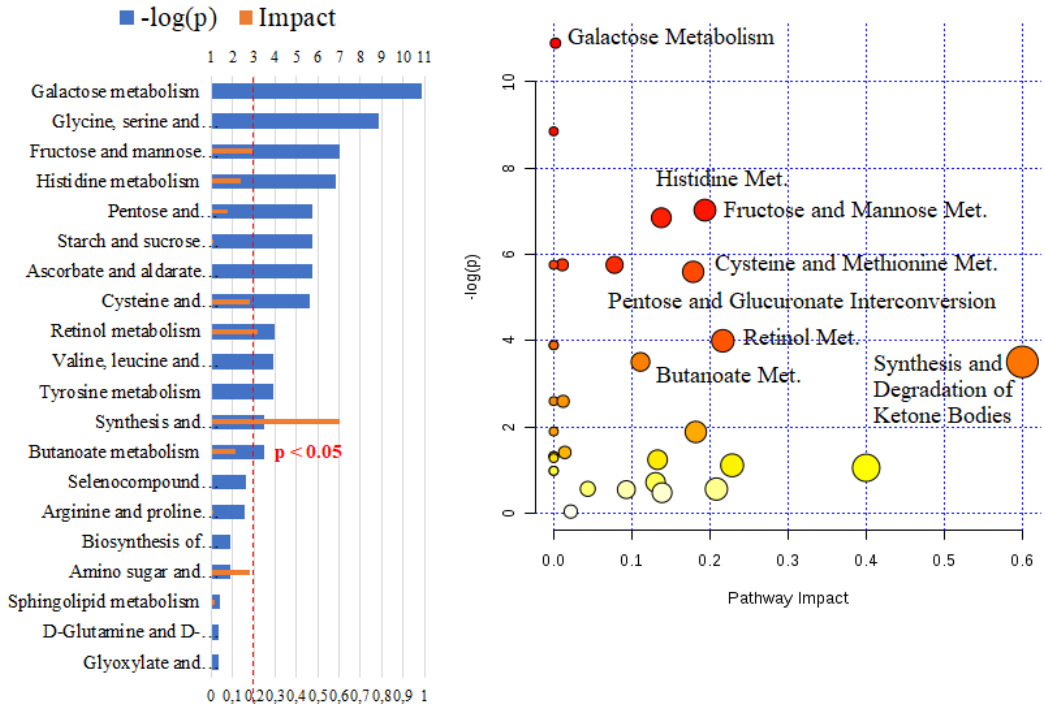

Figure 27. Pathway Analysis of C vs BPA+P
These graphics plotted the impact of the metabolites found in the pathway analized with the logaritm of the p-value. In the lesft graph, the dotted red line represents the cut-off of the p-value. In the right graph the color of the dots represents the statistically significance, where red is the most significant and the white is the least significant; the diameter of the dots represents the impact of the metabolites, where the largest has the biggest impact and the smallest has the least impact.

Pathways analyzed in the BPA+P group are shown in **Figures 11 – 14** and **Figures 28 and 29**, then described in the discussion.

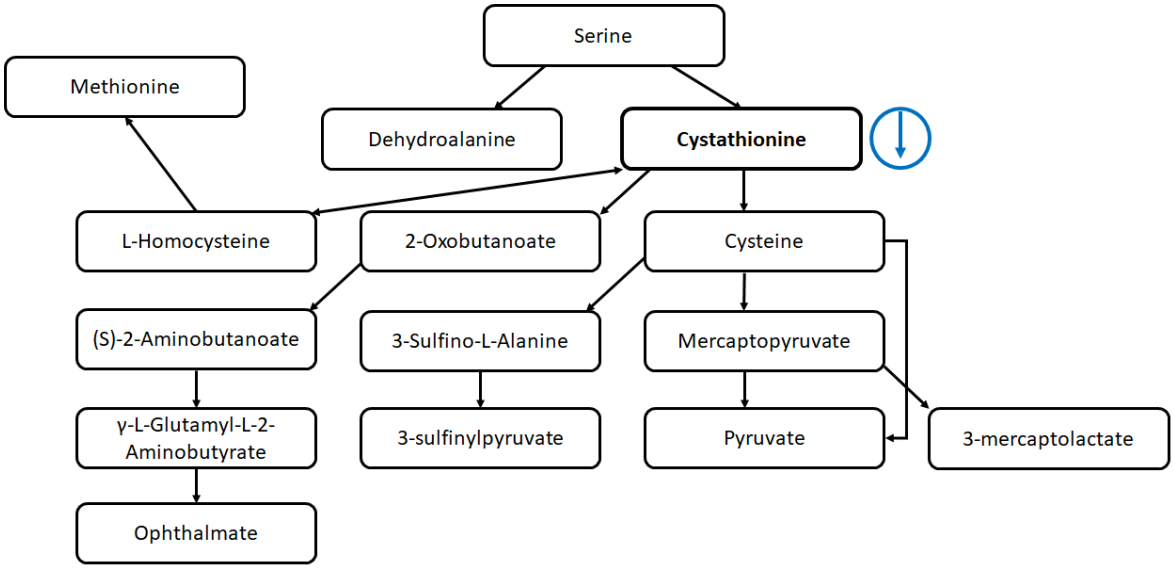

Figure 28. Cysteine and Methionine Metabolism.
In this pathway there is a significant decrease of Cystathionine levels (p-value and FDR < 0.05; blue arrow pointing down).

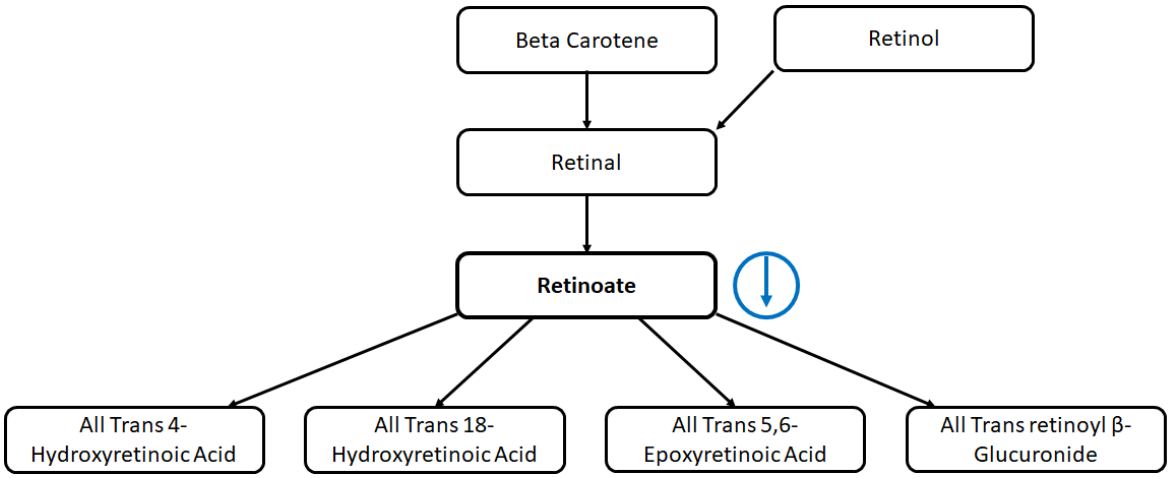

Figure 29. Retinol Metabolism.
In this pathway there is a decrease in the levels of Retinoic Acid (p-value < 0.05; blue arrow pointing down).

3.1.6 Comparison of BPA+P with BPA and P groups

To better assess differences between BPA and BPA+P groups, a T-TEST and a Heatmap were performed. **Table 9** show the T-Test results, while the T-Test and Heatmap graphs are reported in **Figures 30** and **31**.

Table 9 .T-TEST Metabolites of BPA vs BPA+P

Name	p.value	FDR
L-Cystathionine	2,56E+00	0.010992
O-Phosphoethanolamine	0.0012085	0.025983
Glutaric acid	0.0065745	0.086828
Hypoxanthine	0.008077	0.086828
Anserine	0.013078	0.11247
Retinoic acid	0.020296	0.12521
L-Cystine	0.02218	0.12521
N-methyl-L-glutamic Acid	0.023294	0.12521
Guanosine	0.040494	0.17975
Petroselinic acid	0.041803	0.17975

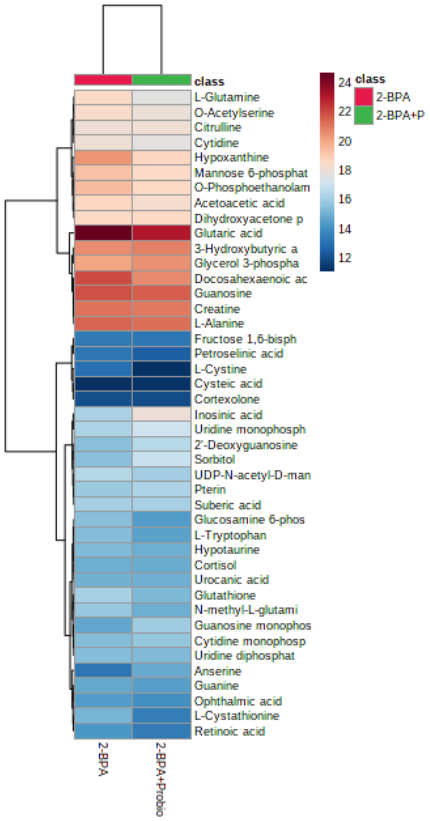

Figure 30. Heatmap of BPA vs BPA+P

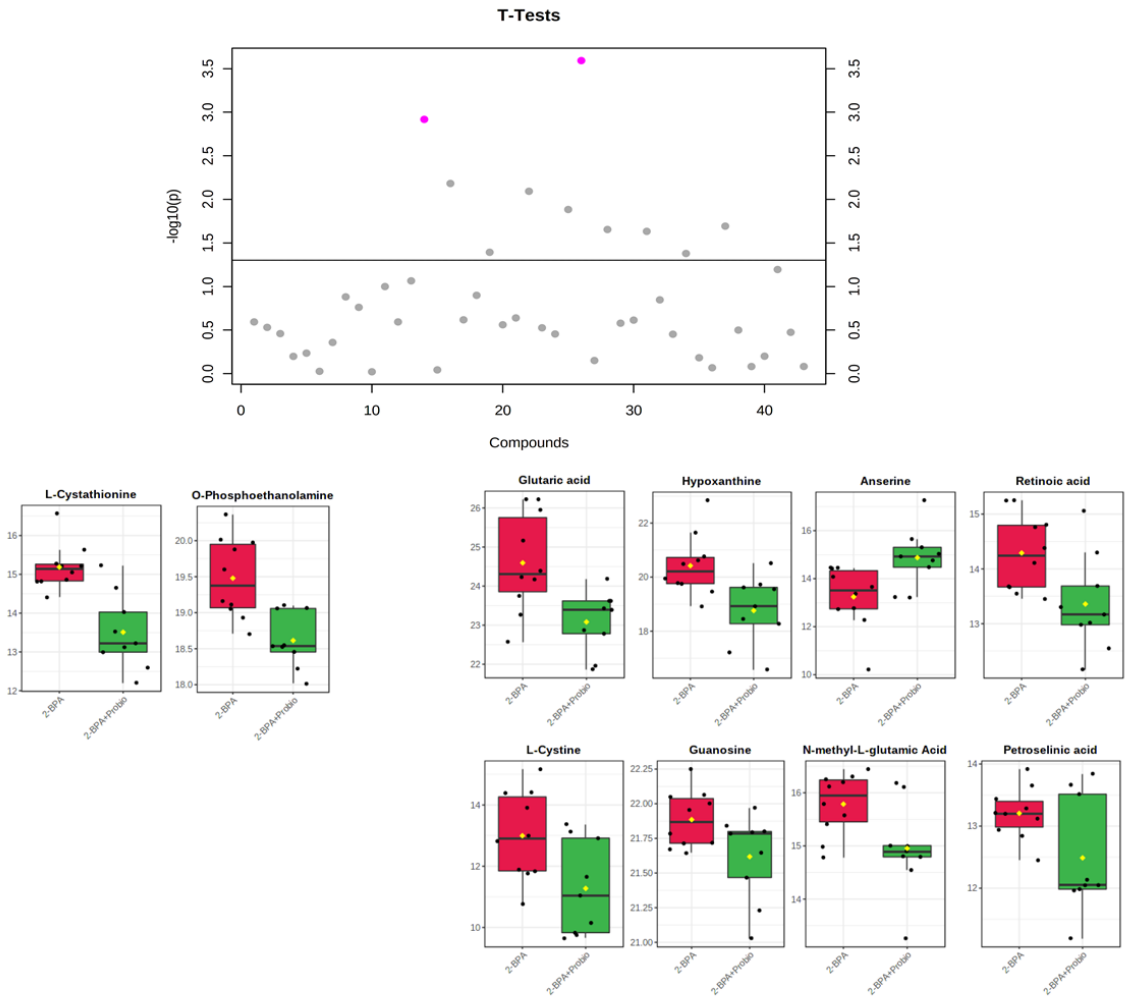

Figure 31. T-TEST of BPA vs BPA+P

In the upper figure the pink dots show the statistically significant changes (p-value and FDR < 0.05). In the figures below the metabolites changes are visible. The left figure show the metabolites with p-value and FDR < 0.05; in the right figure the metabolites with p-value < 0.05 but FDR > 0.05 are shown.

The T-Test show a significant decrease in the levels of Cystathionine and O-Phosphoethanolamine in the BPA+P group compared to BPA. Also, Glutaric Acid, Hypoxanthine, Retinoic Acid, Cystine, Guanosine, N-Methyl-L-Glutamic Acid and Petrosenilic Acid showed a decrease, while Anserine showed an increased level.

The BPA+P and P comparison is shown in **Figure 32**.

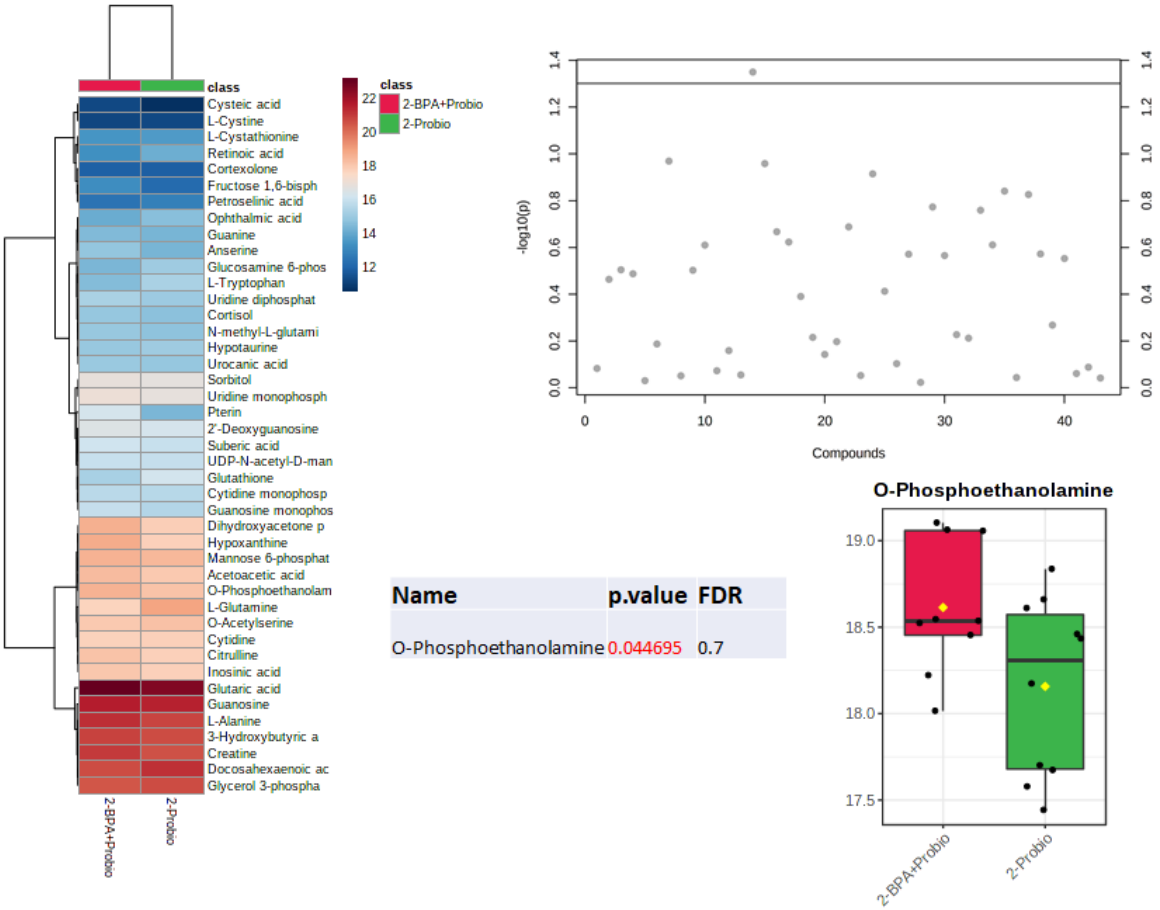

Figure 32.
Heatmap and T-TEST Analysis of BPA+Probiotic (red) vs Probiotic (green) comparison. There is a decreased levels of O-Phosphoethanolamine with p-value < 0.05.

The T-Test found only one change between the two group in the O-Phosphoethanolamine level.

3.1.7 Pathway Analysis Comparison

Pathway Analysis comparison was performed to visualize the differences between pathways in the treated groups. **Table 10** shows the comparison.

Table 10. Pathway Analysis Comparison.
Pathway Analysis of C compared to treatments and treatments compared to each other are shown. Colors found in different comparison mean shared pathways, colors found only in one comparison means unique pathways

C vs BPA			
Pathway Name	**p**	**FDR**	**Impact**
Galactose metabolism	3,25E-01	0.0010724	0,00228
Selenocompound metabolism	0.0016031	0.017905	0
Arginine and proline metabolism	0.0025268	0.017905	0,01206
Pentose and glucuronate interconversions	0.0032555	0.017905	0,07792
Starch and sucrose metabolism	0.0032555	0.017905	0,011
Ascorbate and aldarate metabolism	0.0032555	0.017905	0
Fructose and mannose metabolism	0.0038853	0.018317	0,19359
Biosynthesis of unsaturated fatty acids	0.005008	0.020658	0
Glycine, serine and threonine metabolism	0.0076061	0.024015	0
Valine, leucine and isoleucine degradation	0.0080048	0.024015	0
Tyrosine metabolism	0.0080048	0.024015	0
Synthesis and degradation of ketone bodies	0.010045	0.025499	0,6
Butanoate metabolism	0.010045	0.025499	0,11111
Amino sugar and nucleotide sugar metabolism	0.015882	0.037435	0,1819

P vs BPA+P			
Pathway Name	**p**	**FDR**	**Impact**
Sphingolipid metabolism	0.044695	0.48011	0.0142

C vs P			
Pathway Name	**p**	**FDR**	**Impact**
Galactose metabolism	3,39E-01	0.0011183	0,00228
Fructose and mannose metabolism	3,02E+00	0.0049894	0,19359
Glycine, serine and threonine metabolism	5,21E+00	0.0057349	0
Cysteine and methionine metabolism	0.0077859	0.064234	0,17854
Sphingolipid metabolism	0.014856	0.098053	0,0142
Histidine metabolism	0.032125	0.12479	0,13776
Pentose and glucuronate interconversions	0.034033	0.12479	0,07792
Starch and sucrose metabolism	0.034033	0.12479	0,011
Ascorbate and aldarate metabolism	0.034033	0.12479	0

BPA vs BPA+P			
Pathway Name	**p**	**FDR**	**Impact**
Glycine, serine and threonine metabolism	2,93E+00	0.0099765	0.0
Cysteine and methionine metabolism	0.0011358	0.013697	0.17854
Sphingolipid metabolism	0.0012085	0.013697	0.0142
Retinol metabolism	0.020296	0.13868	0.21649
Histidine metabolism	0.020394	0.13868	0.13776

C vs BPA+P			
Pathway Name	**p**	**FDR**	**Impact**
Galactose metabolism	1,87E-01	6,19E+00	0,00228
Glycine, serine and threonine metabolism	1,44E+00	0.0023775	0
Fructose and mannose metabolism	8,93E+00	0.0087836	0,19359
Histidine metabolism	0.0010647	0.0087836	0,13776
Pentose and glucuronate interconversions	0.0031668	0.014929	0,07792
Starch and sucrose metabolism	0.0031668	0.014929	0,011
Ascorbate and aldarate metabolism	0.0031668	0.014929	0
Cysteine and methionine metabolism	0.0037225	0.015355	0,17854
Retinol metabolism	0.01835	0.060991	0,21649
Valine, leucine and isoleucine degradation	0.02033	0.060991	0
Tyrosine metabolism	0.02033	0.060991	0
Synthesis and degradation of ketone bodies	0.029918	0.075947	0,6
Butanoate metabolism	0.029918	0.075947	0,11111

BPA vs P			
Pathway Name	**p**	**FDR**	**Impact**
Sphingolipid metabolism	3,16E-01	0.0010733	0.0142
Glycine, serine and threonine metabolism	1,10E+00	0.0018772	0.0
Glycerophospholipid metabolism	0.0013746	0.015579	0.13042
Selenocompound metabolism	0.0051424	0.03898	0.0
Arginine and proline metabolism	0.0057324	0.03898	0.01206
Cysteine and methionine metabolism	0.0071336	0.040424	0.17854
Fructose and mannose metabolism	0.045831	0.22261	0.19359

Pathways highlighted in light blue contain metabolites that do not present a change among treated groups so discussion will not focus on them. BPA+P and P share two pathways, highlighted in green, where Cystathionine and Anserine are present. Moreover, this two pathways and the Retinol Metabolism and Sphingolipid Metabolism pathways differentiate the BPA+P group from the BPA one. Also, in the BPA+P group there is not an interest in the Selenocompound Metabolism, Biosynthesis of Unsaturated Fatty Acids and the Arginine and Proline Metabolism, all pathways that belong to BPA group.

3.2 RT-PCR Transcript Analysis

In this study, the expression of 7 genes was evaluated by Real Time-PCR analysis. These genes are involved in the inflammation process, lipid and glucose metabolism and the regulation of appetite.

The first gene analyzed is *ptgs2a* codifying for the Prostaglandin-Endoperoxide Synthase 2, also known as Cyclooxygenase-2 (COX-2), which is induced by an inflammatory state and is involved in the conversion of Arachidonic Acid in Prostaglandins, an important precursor of Prostacyclin. Expression in male resulted significantly upregulated in BPA fish, as already shown in Seabream (Maradonna et al., 2015), while in the BPA+P group was significantly

downregulated respect to other groups. No statistically significant changes of *ptgs2a* expression were found among female experimental groups (**Figure 33**).

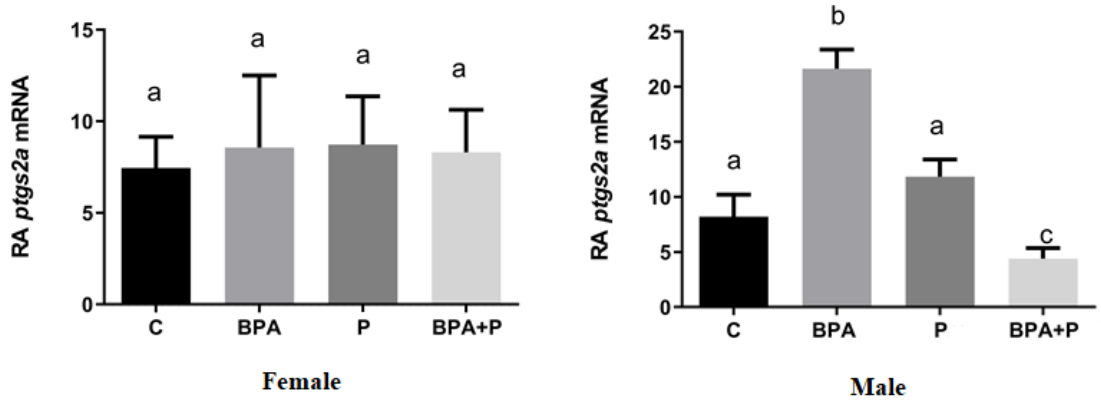

Figure 33. RT-PCR analysis of *ptgs2a* gene in Females (left) and Males (right).
Different letter means a statistically significant change (p-value < 0.05).

The *gpr55bp* and *pparα* genes expression were assessed. The first gene codify for the G Protein-Coupled Receptor 55, a G protein-coupled receptor which has been listed as a new endocannabinoid receptor (Ryberg et al., 2007). The second gene codify for PPAR alpha, that belongs to the class of Peroxisome Proliferator-Activated Receptor and was the first to be discovered in this class (Hong et al., 2019). PPAR alpha is mainly present in the liver and tissues with high lipidic catabolism. This class of receptors use as ligands the dietary and endogenous lipids instead of lipidic and protein hormones, so they can act as endogenous lipids sensor. So, among the ligands for this receptor is possible to

find medium and long chain fatty acids but also eicosanoids and prostaglandins. Also, endocannabinoids can act through PPAR receptors because they derive from Arachidonic Acid and are fatty acids derivatives (O'Sullivan, 2007) PPAR mediates a series of biological effect including:

- Increased beta and omega fatty acids oxidation; (Hong et al., 2019)
- Increased ketogenesis; (Hong et al., 2019)
- Activation of genes involved in lipid metabolism, including transport, lipogenesis, cholesterol metabolism and adipocyte differentiation; (Maradonna and Carnevali, 2018)

Significantly changes were only detected in male BPA group. No statistically significant changes in the expression of these genes were found in females. (**Figure 34**).

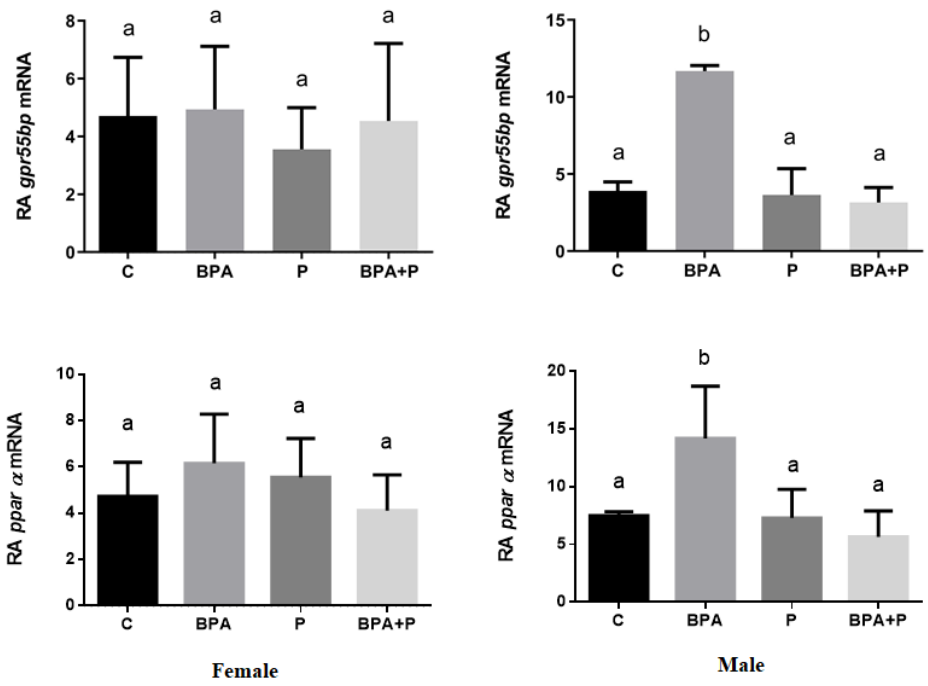

Figure 34. RT-PCR analysis of *gpr55bp* (upper graphs) and *pparα* (lower graphs) in Females (left) and Males (right).
Different letter means a statistically significant change (p-value < 0.05).

cebpα, a gene coding for a trascription factor called CCAAT-Enhancer-Binding Protein alpha that promote the gene expression of downstream adipocytes-related genes (Maradonna and Carnevali, 2018) through the interaction with the CCAAT box motif on the promoter of these genes, recruiting the basal transcription factors. Gene expression was investigated. In females significantly changes of mRNA levels were detected only between BPA and in BPA+P groups. Nevertheless, their expression is not different from that of C fish. In males, both BPA and P groups present an upregulation compared to

BPA+P and C groups, which present similar levels (**Figure 35**).

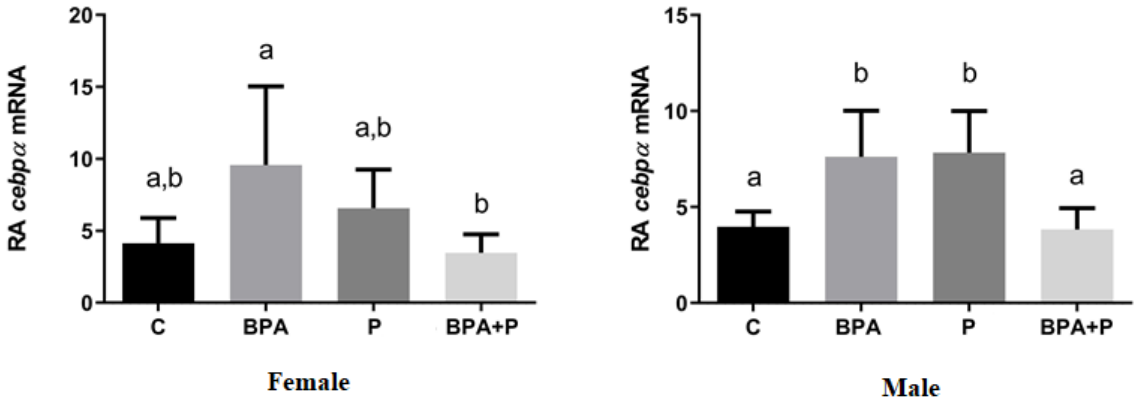

Figure 35. RT-PCR analysis of *cebpa* gene in Females (left) and Males (right).
Different letter means a statistically significant change (p-value < 0.05).

The expression of *gcga*, codifing for a peptide hormone belonging to the family of incretin, that increase the levels of glucose and fatty acids in the bloodstream, was studied. This gene is significantly upregulated in female only by the P treatment, while in males by BPA exposure (**Figure 36**).

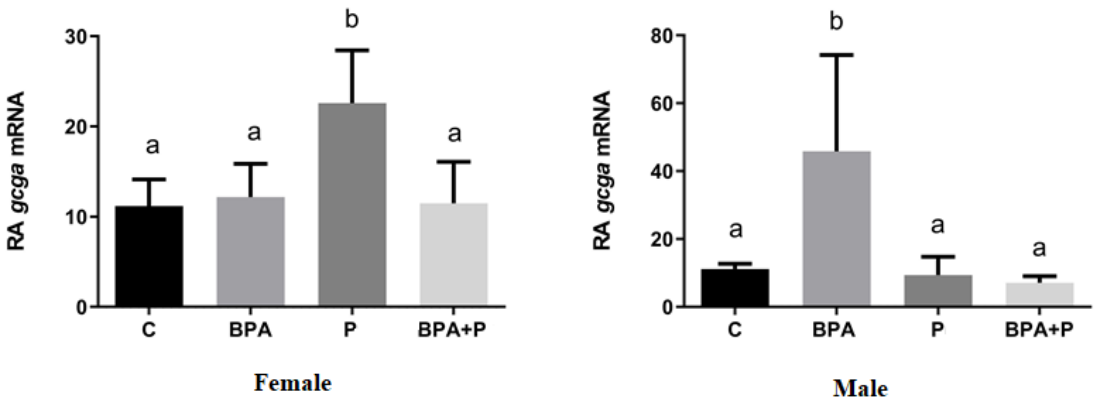

Figure 36. RT-PCR analysis of *gcga* gene in Females (left) and Males (right).
Different letter means a statistically significant change (p-value < 0.05).

Another gene indagated is *mboat4* codifying for Membrane bound O-acyltransferase domain containing 4 (*mboat4*) that catalyzes the binding of the fatty acid Hexanoil-CoA or Octanoyl-CoA to the Serine residue in position 3. This reaction leads to the activation of the Ghrelin hormone that stimulate the appetite. Evidences highlight an important role of ghrelin as a key regulator of glucose metabolism. In females, all experimental groups display a significant downregulation respect to C, while in males, in BPA group a mRNA upregulation was detected. In P group a significant downregulation respect to all group was observed. No changes were observed between BPA+P and C fish (**Figure 37**).

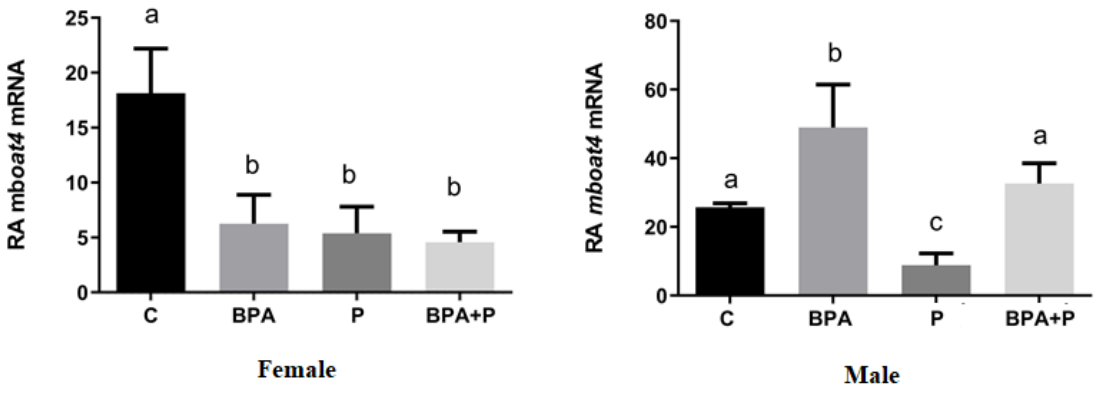

Figure 37. RT-PCR analysis of *mboat4* gene in Females (left) and Males (right).
Different letter means a statistically significant change (p-value < 0.05).

The last gene investigated in this study was *nucb2a* that codify the Nucleobindin protein that is the precursor of Nefastin-1, a protein hormone that reduce the appetite and gives a sense of fullness and also stimulate the use of stored fat with a potential loss of body fat and weight. This gene presents an upregulation in the P group of females compared to other treatments and C, while no statistically significant changes are presents in males (**Figure 38**).

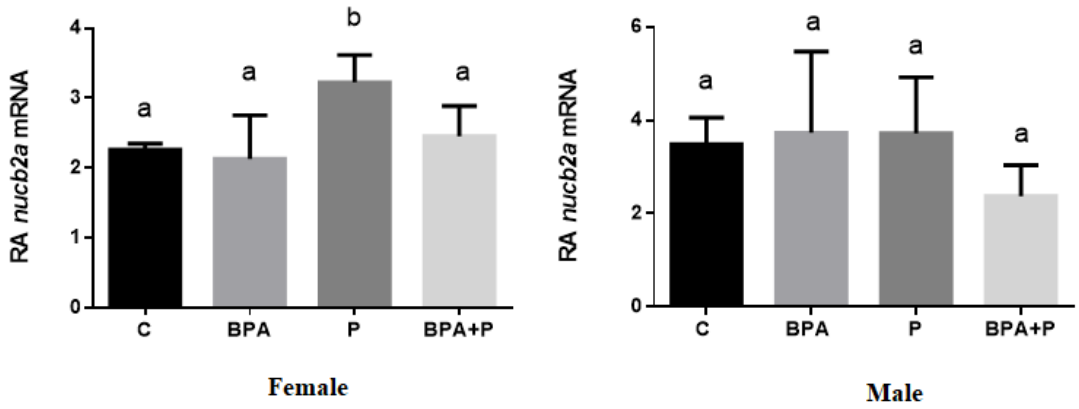

Figure 38. RT-PCR analysis of *nucb2a* gene in Females (left) and Males (right).
Different letter means a statistically significant change (p-value < 0.05).

3.3 Metagenomic Analysis

To analyze the samples, a PCA was first performed and is shown in **Figure 39**. This graph show that the metagenomic profiles of BPA and BPA+P cluster together and overlapped, while C and P group clusters are well separated from each other and from the other groups.

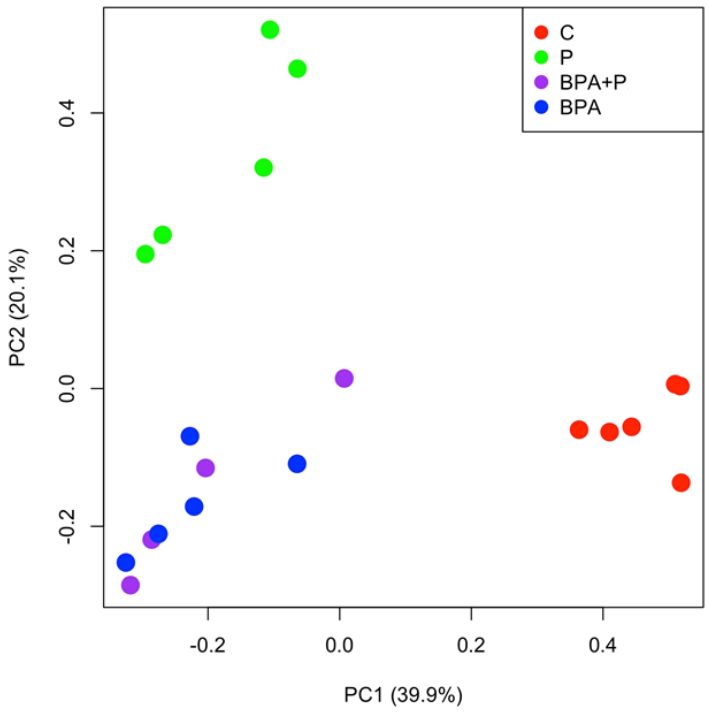

Figure 39. PCA Analysis of all groups.
BPA (blue) and BPA+P (purple) groups cluster together and overlapped.

This is also shown in **Figure 40**, where a cluster analysis is provided.

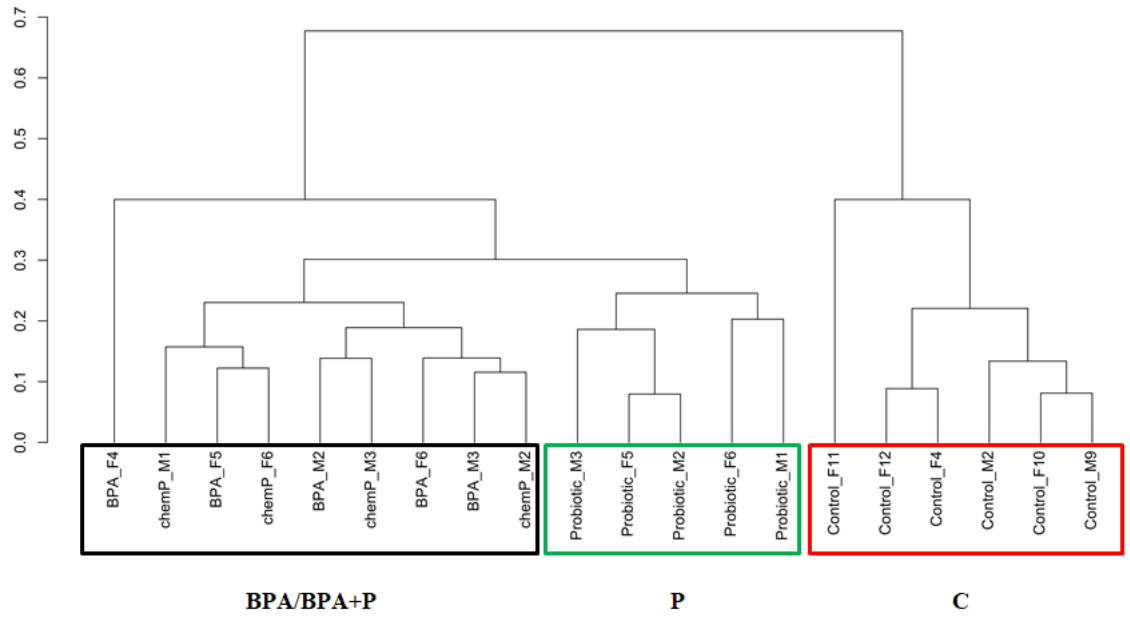

Figure 40. Cluster Analysis of all groups.
The cluster analysis show that BPA and BPA+P groups cluster together.

Alpha Diversity shows the mean species diversity per site or habitat at a local scale and is shown in **Figure 41**. The alpha diversity was performed with the observed diversity, that uses only the species richness counting the number of different species presents and with the Shannon Index and the Inv Simpson Index that both take also into account the number of units presents for each species. This analysis found that only C presents statistically significant differences respect to other groups. In particular, the observed diversity show that BPA and P groups differ from C while BPA+P does not show differences respect to all other groups.

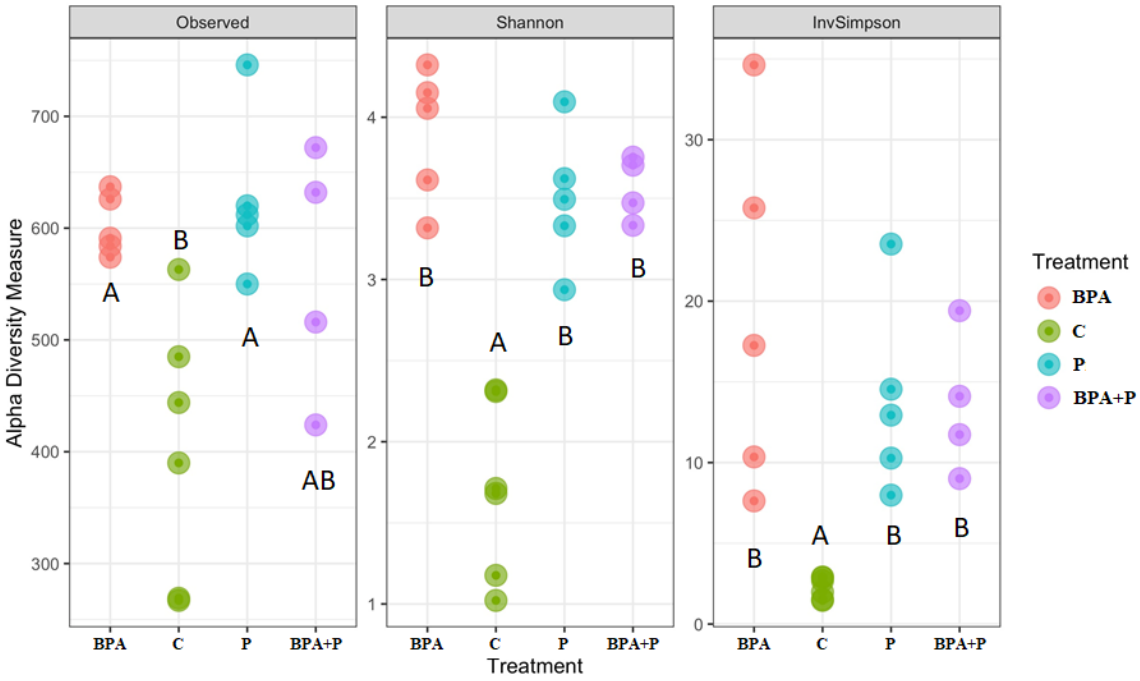

Figure 41. Alpha Diversity Analysis.
This analysis was performed using the Observed Diversity, the Shannon Index and the InvSimpson Index. Different letters means statistically significant changes, while same letters means no changes between groups.

The Beta Diversity shows the ratio between the species diversity at regional and local levels and is provided in **Figure 42**. This analysis confirms the difference between C and all the other groups and also show a difference between the P group and the BPA and BPA+P groups.

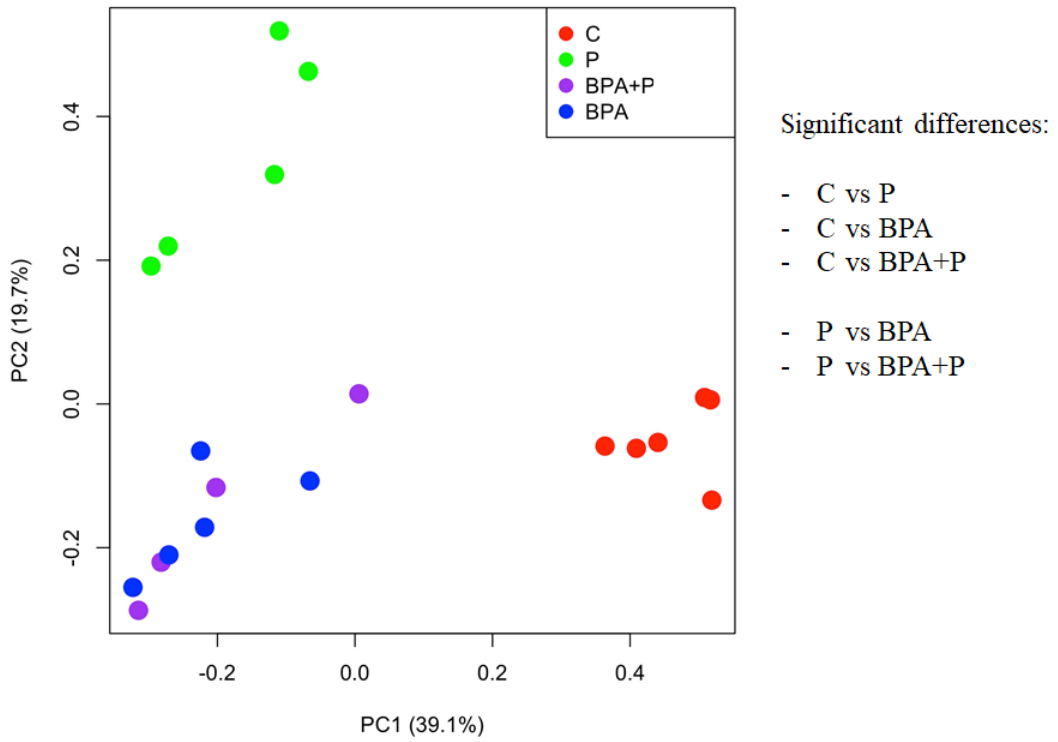

Figure 42. Beta Diversity Analysis.
C group (red) show significant changes compared with all the other groups, P group (green) show changes compared to BPA (blue) and BPA+P (purple) groups.

Microbial Abundances was assessed at phylum, class and order levels. Main differences among groups were evident when analyzing the results focusing on differences of orders (**Figure 43 - Table 11**).

In control fish, the most abundant order is represented by Enterobacteriales. Similar abundances of Flavobacteriales, Pseudomondales and Fusobacteriales are present. This pattern is very different from that of treated groups, which share many similarities among them. The decrease of Enterobacteriales is associated to the increase of Pseudomonadales in BPA and BPA+P groups,

which results the most abundant order. In probiotic group, to the decrease of Enterobacteriales corresponds an increase of Pseudomonadales and Fusobacteriales. In BPA group also Chlamydiales and Pirellulares, which are almost undetected in the other guops are present. BPA presents also significant presence of Sphingomonadales, which lack in Control fish (**Figure 43**).

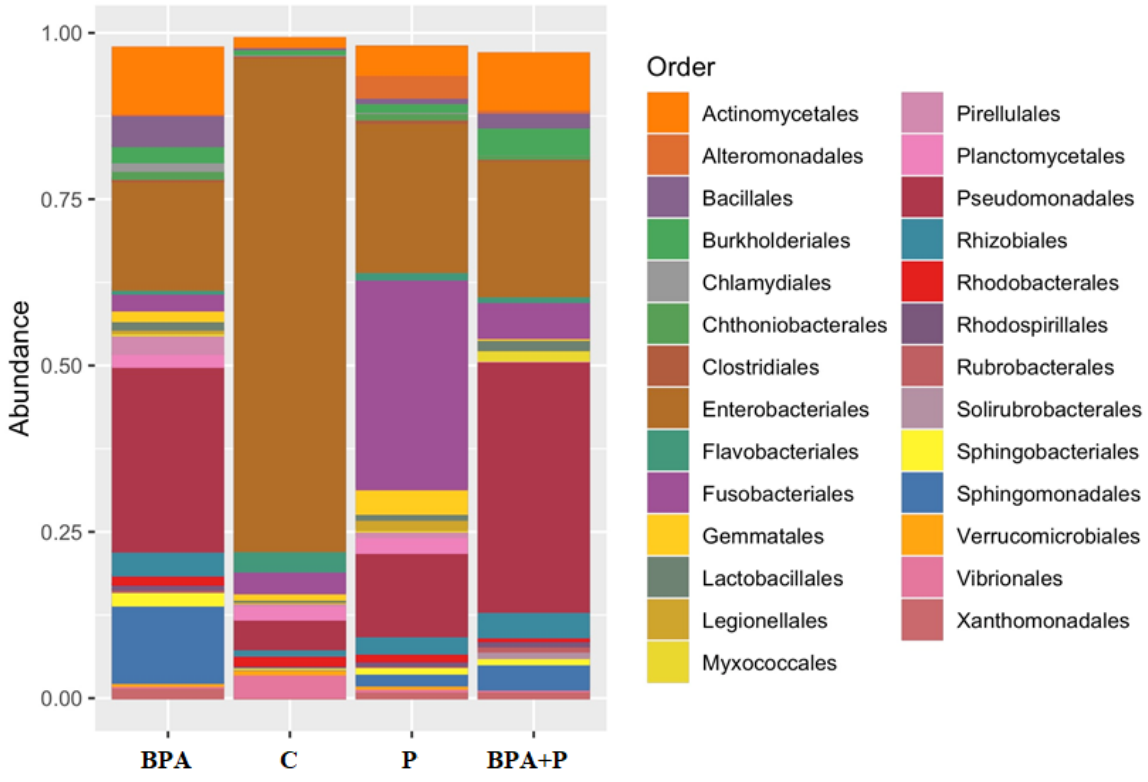

Figure 43. Microbial Abundances - Order.
At order level, P group shows an increased abundance of Fusobacteriales and a reduced abundance of Pseudomonadales, while C group shows a big abundance of Enterobacteriales.

Table 11. Microbial Abundance - Order.

CONDITION	Order	LOG2FC	FDR
C - P	Enterobacteriales	3.6	6.17x10-11
	Bifidobacteriales	23.7	1.76x10-7
	Coriobacteriales	23.6	1.76x10-7
	Gemellales	21.1	3.67x10-7
	Rubrobacterales	-7.4	1.62x10-3
	Fusobacteriales	-2.8	0.01
	Alteromonadales	-3.9	0.01
C - BPA+P	Enterobacteriales	4.5	1.27x10-14
	Roseiflexales	24.7	1.08x10-13
	Bdellovibrionales	21.7	7.28x10-7
	Coriobacteriales	24.1	1.06x10-6
	Bifidobacteriales	23.7	1.26x10-6
	Rubrobacterales	-9.8	2.96x10-5
	Solirubrobacterales	-9.3	9.89x10-4
	Pirellulales	6.1	2.76x10-3
	Rhodobacterales	4.2	3.18x10-3
	Gemmatimonadales	-9.7	3.39x10-3
	Planctomycetales	5.2	4.05x10-3
	Fusobacteriales	2.7	0.01
	Clostridiales	3.9	0.02
	Chlamydiales	5	0.04
	Sphingomonadales	-2.7	0.04
C - BPA	Enterobacteriales	4.5	6.96x10-18
	Coriobacteriales	23.6	1.40x10-7
	Bifidobacteriales	23.1	2.05x10-7
	Anaerolineales	-22.4	3.88x10-7
	Sphingomonadales	-4.6	7.08x10-5
	Oceanospirillales	-12.9	4.00x10-4
	Rubrobacterales	-6.9	2.13x10-3
	Flavobacteriales	3.2	0.01
	Chthoniobacterales	-5.3	0.02
	Fusobacteriales	2.3	0.02
P - BPA+P	Roseiflexales	24.6	1.72x10-13
	Gemellales	-24.6	3.87x10-8
	Bdellovibrionales	23.8	3.87x10-8
	Fusobacteriales	5.5	8.84x10-8
	Pirellulales	7.1	5.47x10-4
	Legionellales	5.4	0.01
	Planctomycetales	5.5	0.03
P - BPA	Anaerolineales	-28.2	1.85x10-9
	Fusobacteriales	5.1	3.09x10-7
	Alteromonadales	5.7	1.63x10-4
	Gemellales	-16.5	4.80x10-4
	Oceanospirillales	-10.4	0.02
BPA+P - BPA	Anaerolineales	-28	3.12x10-8
	Roseiflexales	-19.6	3.12x10-8
	Bdellovibrionales	-20.9	4.15x10-6
	Pirellulales	-8.8	1.17x10-5
	Chlamydiales	-6.9	7.88x10-3
	Solirubrobacterales	7.9	8.38x10-3
	Oceanospirillales	-9.9	0.04

Comparison between two experimental groups are presented, showing the differences on order composition (**Table 11**). A Linear Discriminant Analysis Effect Size (LEfSa) was assessed at pathway levels to assess differences between groups. The LEfSa analysis is shown in **Figure 44**.

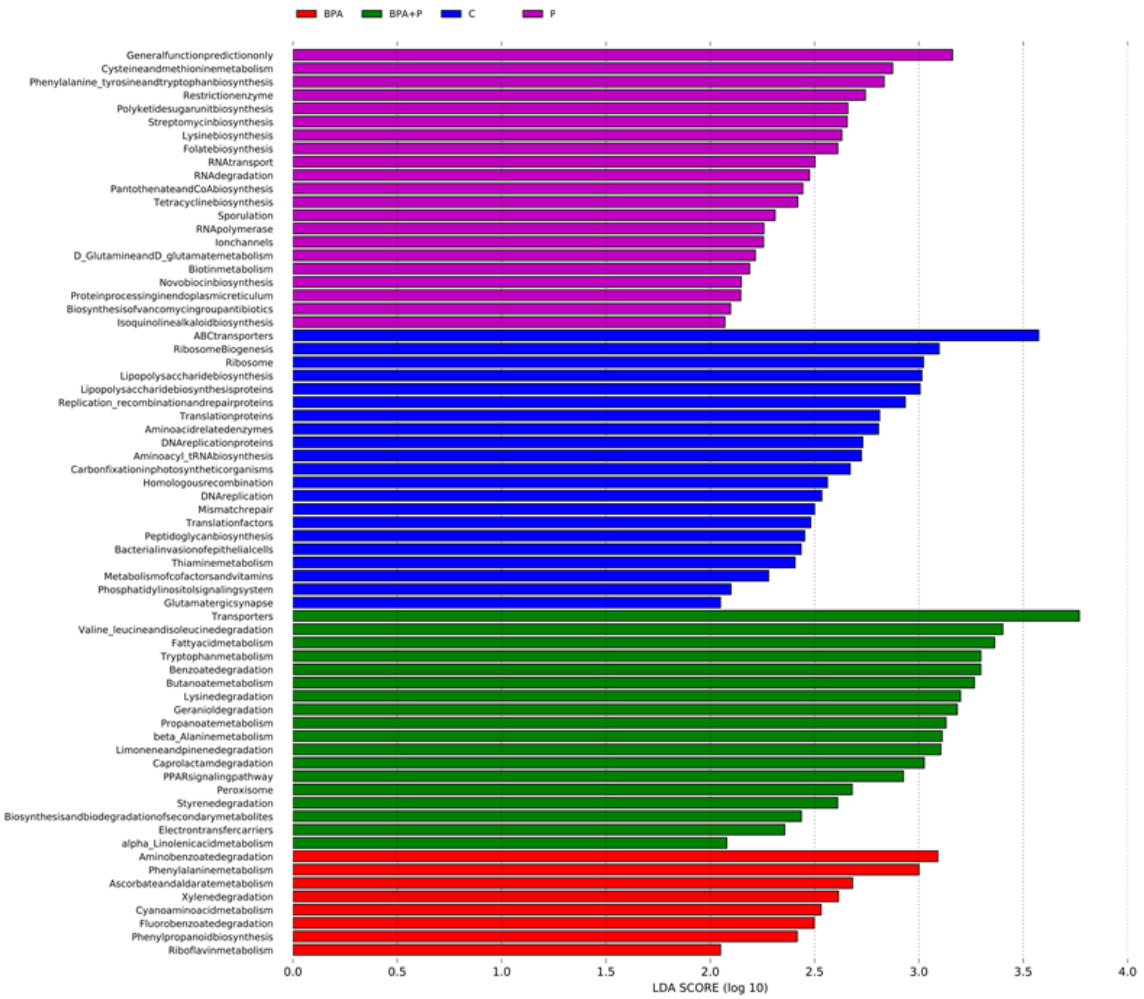

Figure 44. Linear Discriminant Analysis Effect Size - Pathway.
This analysis shows the features, pathways in this case, that allow to distinguish the different groups.

DISCUSSION

Despite several metabolites have been found to be differently modulated by treatments respect to control, only those changes which are characteristic of a specific treatment, will be discussed and not those commonly modified in all experimental groups respect to C, probably caused by changes in fish habits. Among metabolites significantly modulated by BPA treatment is Acetoacetic Acid, which presents an impact of 0 in the Valine, Leucine and Isoleucine Degradation and the Tyrosine Metabolism pathways, providing information that with BPA treatment, Acetoacetic Acid is produced as result of the metabolic catabolism of amino acids. Amino acids in the liver can be used, after deamination, for gluconeogenesis, production of Citric Acid Cycle intermediates or for production of ketone bodies.

More specifically, Tyrosine and Isoleucine participate in gluconeogenesis and ketogenesis. In relation to ketogenesis, from these amino acids carbon backbone, derives Acetyl-CoA or Aceto-acetyl-CoA that are both used for Acetoacetic Acid production. In the present study the picture that emerges from the analysis of pathways in which this metabolite is involved makes speculate on an augmented amino acids degradation in BPA group, resulting in increasing production of ketone bodies that can be used as energy source in starving

condition or in case of high energy demand (McCue, 2010). In our case we can speculate that BPA, acting as stressor, can induce an alert condition similar to those activating in case of starving and thus activating the pathways involving Acetoacetic acid.

Acetoacetic acid is also related to the hepatic production of main ketone bodies. Ketone bodies are water soluble transport form of acetyls. They are produced when excessive acetyl-CoA is present as in case of hepatic β-oxidation, i.e. the liver pre-arranges fatty acids and provides ketone bodies as an alternative source of energy (McCue, 2010). Considering our result, we can speculate that the increase of acetoacetic acid as consequence of acetyl-CoA increase, derives from the upregulation of lipid β oxidation, a process that was previously observed to occur in case of BPA exposure (Wang et al., 2018) concomitant with the enhancement of PPAR α which results upregulated in liver.

Ketogenesis is commonly evident in the diabetes mellitus where there is a strong ketoacidosis, so the augmented production of ketone bodies could be also seen as a sign of this disease (Preeti and Sushil, 2016).

Creatine, also known as α-Methylguanidino Acetic Acid, results among molecules upregulated by BPA treatment and is synthetized in the liver from the Guanidinoacetate which in turn is produced in the kidney from the amino acids Arginine and Glycine and using the amino acid Methionine as methyl

group donor. Creatine supplementation increases homocysteine production in liver, diminishing fat accumulation and resulting in beneficial effects in fatty liver and non-alcoholic liver disease, suggesting that the organism tries to respond to the toxic effect of BPA.

The involvement of Glycine, Serine and Threonine Metabolism and Arginine and Proline Metabolism pathways show that Creatine is synthetized from Glycine and Arginine and then Creatine could be released in the bloodstream and used as primary energy source in skeletal muscle after the conversion in Phospho-Creatine that is usually used in the regeneration of ATP from the ADP proving that in fish exposed to BPA the amino acids are used for energy generation through their degradation. In addition, different studies have shown that creatine supplementation could supply brain energy, presenting neuroprotective effects against those pathologies associated to acute liver failure (Barcelos et al., 2016).

Docosahexanoic-acid, a fatty acid belonging to the omega-3 class, has a central role in the pathway involved in the Biosynthesis of Unsaturated Fatty Acids. Among the PUFA, docosahexaenoic acid (DHA, C22:6*n*-3), eicosapentaenoic acid (EPA, C20:5*n*-3), and arachidonic acid (ARA, C20:4*n*-6), also collectively known as highly unsaturated fatty acids (HUFA) have been shown to play pivotal role in different physiological processes (Vargas et al., 2018) from

reproduction (Tahara and Yano., 2004; Izquierdo et al., 2001) to immune response (Wischhusen et al., 2020) and lipid metabolism (Garrido et al., 2020). The involvement of the Biosynthesis of Unsaturated Fatty Acids pathway suggests an increased biosynthesis of highly unsaturated fatty acids biosynthesis caused by BPA, potentially to supply the increased production/request of ketone bodies above discussed.

The metabolic changes so far described are involved in the degradation of amino acids and fatty acid synthesis, and similar changes, mainly of Acetoacetic Acid and Creatine, have been reported in a study where zebrafish were treated with ethyl alcohol inducing Alcoholic Fatty Liver (Jang et al., 2012).

In addition, O-Phosphoethanolamine is involved in the Sphingolipid Metabolism and is a precursor of Phosphatidylethanolamine. Despite in BPA group, this metabolite increase seems not to have a central role in any metabolic Pathway, the increased bioavailability of this metabolite can be used in the production of Phosphatidylethanolamine which is reported at high levels in patient with Non-Alcoholic Fatty Liver Disease (NAFLD) and Non-Alcoholic Steatohepatitis (NASH), presenting a decrease in the Phosphatidylcholine/Phosphatidylethanolamine ratio in the liver (Van der Veen et al., 2017). To confirm this hypothesis, in this group there is also a

statistically significant up regulation of Cysteine, Guanosine and Guanine; Cysteine is used as a precursor for Cysteine-Triphosphate (CTP) that is used in the Phosphatidylethanolamine production, while Guanine and Guanosine are involved, in the form of Guanosine-Triphosphate (GTP) in the activation of the Phospholipases C and A2 that breaks down the Phosphatidylcholine and so, these metabolites can be involved in the decrease of the Phosphatidylcholine/Phosphatidylethanolamine ratio. From the biological side, Sphingolipids are essential components of the cell structure, such as cellular membranes, and play a role in cellular signaling and cellular immune processes and inflammation (Bartke and Hannun, 2009; Maceyka and Spiegel, 2014). New perspectives on the biological functions of sphingolipids have revealed their roles as regulators of inflammation (Bartke and Hannun, 2009). Pathogens, oxidative stress, and cytokines are the key stimuli that affect sphingolipid biosynthesis and metabolism (Hannun and Obeid, 2008). Recently, several BPA studies have demonstrated the potential role of BPA as a disruptor of the immune response (Kristensen et al., 2011; Xu et al., 2015; Cho 2018), and let us speculate that also in this study the immune system can be alerted by the treatment.

Considering the Selenocompound Metabolism, a raise in the Alanine level in BPA exposed fish can be associated to an augmented level of Alanine

Transaminase (ALT) that produce Alanine and is an index of liver damage. An increased level of this enzyme is found in a study on the BPA hepatotoxicity in rats (Khan et al., 2015) and in a study where human urinary BPA levels were associated with NAFLD (Kim et al., 2019). An elevated concentration of ALT is also found in association with the decrease of Phosphatidylcholine/Phosphatidylethanolamine ratio in the liver (Van der Veen et al., 2017).

Therefore, the metabolic profile of BPA group let speculate on a possibly steatosis and liver injury state, predisposing to the development of the metabolic syndrome that is characterized by obesity and diabetes (Kim et al., 2018). Nevertheless, hepatic steatosis has been clearly demonstrated in previous trials preformed in this lab, exposing zebrafish to the same BPA concentration (Forner-Piquer et al., 2018; Martella et al., 2016).

In the P treatment, Anserine, a metabolite involved in the Histidine Metabolism pathway, synthetized from Carnosine through the Carnosine N-Methyl Transferase enzyme, is present. Anserine, N-β-Alanyl-3-Methyl-L-Histidine, is therefore a methylated Carnosine (β-Alanyl-L-Histidine) analog. Histidine containing dipeptides, can act as direct antioxidants through peroxide radicals and singlet oxygen scavenging or as indirect antioxidants through a chelating

action in which they inactivate the pro-oxidant effects of the transition metals (Wu, 2020). They also act as scavenger of carbon reactive species produced from glucose and lipids oxidation. Their integration into the diet has been seen to improve the prognosis and reducing the probability to develop the metabolic syndrome (Wu, 2020). Moreover, differently from other antioxidants that acts as pro-oxidants and/or produce toxic metabolites, the histidine containing dipeptides produce non-toxic metabolites that are safe at high doses, supporting the positive role of probiotics in their synthesis and in modulation of the immune response and suggesting the use of this probiotic formula as dietary nutritional supplement. A positive role of probiotic formulations on the immune system was observed in zebrafish (Gioacchini et al., 2017) where its potential to regulate immune cell functioning, decreasing detrimental effects and consequent inflammatory events, was clearly demonstrated and thus suggesting its possible role to counter act the negative effect of BPA or other endocrine disruptor exposure.

Cystathionine and ophthalmic acid are both involved in the Cysteine and Methionine Metabolism. Cystathionine is synthetized from Serine and Homocysteine through the enzyme Cystathionine-β-Synthase and is break through the action of the Cystathionine-γ-Lyase enzyme producing Cysteine and 2-Oxobutanoate, so Cystathionine can be used as Cysteine donor used for

the Glutathione production. Glutathione is a tripeptide formed by Glycine, Glutamate and Cysteine that have strong antioxidant power at cellular level. The metabolite Ophthalmic Acid is a tripeptide Glutathione analog in which there is a L-2-Aminobutyrate instead of Cysteine. This metabolite production follows the Glutathione consumption so it can be used as a biomarker for Glutathione depletion, representing a clear biomarker of oxidative stress (Soga et al., 2016). In this study, the lack of glutathione consumption suggest that ophthalmic acid is not involved in the increase of oxidative stress but is only related to the increase of amino acid catabolism or its involvement in the gluconeogenesis instead of in the production of Glutathione (Kobayashi et al., 2017; Teruya et al., 2019). A fasting state is promoted by probiotic formulation use (Sivamaruthi et al., 2019) and is therefore investigated and will further discussed considering transcriptomic results.

As observed in BPA exposure, Sphingolipid Metabolism pathway is also affected by probiotic treatment, showing a decrease of the metabolite O-Phospoethanolamine. As previously discussed, high level of this metabolite can lead to the increase of Phosphatidylethanolamine that is involved in NAFLD and NASH diseases. Then, the fact that in the P group there is a decreased level of this metabolite let speculate on a lower bioavailability for the

Phosphatidylethanolamine production and its administration can have a role in counteracting the development of the NAFLD and NASH conditions.

The metabolic profile of P treatment shows that the Slab51 probiotic formulation stimulated the cellular antioxidant power in a positive way through the increase of metabolites like Anserine and Ophthalmic Acid. This probiotic formulation can also stimulate the reduction of O-Phosphoethanolamine opening the possible use of this supplement in reducing the probability of NAFLD and NASH development and improving their prognosis.

In the BPA+P treatment, pathways containing the Acetoacetic Acid metabolite (Tyrosine Metabolism and the Valine, Leucine and Isoleucine Degradation, Synthesis and Degradation of Ketone Bodies and the Butanoate Metabolism) are affected. Despite this is a common point between the BPA and the BPA+P groups, differently from the BPA group, the co-treated one does not show an increase in the Creatine, Docosahexaenoic Acid and O-Phosphoethanolamine levels, suggesting that lipid metabolism and the energy state of BPA+P fish is more closely to C group than to BPA. Moreover, the presence of Anserine, previously discussed also about the P group, confirms the antioxidant role of the probiotic formulation and its ability to counteract the BPA toxic effect. From the Pathway Analysis is possible to see that, as for the P group, in BPA+P

group is present the Cysteine and Methionine Metabolism, pathway where there is a decrease in the Cystathionine level but, unlike the P group, there is not an increased level of Ophthalmic Acid. This let speculate that, in BPA+P group, similarly to P group, the Cysteine is used in the gluconeogenesis pathway, but the 2-Oxobutanoate product from Cystathionine, in equimolar quantities compared to those of Cysteine, in this group are not processed until the production of L-2-Aminobutyrate. So, we can imagine that the process triggers the oxidative decarboxylation of 2-Oxobutanoate that produce Propionyl-CoA. This occurs since in the co-treatment with BPA, there is not a true fasting state, as supported by molecular results concerning *mboat4* and *nucb2a*, but only an amino acids degradation which leads to the production of ketone bodies. Indeed, the 2-Oxobutanoate is converted through amination in L-2-Aminobutyrate only in the fasting state (Kobayashi et al., 2017) The Propionyl-CoA then, can enter the Citric Acid Cycle or the gluconeogenesis.

A last pathway found in the BPA+P group is the Retinol Metabolism, where a depletion of Retinoic Acid, an All-Trans-Retinoic Acid produced from Vitamin A_1 (Retinol) and that mediates the functions of Retinol, is present. It has been found that the BPA elimination process is Retinol-dependent (Shmarakov et al., 2017). So, decreased level of this metabolite only in the BPA+P group

compared to the BPA group let speculate on a possible role of probiotic in the higher elimination of the BPA from the organism.

Summarizing, the probiotic formula SLAB51 favors the elimination of the contaminant and enhances the hepatic antioxidant power suggesting its ability in counteracting the metabolic negative effects of BPA.

Moving to transcriptomic analysis, the study was carried on considering possible gender differences.

Among gene analyzed, the upregulation of *ptgs2a* gene only in the BPA group agrees with metabolomic results of BPA group that well correlate with the increase of Docosahexaenoic Acid. This metabolite is a fatty acid acting as substrate for the Prostaglandin-Endoperoxide Synthase 2 a, that finally produces eicosanoids, resolvins and the neuroprotectin D1, molecules involved in regulation of cellular functions important for the anti-inflammatory response (Gdula-Argasińka et al., 2016; Yee-Ki Lee et al., 2010). These analyses let speculate that in the BPA group, the *ptgs2a* raise is due to the presence of inflammation and to an increased liver fatty acids production. In the BPA+P group instead, there is a depletion of *ptgs2a* and there is not a significant change

in the Docosahexaenoic Acid level confirming previous results obtained in *Dicentrarchus labrax* larvae (Abelli et al., 2009). Therefore, we can suggest that this probiotic formulation is responsible for the decrease of inflammation and fat liver.

Focusing on *gpr55bp* gene, a study conducted on Human and animal models, showed that the *gpr55bp* level are increased in obese subjects which have NASH, NAFLD and Non-Alcoholic Fatty Liver (NAFL) (Fondevila et al., 2020). Both *gpr55bp* and *pparα* are involved in the up regulation of hepatic lipids production and in the present study, high levels of these transcripts in the BPA group of male zebrafish respect to C evidencing a clear sex specificity in the way the contaminant can induce these genes production. Similar results were previously described in different experimental models (Maradonna et al., 2015; O'Sullivan, 2007). As already reported in a previous work (Forner-Piquer et al., 2018), female does not show changes in the expression of the *gpr55bp* expression. These data are in agreement with those found in the BPA Metabolomic Analysis where the raise in the levels of Acetoacetic Acid, Docosahexaenoic Acid, Creatine and O-Phosphoethanolamine are indicators of the increased hepatic fat and of the consequent higher probability of hepatic steatosis development supporting previous results in different models showing a fat liver in fish exposed to BPA (Forner et al., 2018; Martella et al., 2016).

In the present study, the expression trend of *cebpα* gene shows an increase in BPA and P group suggesting a role in the hepatic lipid pattern. BPA treatment was previously observed to cause *cebpα* upregulation when administrated to perinatal rats (Somm et al., 2009). The augmented expression of this transcription factor, that in turn enhances the expression of adipogenesis genes, in BPA group is in line with others metabolomic and transcriptomic results previously discussed.

Regarding Glucagon A, the increase observed in male zebrafish exposed to BPA, can be due to the presence of Acetoacetic Acid, which is part of the short chain fatty acids (SCFA) family, and that can stimulate its production by activating the G-protein-coupled receptor. This hypothesis is confirmed in BPA males by the up regulation of the *gpr55bp* gene expression. Glucagon level was observed to be upregulated by prenatal exposure in mouse (Whitehead et al., 2016) and this increase is possibly correlated with an impairment in the ratio of α:β pancreatic cells, which causes a pancreatic morphology alteration. Conversely, in female exposed to P, the upregulation of this gene may suggest a beneficial effect of this probiotic formulation on the glucose metabolism (Falcinelli et al., 2016; Kim et al., 2018) letting us hypothesize an anti-diabetes effect.

Ghrelin plasma levels were upregulated in a BPA exposure on *Microtus agrestis* while Leptin was downregulated showing an appetite stimulation (Nieminem et al., 2002). In the present study, an up regulation of *mboat4* gene expression in the male BPA group and a concomitant down regulation in P group together with no changes compared to C in the BPA+P group, let speculate on the possibility that in the BPA group, where accumulation fatty acids occur, the production of this enzyme is enhanced and then there is a greater activation of Ghrelin with a consequent increase of appetite. In the P group instead, this enzyme expression is downregulated, as previously reported in zebrafish and also in rats and goldfish (Falcinelli et al., 2016; Carreras et al., 2018; Hosseini et al., 2016), letting speculates on a decrease in the appetite stimulation and a lower food intake. This is also shown in the co-treated group, where the levels of this gene expression return to the control situation.

nucb2a gene upregulation only in female of the P group, confirm previous study on zebrafish larvae (Falcinelli et al., 2016). So, after the probiotic administration, there is a downregulation of *mboat4* and an up regulation of *nucb2a* in the probiotic feed fish and this let speculate on the possibility that this probiotic formulation, as others, has anorexigenic effects down regulating the appetite and the food intake. This data confirms that this formulation can be used in the treatment of subjects affected by metabolic syndrome.

Recently was also demonstrated the ability of BPA to alter gut microbiota, our preliminary metagenomic results, such as PCA and Hierarchical Cluster Analysis show no differences between the BPA and BPA+P groups. Microbial differences ascribable to treatments, are evident at order level, specifically in the C gut, high abundance of *Enterobacteriales* that belong to the *Gamma-proteobacteria* class was observed, the exposure to BPA or feed with P, a decrease of this bacterial facilitated the enrichment of others bacteria e.g. in P group increase of *Fusobacteriales* was recorded. The highly presence of this strain, registered only in few samples belonging to the other experimental groups, let imagine that this strain could be tightly associated to probiotic treatment. Despite most of fusobacteria presents negative effects, being responsible of a wide variety of infections, the OTU analysis revealed that 99% of them belong to the specie *Cetobacterium*, which commonly occurs in intestinal tracts of freshwater fish such as *Oreochromis niloticus* (Tsuchiya et al., 2008) and *Cyprinus carpio* (Van Kessel et al., 2011. Interestingly, *Cetobacterium* has been observed in high relative abundance (72–94%) in *Lepomis macrochirus*, *Micropterus salmoides*, and *Ictalurus punctatus (*Larsen et al., 2014. It has been reported that *Cetobacterium* isolated from the intestine of freshwater fish produces vitamin B-12 (Tsuchiya et al.,2008). The importance of this genus in the production of vitamin B-12 in

fish was revealed in a study of microbiota of the Japanese eel, ayu, carp, tilapia, goldfish, and catfish — where specifically, carp and tilapia did not require dietary vitamin B-12 supplement since they showed high levels of *Cetobacterium* namely, *Bacteroides* type A) and vitamin B-12 in their intestinal content (Sugita et al., 1991). These results strongly suggest the ability of this probiotic strain to favor the colonization by B12 producing bacteria and could be used in case of vitamin B-12 deficiencies.

Moving to BPA treatment, an augmented presence of *Chlamydiales*, an order that contain pathogen bacteria, is visible. The BPA and BPA+P groups show an increase of *Pseudomonadales* compared to P and C that show similar abundances. The order *Pseudomonadales* contain species that can act as opportunistic pathogens, like some species of *Pseudomonas*, *Moraxella* and *Acinetobacter*. There are evidences that *Pseudomonas* can biodegrade BPA suggesting a possible activation of the organism to counteract BPA toxicity (Matsumura et al., 2015). At order level is also present an increase of *Sphingomonadales* in BPA group while BPA+P and P groups show similar abundances to each other and lower levels compared to BPA. This order contains species that can degrades organic compounds and are used in the environmental remediation to decrease the pollution (Villemur et al., 2013;

Matsumura et al., 2015). So, an increased colonization of this order in the BPA group can highlight the necessity of bacteria that allow the degradation and elimination of the contaminant.

The LEfSa analysis show the metabolic features that most characterize the different treatments. In the P group, the gut microbial metabolism is mainly focused in the production of antimicrobial and antibiotics compounds as it is shown by the presence of Polyketide sugar biosynthesis, Streptomycin biosynthesis, Tetracycline biosynthesis, Novobiocin biosynthesis and Biosynthesis of Vancomycin group antibiotics pathways. All these antibiotics are used in the treatment of infections caused by pathogens bacteria. Usually the bacterial antibiotics production is used as mechanism to control the composition and the abundance of the microbial community. Possibly the probiotic produces a major richness in the microbiota composition and the bacterial interaction can trigger the production of antibiotics by which the microbial community are regulated and controlled (Plaza-Diaz et al., 2019). Other pathways found in the P group are the biosynthesis of amino acids with the Phenylalanine, Tyrosine and Tryptophan biosynthesis and Lysine biosynthesis pathways in which the first is formed by the shikimate pathway that is involved in the production of folate and aromatic amino acids, the second produces the amino acid lysine that is a precursor of the Carnitine, metabolite

used in the complete degradation of fatty acids that need to be linked to carnitine to cross the mitochondrial membrane. Aside the amino acids production, in the P group there is a presence of group B vitamins production with the involvement of Folate biosynthesis, previously reported in *Lactobacillus plantarum* (Rossi et al., 2011), Pantothenate and CoA biosynthesis and Biotin biosynthesis pathways that leads to the production of B_9, B_5 and B_8 vitamins production respectively. Group B vitamin production was already reported and reviewed for probiotics (LeBlanc et al., 2011). The group B vitamin represents a class of compound important for growth, development and cellular metabolism. All these pathways are mechanisms by which this probiotic formulation positively act on the intestine microbial metabolism

In the BPA group, the LEfSa found pathways involved in the degradation of aromatic compounds that could derives from the BPA degradation, such as Aminobenzoate degradation and Fluorobenzoate degradation both involved in the degradation of benzoic acid forms that are produced in the catabolism of phenols compounds. This confirm the role of *Pseudomonadales* and *Sphingomonadales* orders in biodegrading BPA and also other EDCs, let us speculate on the possibility that BPA, because of its use as nutrient by these bacteria, induces a colonization of bacteria able to enhance its elimination. In

this group is also found activation of the Xylene degradation pathway that is involved in the production of propanoyl-CoA that can both enter the Citric Acid Cycle or the Gluconeogenesis. *Pseudomonas* was observed to degrade xylene and was isolated from this compound as sole carbon and energy source (Davey and Gibson, 1974). Aside this, there is the Phenylalanine metabolism and the Riboflavin metabolism pathways where these metabolites are catabolized.

The BPA+P group present changes in pathways like Tryptophan metabolism, Lysine degradation and Beta-alanine metabolism, in the Fatty acids metabolism and in the Propanoate metabolism where there is a presence of acetoacetic acid that derives from the Styrene metabolism. The Propanoate metabolism is involved in the production of fatty acids from the amino acid catabolism. So, the BPA+P microbial features confirm what was found in the metabolomic analysis were Cystathionine is processed only till the Propanoyl-CoA, the CoA conjugated form of propanoate, without leading to the production of Ophthalmic Acid. Aside these pathways and like in the BPA group, the Benzoate metabolism is present in the BPA+P group, probably used for the BPA degradation by *Pseudomonadales*.

CONCLUSION

In conclusion, using an “omic” approach, the present study provides evidences on the ability of the SLAB51 a mix of probiotic to counteract BPA toxicity. As it is possible to see from the metabolomic results and the discussion of this study, SLAB51 probiotic formulation produces a metabolic shift, in zebrafish liver metabolism, enhancing the antioxidant power of the organism. From the metabolomic point of view, when fish are co-exposed to BPA and probiotics, their metabolic profile results closer to P than to BPA, suggesting the ability of beneficial bacteria to counteract the contaminant effects at hepatic level. Transcriptomics revealed that this formula reduces the appetite exerting an anorexigenic power, counteracting the effects of BPA which instead induces appetite and lipid accumulation. Moreover, the probiotic mix counteract the BPA inflammatory activity when co-administered together with the contaminant. The metagenomic profile shows that in groups receiving SLAB51 enrichment of bacteria producing antibiotics and bacteriocins, amino acids and group B vitamins occurs. This means that this probiotic formula exerts a control of the microbial composition and also produces a positive effect on the gut environment, making it effective as a supplement in the treatment of dysbiosis and group B vitamins deficiencies. From the metagenomic profile of BPA

exposure is possible to see that the contaminant induces a colonization of bacteria able to accelerate BPA degradation and elimination process.

These data provide evidences of the SLAB51 probiotic effectiveness as “therapy” to reduce the toxic effects linked to BPA exposure and opens new perspectives towards the use of this formulation to counteract the toxic effects of environmental contaminants.

BIBLIOGRAPHY

- Abelli L, Randelli E, Carnevali O, Picchietti S, (2009), Stimulation of gut immune system by early administration of probiotic strains in Dicentrarchus labrax and Sparus aurata. Ann N Y Acad Sci. 1163:340-2.

- Abràmoff MD, Magalhães PJ, Ram SJ, (2004), Image processing with ImageJ. Biophotonics international. 11:36–42.

- Allard P, (2014), Bisphenol A. Biomarkers in Toxicology. 459-474.

- Barcelos RP, Stefanello ST, Mauriz, Gonzalez-Gallego J, Soares FAA, (2016), Creatine and the Liver: Metabolism and Possible Interactions, Mini Rev Med Chem. 16(1):12-8.

- Bartke N,Hannun YA, (2009), Bioactive Sphingolipids: Metabolism and Function. J Lipid Res. 50:S91-6.

- Beyer J, Petersen K, Song Y, Ruus A, Grung M, Bakke T, Tollefsen KE, (2014), Environmental risk assessment of combined effects in aquatic ecotoxicology: A discussion paper. Marine Environmental Research. 96:81-91.

- Bonfili L, Cecarini V, Berardi S, Scarpona S, Suchodolski JS, Nasuti C, Fiorini D, Boarelli MC, Rossi G, Eleuteri AM, (2017), Microbiota Modulation

Counteracts Alzheimer's Disease Progression Influencing Neuronal Proteolysis and Gut Hormones Plasma Levels. Sci Rep. 7(1):2426.

• Bonfili L, Cecarini V, Cuccioloni M, Angeletti M, Berardi S, Scarpona S, Rossi G, Eleuteri AM, (2018), SLAB51 Probiotic Formulation Activates SIRT1 Pathway Promoting Antioxidant and Neuroprotective Effects in an AD Mouse Model. Mol Neurobiology. 55(10):7987-8000.

• Brockmeier EK, Hodges G, Hutchinson TH, Butler E, Hecker M, Tollefsen KE, Garcia-Reyero N, Kille P, Becker D, Chipman K, Colbourne J, Collette TW, Cossins A, Cronin M, Graystock P, Gutsell S, Knapen D, Katsiadaki I, Lange A, Marshall S, Owen SF, Perkins EJ, Plaistow S, Schroeder A, Taylor D, Viant M, Ankley G, Falciani F, (2017), The Role of Omics in the Application of Adverse Outcome Pathways for Chemical Risk Assessment. Toxicological Sciences. 158(2):252-262.

• Calafat AM, Ye X, Wong L-Y, Reidy JA, Needham LL (2008), Exposure of the U.S. Population to Bisphenol A and 4-tertiary-Octylphenol: 2003–2004. EnvironmentalHealthPerspectives 116, 39–44.

• Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R, (2010), PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics. 26, 266–267.

• Caporaso, JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R, (2010), QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7:335–336.

• Carreras NL, Martorell P, Chenoll E, Genovés S, Ramón D, Aleixandre A, (2018), Anti-obesity Properties of the Strain Bifidobacterium Animalis Subsp. Lactis CECT 8145 in Zücker Fatty Rats. Beneficial Microbes. 9(4): 629-641.

• Chapman TM, Plosker GL, Figgitt DP, (2006), VSL#3Probiotic Mixture; A Review of its Use in Chronic Inflammatory Bowel Diseases. Drugs. 66:1371–1387.

• Cho S, Khan A, HA Jee S, Lee HS, Hwang MS, Koo YE, Park YH, (2018), High resolution metabolomics to determines the risk associated with bisphenol A exposure in humans. Environmental Toxicology and Pharmacology. 58:1-10.

• Cousins I, Staples C,Klečka G, Mackay D (2002), A multimedia assessment of the environmental fate of bisphenol A. Human and Ecological Risk Assessment 8, 1107–1135.

• Davey JF, Gibson DT, (1974), Bacterial metabolism of para- and meta-xylene: oxidation of a methyl substituent. J Bacteriol. 119(3):923-9.

• Desantis S, Mastrodonato M, Accogli G, Rossi G, Crovace AM, (2019), Effects of a Probiotic on the Morphology and Mucin Composition of Pig Intestine. Histology Histopathology. 34(9):1037-1050.

• DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL, (2006), Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microb. 72:5069–5072.

• Dettmer K, Aronov PA, Hammock BD, (2007), Mass spectrometry-based metabolomics. Mass Spectrom Rev. 26 (1):51–78.

• Edgar RC, (2010), Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 26:2460–2461.

• Europe Commission, (13 December 2016), Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards bisphenol A.

• Europe Commission, 31 May 2011), Bisphenol A: EU ban on baby bottles to enter into force tomorrow.

• Falcinelli S, Picchietti S, Rodiles A, Cossignani L, Merrifield DL, Taddei AR, Maradonna F, Olivotto I, Gioacchini G, Carnevali O, (2015), Lactobacillus rhamnosus lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. Scientific Reports. 5:9336.

• Falcinelli S, Rodiles A, Unniappan A, Picchietti S, GioacchiniG, Merrifield DL, Carnevali O, (2016), Probiotic treatment reduces appetite and glucose level in the zebrafish model. Scientific Reports. 6:18061.

• Fondevila MF, Fernandez U, Gonzalez-Rellan MJ, Lima NDS, Buque X, Gonzalez-Rodriguez A, Alonso C, Iruarrizaga-Lejarreta M, Delgado TC, Varela-Rey M, Senra A, Garcia-Outeiral V, Novoa E, Iglesias C, Porteiro B, Beiroa D, Folgueira C, Tojo M, Torres JL, Hernández-Cosido L, Blanco Ó, Arab JP, Barrera F, Guallar D, Fidalgo M, López M, Dieguez C, Marcos M, Martinez-Chantar ML, Arrese M, Garcia-Monzon C, Mato JM, Aspichueta P, Nogueiras R, (2020), The L-α-lysophosphatidylinositol/GPR55 system induces the development of non-alcoholic steatosis and steatohepatitis. Hepatology.

• Forner-Piquer I, Santangeli S, Maradonna F, Verde R, Piscitelli F, di Marzo V, Habibi HR, Carnevali O, (2018). Role of Bisphenol A on the

Endocannabinoid System at central and peripheral levels: Effects on adult female zebrafish. Chemosphere. 205:118-125.

• Garrido D, Monroig Ó, Galindo A, Marrero M, Rodríguez C, (2020), Lipid metabolism in Tinca tinca and its n-3 LC-PUFA biosynthesis capacity. Aquaculture. 523:735147.

• Gdula-Argasińka J, Bystrowska B, (2016), Docosahexaenoic Acid Attenuates in Endocannabinoid Synthesis in RAW 264.7 Macrophages Activated With Benzo(a)pyrene and Lypopolysaccharide. Toxicology Letters. 258:93-100.

• Geens T, Neels H, Covaci A (2012), Distribution of bisphenol-A, triclosan and n-nonylphenol in human adipose tissue, liver and brain. Chemosphere. 87:796–802.

• Gioacchini G, Giorgini E, Merrifield DL, Hardiman G, Borini A, Vaccari L, Carnevali O, (2012), Probiotics can induce follicle maturation competence: The Danio rerio case. Biology of Reproduction. 86(3):65, 1-11.

• Gioacchini G, Giorgini E, Olivotto I, Maradonna F, Merrifield DL, Carnevali O, (2014), The Influence of Probiotics on Zebrafish Danio Rerio Innate Immunity and Hepatic Stress. Zebrafish. 11(2).

• Gioacchini g, Maradonna F, Lombardo F, Bizzaro D, Olivotto I, Carnevali O, (2010), Increase of fecundity by probiotic administration in zebrafish. Reproduction. 140:953-959.

• Gioacchini G, Rossi G, Carnevali O, (2017), Host-probiotic interaction: new insight into the role of the endocannabinoid system by in vivo and ex vivo approaches. Scientific Reports. 7:1261.

• Guideline for the treatment of animals in behavioural research and teaching. Animal Behaviour. 53(1)229-234.

• Guoyao Wu (2020), Important roles of dietary taurine, creatine, carnosine, anserine and 4-hydroxyproline in human nutrition and health. Amino Acids. 52(3):329-360.

• Hamilton-Miller JMT, Gibson GR, Bruck W, (2003), Some insights into the derivation and early uses of the word ‘probiotic’. British Journal of Nutrition. 90:845.

• Hannun YA, Obeid LM, (2008), Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Bio. 9(2):139-50.

• Ho CS, Lam CWK, Chan MHM, Cheung RCK, Law LK, Lit LCW, Ng KF, Suen MWM, Tai HL, (2003), Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications. Clinical Biochemistry Rev. 24.

• Hong F, Pan S, Guo Y, Xu P, Zhai Y, (2019), PPARs as Nuclear Receptors for Nutrient and Energy Metabolism. Molecules. 24(14): 2545.

• Hosseini M, Miandare HK, Hoseinifar SH, Yarahmadi P, (2016), Dietary Lactobacillus acidophilus modulated skin mucus protein profile, immune and appetite genes expression in goldfish (Carassius auratus gibelio). Fish and Shellfish Immunology. 59:149-154.

• Howe K, Clark MD et al., (2013), The zebrafish reference genome sequence and its relationship to the human genome. Nature. 496:498–503.

• Huang X, Cang X, Liu J, (2019), Molecular Mechanism of Bisphenol A on Androgen Receptor Antagonism. Toxicology in Vitro. 61:104621.

• Hwang S, Lim JE, Choi Y, Jee SH, (2018), Bisphenol A exposure and type 2 diabetes mellitus risk: a meta-analysis. BMC Endocrine Disorders. 18:81.

• Izquierdo MS, Fernandez-Palacios H, Tacon AGJ, (2001), Effect of broodstock nutrition on reproductive performance of fish. Aquaculture. 197(1–4):25-42.

• Jang JH, Chung HC, Ahn YG, Know YK, Kim JS, Ryu JH, Ryu DH, Kim CH, Hwang GS, (2012), Metabolic Profiling of an Alcoholic Fatty Liver in Zebrafish (Danio Rerio). Molecular BioSystems. 7.

• Khan S, Beigh S, Chaudhari BP, Sharma S, Abdi SAH, Ahmad S, Ahmad F, Parvez S, Raisuddin S, (2015), Mitochondrial dysfunction induced by Bisphenol A is a factor of its hepatotoxicity in rats. Environmental Toxicology. 31(12).

• Kim D, Touros A, Kim WR, (2018), Nonalcoholic Fatty Liver Disease and Metabolic Syndrome. Clinics in Liver Disease. 22(1)133-140.

• Kim D, Yoo ER, Li AA, Cholankeril G, Tighe SP, Kim W, Harrison SA, Ahmed A, (2019), Elevated urinary bisphenol A levels are associated with non-alcoholic fatty liver disease among adults in the United States. Liver International. 39(7).

• Kim YA, Keogh JB, Clifton PM, (2018), Probiotics, Prebiotics, Synbiotics and Insulin Sensitivity. Nutrition Research Reviews. 31(1).

• Kobayashi S, Lee J, Takao T, Fujiia J, (2017), Increased ophthalmic acid production is supported by amino acid catabolism under fasting conditions in mice. Biochemical and Biophysical Research Communications. 491(3)649-655.

• Kristensen DM, Skalkam ML, Audouze K, Lesné L, Desdoits-Lethimonier C, Frederiksen H, Brunak S, Skakkebæk NE, Jégou B, Hansen JB,

Junker S, Leffers H, (2011), Many Putative Endocrine Disruptors Inhibit Prostaglandin Synthesis. Env Health Perspective. 11(4).

- Larsen A, Mohammed H, Arias C, (2014), Characterization of the gut microbiota of three commercially valuable warmwater fish species. J. Appl. Microbiol. 116:1396–1404.

- LeBlanc JG, Laiño JE, del Valle MJ, Vannini V, van Sinderen D, Taranto MP, de Valdez GF, de Giori GS, Sesma F, (2011), B-group vitamin production by lactic acid bacteria--current knowledge and potential applications. J Appl Microbiol. 111(6):1297-309.

- Legeay S, Faure S, (2017), Is bisphenol A an invironmental obesogen? Foundamental and Clynical Pharmacology. 31:594-609.

- Lu X, Zhao X, Bai C, Zhao C, Lu G, Xu G, (2007), LC-MS-based metabolomics analysis. Journal of Chromatography B. 866:64-76.

- Lu X, Zhao X, Bai C, Zhao C, Lu G, Xu G, (2007), LC-MS-based metabonomics analysis. J Chromatogr B Analyt Technol Biomed Life Sci. 866(1-2):64-76.

- Maceyka M, Spiegel S, (2014), Sphingolipid metabolites in inflammatory disease. Nature. 510(7503):58-67.

• Maradonna F, Carnevali O, (2018), Lipid Metabolism Alteration by Endocrine Disruptors in Animal Models: An Overview. Frontiers in Endocrinology. 654(9).

• Maradonna F, Nozzi V, Dalla Valle L, Traversi I, Gioacchini G, Benato F, Colletti E, Gallo P, Di Marco Pisciottano I, Mita DM, Hardiman G, Mandich A, Carnevali O, (2014), A Developmental Hepatotoxicity Study of Dietary Bisphenol A in Sparus Aurata Juveniles. Comp Biochem Physiol C Toxicol Pharmacol. 166:1-13.

• Maradonna F, Nozzi V, Santangeli S, Traversi I, Gallo P, Fattore E, Mita DG, Mandich A, Carnevali O, (2015), Xenobiotic-Contaminated Diets Affects Hepatic Lipid Metabolism: Implications for Liver Steatosis in Sparus aurata Juveniles. Acquatic Toxicology. 167:257-264.

• Martella A, Silvestri C, Maradonna F, Gioacchini G, Allara M, Radaelli G, Overby DR, Di Marzo V, Carnevali O, (2016) Bisphenol A Induces Fatty Liver by an Endocannabinoid-Mediated Positive Feedback Loop. Endocrinology. 157(5):1751-63.

• Matsumura Y, Hosokawa C, Sasaki-Mori M, Akahira A, Fukunaga K, Ikeuchi T, Oshiman KI, Tsuchido T, (2015), Isolation and Characterization of

Novel bisphenol-A-degrading Bacteria From Soils. Biocontrol Sci. 14(4):161-9.

• McCue MD, (2010), Starvation Physiology: Reviewing the Different Strategies Animals Use to Survive a Common Challenge. Comp Biochem Physiol A Mol Integr Physiol. 156(1):1-18.

• Morelli L, Salari P, (2007), Handbook dei Probiotici. Mediserve Editoria & Formazione.

• Nieminen P, Lindström-Seppä P, Mustonen AM, Mussalo-Rauhamaa H, Kukkonen JVK, (2002), Bisphenol A Affects Endocrine Physiology and Biotrasformation Enzyme Activities of the Field Vole (Microtus agrestis). General and Comparative Endrocrinology. 126(2):183-189.

• Nunez A, Kannan K, Giesy J, Fang J, Clemens L (2001), Effects of bisphenol A on energy balance and accumulation in brown adipose tissue in rats. Chemosphere. 42:917–922.

• O'Sullivan SE (2007), Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. Br J Pharmacol 152:576–82.

• Oishi K, Sato T, Yokoi W, Yoshida Y, Ito M, Sawada H, (2008), Effect of Probiotics, Bifidobacterium Breve and Lactobacillus Casei, on Bisphenol A Exposure in Rats. Bioscience Biotechnology Biochemistry. 72(6):1409-15.

• Plaza-Diaz J, Ruiz-Ojeda FJ, Gil-Campos M, Gil A, (2019), Mechanisms of Action of Probiotics. Advances in Nutrition.10(1):S49–S66.

• Preeti KM, Sushil KJ, (2016), Hyperketonemia and Ketosis Increase the Risk of Complications in Type 1 Diabetes. Free Radic Biol Med. 95:268-77.

• Roeselers G, Mittge EK, Stephens WZ, Parichy DM, Cavanaugh CM, Guillemin K, Rawls JF, (2011), Evidence for a core gut microbiota in the zebrafish. ISME J. 5, 1595–1608.

• Rossi G, Gioacchini G, Pengo G, Suchodolski JS, Jergens AE, Allenspach K, Gavazza A, Scarpona S, Berardi S, Galosi L, Bassotti G, Cerquetella M, (2020), Enterocolic Increase of Cannabinoid Receptor Type 1 and Type 2 and Clinical Improvement After Probiotic Administration in Dogs With Chronic Signs of Colonic Dysmotility Without Mucosal Inflammatory Changes. Neurogastroenterology Motil. 32(1):e13717.

• Rossi G, Jergens A, Cerquetella M, Berardi S, Di Cicco E, Bassotti G, Pengo G, Suchodolski JS, (2018), Effects of a Probiotic (SLAB51™) on Clinical and Histologic Variables and Microbiota of Cats With Chronic constipation/megacolon: A Pilot Study. Benef Microbes. 9(1):101-110.

• Rossi M, Amaretti A, Raimondi S, (2011), Folate Production by Probiotic Bacteria. Nutrients. 3(1):118-34.

• Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ, (2007), The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol. 152(7):1092-101

• Sauer UG, Deferme L, Gribaldo L, Hackermüller J, Tralau T, Ravenzwaay B, Yauk C, Poole A, Tong W, Gant TW, (2017), The challenge of the application of 'omics technologies in chemicals risk assessment: Background and outlook. Regulatory Toxicology and Pharmacology. 91(1):S14-S26.

• SCF. Opinion of the Scientific Committee on Food on BisphenolA. European Commission. Health, Consumer Protection Directorate General. Brussels 2002.

• Segner H, (2009), Zebrafish (Danio rerio) as a model organism for investigating endocrine disruptor. Comparative Biochemistry and Physiology, part C. 149, 187-195.

• Shmarakov IO, Borschovetska VL, Blaner WS, (2017), Hepatic Detoxification of Bisphenol A is Retinoid-Dependent. Toxicological Sciences. 157 (1): 141-155.

• Sivamaruthi BS, Kesika P, Suganthy N, Chaiyasut C, (2019), A Review on Role of Microbiome in Obesity and Antiobesity Properties of Probiotic Supplements. BioMed Research International. Volume 2019.

• Soga T, Baran R, Suematsu M, Ueno Y, Ikeda S, Sakurakawa T, Kakazu Y, Ishikawa T, Robert M, Nishioka T, Tomita M, (2006) Differential Metabolomics Reveals Ophthalmic Acid as an Oxidative Stress Biomarker Indicating Hepatic Glutathione Consumption. J Biol Chem. 281(24):16768-76

• Somm E, Schwitzgebel VM, Toulotte A, Cederroth CR, Combescure C, Nef S, Aubert ML, Hüppi PS, (2009), Perinatal exposure to bisphenol a alters early adipogenesis in the rat. Environ Health Perspect. 117(10):1549-55.

• Spence R, Gerlach G, Lawrence C, Smith C, (2007), The behaviour and ecology of the zebrafish, Danio rerio. Biol. Rev. 83, 13-34.

• Standen BT, Rodiles A, Peggs DL, Davies SJ, Santos GA, Merrifield DL, (2015), Modulation of the intestinal microbiota and morphology of tilapia, Oreochromis niloticus, following the application of a multi-species probiotic. Appl. Microbiol. Biotechnol. 1–15.

• Sugita H, Miyajima C, Deguchi Y, (1991), The vitamin B12-producing ability of the intestinal microflora of freshwater fish. Aquaculture. 92:267–276.

• Tahara D, Yano I, (2004), Maturation-related variations in prostaglandin and fatty acid content of ovary in the kuruma prawn (Marsupenaeus japonicus). Comp Biochem Physiol A.137 (4): 631-637.

• Teruya T, Chaleckis R, Takada J, Yanaguda M, Kondoh H, (2019), Diverse Metabolic Reactions Activated During 58-hr Fasting are Revealed by Non-Targeted Metabolomic Analysis of Human Blood. Scientific Reports. 9:854.

• Thomson BM, Cressey PJ, Shaw IC (2003), Dietary exposure to xenoestrogens in New Zealand. Journal of Environmental Monitoring. 229–235.

• Tsuchiya C, Sakata T, Sugita H, (2008), Novel ecological niche of Cetobacterium somerae, an anaerobic bacterium in the intestinal tracts of freshwater fish. Lett. Appl. Microbiol. 46:43–48.

• Van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL, (2017), The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. Biochimica et Biophysica Acta (BBA) – Biomembranes. 1859(9)B:1558-1572.

• Van Kessel MA, Dutilh BE, Neveling K, Kwint MP, Veltman JA, Flik G, Jetten MS, Klaren PH, Den Camp HJ, (2011), Pyrosequencing of 16s rRNA

gene amplicons to study the microbiota in the gastrointestinal tract of carp (Cyprinus carpio L.). AMB Express. 1:41.

• Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV (2007). Human exposure to bisphenol A (BPA). Reproductive Toxicology. 24:139–177.

• Vargas A, Randazzo B, Riolo P, Truzzi C, Gioacchini G, Giorgini E, Loreto N, Ruschioni S, Zarantoniello M, Antonucci M, Polverini S, Cardinaletti G, Sabbatini S, Tulli F, Olivotto I, (2018), Rearing Zebrafish on Black Soldier Fly (Hermetia illucens): Biometric, Histological, Spectroscopic, Biochemical, and Molecular Implications. Zebrafish. 15(4)404-419.

• Villemur R, Cunha Dos Santos SC, Ouellette J, Juteau P, Lépine F, Déziel E, (2013), Biodegradation of Endocrine Disruptors in Solid-Liquid Two-Phase Partitioning Systems by Enrichment Cultures. Appl Environ Microbiol. 79(15):4701-11.

• Wang M, Rang O, Liu F, Xia W, Li Y, Zhang Y, Lu S, Xu S, (2018), A Systematic Review of Metabolomics Biomarkers for Bisphenol A Exposure. Metabolomics. 4(4):45.

• Wang Q, Garrity GM, Tiedje JM, Cole JR, (2007), Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microb. 73:5261–5267.

• Whitehead R, Guan H, Arany E, Cernea M, Yang K, (2016), Prenatal Exposure to Bisphenol A Alters Mouse Fetal Pancreatic Morphology and Islet Composition. Horm Mol Biol Clin Investig. 25(3):171-9.

• Wischhusen et al, (2020), Oxidative stress and antioxidant response in rainbow trout fry exposed to acute hypoxia is affected by selenium nutrition of parents and during first exogenous feeding. Free Radic Biol Med. 1;155:99-113

• Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, et al. (January 2009), HMDB: a knowledgebase for the human metabolome. Nucleic Acids Research. 37.

• Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, et al., (January 2007), HMDB: The Human Metabolome Database. Nucleic Acids Research. 35.

• Xu H, Yang M, Qiu W, Pan C, Wu M, (2015), The impact of endocrine-disrupting chemicals on oxidative stress and innate immune response in zebrafish embryos. Environmental Toxicology. 32(8).

• Yee-ki Lee C, Sit WH, Fan ST, Man K, Jor IWY, Wong LLY, Wan MLY, Tan-Un KC, Wan JMF, (2010), The cell cycle effects of docosahexaenoic acid on human metastatic hepatocellular carcinoma proliferation. International Journal of Oncology. 991-998.

• Zang L, Ma Y, Huang W, Ling Y, Sun L, Wang X, Zeng A, Dahlgren RA, Wang C, Wang H, (2019), Dietary Lactobacillus Plantarum ST-III Alleviates the Toxic Effects of Triclosan on Zebrafish (Danio Rerio) via Gut Microbiota Modulation. Fish Shellfish Immunology. 84:1157-1169.

SPECIAL THANKS

Vorrei ringraziare la Professoressa Carnevali per avermi permesso di intraprendere questo viaggio meraviglioso insieme a Lei ed al suo staff. Il giorno in cui sono andato a parlarLe per chiederLe di farmi da relatrice, non solo mi ha fatto appassionare al suo lavoro, mi ha anche proposto di poter lavorare a parte della mia Tesi in Canada. Opportunità veramente importante per cui la ringrazio infinitamente. Quel giorno la Professoressa ha riposto in me una fiducia che io ho sempre cercato di ripagare lavorando al meglio e con passione, dandomi un enorme stimolo a migliorami e a fare sempre meglio. Grazie.

Ringrazio la Professoressa Maradonna per la sua grande pazienza, la sua disponibilità in ogni momento e per tutto ciò che mi ha insegnato in laboratorio. Ha reso il mio lavoro ad Ancona veramente piacevole e divertente anche, rendendomi sempre partecipe e facendomi sempre sentire a mio agio. Ha partecipato alla mia crescita personale e lavorativa preparandomi al meglio in tutte le mansioni di un Biologo. Il suo lavoro costante mi ha permesso di sviluppare un background che porterò con me per tutta la vita. Grazie.

Ringrazio il Professor Habibi per avermi permesso di lavorare nel suo laboratorio per sei mesi. Il Professore è sempre stato gentilissimo e accogliente, non mi ha mai fatto mancare nulla, permettendomi di sentirmi a casa anche dall'altra parte del mondo e incoraggiandomi a migliorare giorno dopo giorno. Insieme a lui vorrei ringraziare sua moglie Shahin, una persona veramente squisita e gentile.

Ringrazio anche lo staff del Professore, Claudia Ladisa e Maya Zanardini, per tutto ciò che mi hanno insegnato. Le ringrazio specialmente per l'amicizia che mi hanno dimostrato in tutta la mia permanenza, che mi ha permesso alle volte di dimenticare di essere lontano da casa, rassicurandomi e facendo scorrere più velocemente il tempo.

Un enorme ringraziamento va alla mia fidanzata Elisa Terenzi, che ha veramente avuto una pazienza infinita con me aiutandomi costantemente in tutto il mio percorso universitario, non solo durante gli anni della magistrale ma anche e soprattutto negli anni della triennale. Elisa è sempre stata un pilastro per me, aiutandomi a migliorare nello studio, facendomi guadagnare dei bei voti e permettendomi di esprimere tutto il mio potenziale. Inoltre, mi è stata d'aiuto anche con tutte le questioni burocratiche, in cui io sono abbastanza inetto. Senza di lei infatti, non sarei riuscito in tutto quello che ho conseguito fino ad ora. Nella vita posso puntare ad ogni tipo di successo ma, nulla è e sarà

mai paragonabile al fatto di avere al mio fianco una donna così stupenda. Ogni problema, se affrontato insieme ad Elisa, diventa piccolo e più facile da superare. Averla al mio fianco è il successo più grande della mia vita, e dedico quindi a lei, la mia musa e la mia amata, questa tesi.

Ringrazio poi tutta la mia Famiglia che mi ha sostenuto negli studi credendo in me e permettendomi di studiare la Biologia, la mia più grande passione. Tornando a casa dopo un esame, il loro orgoglio era sempre evidente negli abbracci di mia Madre, nel sorriso di mio Padre e nelle parole di mia Sorella. Seppure io sia diverso da loro in molti aspetti e ciò abbia creato alle volte delle incomprensioni, mi hanno sempre incoraggiato, aiutato, supportato e sopportato con pazienza, permettendomi di arrivare dove sono ora e sarò sempre grato quindi di avere la mia Famiglia dalla mia parte. Vi voglio molto bene.

Ringrazio anche la famiglia Terenzi per avermi fatto da seconda famiglia in tutto il periodo universitario, accogliendomi sempre in casa loro come un figlio. Degli anni universitari rimarrà sempre la frase di incoraggiamento che mi rivolgeva Anna prima di un esame: “Vai Signor Giommi e fagli vedere chi sei!”. Ora è il tuo turno di far vedere chi è la Signora Orciani Anna, presto tornerà tutto come prima.

Un grazie speciale anche ai miei amici, Denis Toccacieli, Riccardo Rupoli, Silvia Bernardini e Roberto Ghilardi, per avermi sempre permesso di staccare dal mondo universitario passando del piacevole e spensierato tempo insieme. Ricorderò per sempre il calore con cui tutti mi avete riaccolto in Italia.