SCHOOL OF MEDICINE

DEPARTMENT OF HEALTH SCIENCES

MASTER'S DEGREE IN MEDICAL BIOTECHNOLOGIES

EXPERIMENTAL THESIS

INVESTIGATING THE EFFECTS OF PREBIOTICS AND POSTBIOTICS ON NEUROTRANSMITTER AND SCFA LEVELS IN RETT SYNDROME PATIENTS

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Contents

1. Summary

Rational of the study: Rett syndrome is a neurological disorder that affects approximately 1:10000 live female births, making it one of the most common developmental and intellectual impairment disorders in females. To date, there is no cure. Current treatment options are centered on symptom relief. Previous studies have shown that patients with Rett syndrome have lower microbiota α-diversity compared to healthy individuals, which affects levels of neurotransmitters, neuromodulators, and short-chain fatty acids. In this study, we investigated the effects of prebiotic and/or postbiotic products on the levels of neurotransmitters, neuromodulators, and short-chain fatty acids in Rett syndrome patients.

Planning of the study: A randomized crossover clinical trial was conducted at the Policlinico Le Scotte (Siena) under the supervision of Prof. Claudio De Felice, with 28 admitted Rett syndrome patients. The patients were treated with Lalbaay® (Alpha-lattoalbumin + inulin + fructo-oligosaccharides + sodium butyrate) and Bizetaflox Pediatrico (Sodium butyrate + zinc oxide). Fecal and plasma samples were collected before and after treatment. Nuerotransmitters and nueromodulators were extracted, identified and quantified using LC-MS techniques, while short-chain fatty acids were extracted, identified and quantified using GC-MS techniques.

Results: The results showed that prebiotics and/or postbiotics can significantly modulate neurotransmitters, neuromodulators, and short-chain fatty acids in Rett syndrome patients.

Conclusion: Prebiotics and postbiotics offer an effective alternative treatment approach for Rett syndrome patients. Lalbaay® and Bizetaflox Pediatrico modulate neurotransmitters, neuromodulators, and short-chain fatty acids suggesting a shift in the gut microbiota ecology leading to the improvement of α-diversity.

2. Introduction

2.1 RETT syndrome

Rett syndrome is an X-linked neurological disorder with a prevalence of 1 in 10000 live female births. The syndrome is caused by a mutation in the Methyl-CpG-binding protein 2 (MECP2) gene (Ramirez et al., 2022; Borloz et al., 2021). The MECP2 mutation is detectable in 95-97% cases of Rett syndrome. However, detecting the mutation only is insufficient for diagnosis; physicians rely on clinical assessment (Petriti et al., 2023). Other genes involved in Rett syndrome include forkhead box G1 (FOXG1) and cyclin-dependent kinase-like 5 (CDKL5) (Hudu et al., 2023).

2.1.1 MECP2 molecular function

MECP2 gene encodes an epigenetic regulatory protein that operates by binding to methylated DNA. This protein regulates numerous genes associated with neuronal differentiation, maturation, and synaptic maintenance, predominantly expressed in the nervous system. The MECP2 protein has many functional confirmations enabling it to activate or repress transcription, and interact with both RNA splicing machinery and microRNA machinery (Panayotis et al., 2023; Sandweiss et al., 2020).

2.1.2 Clinical features

Rett syndrome symptoms include loss of acquired speech, motor dysfunction, hand stereotypies, development of gait abnormalities, and epilepsy although, patients may display some but not all of these symptoms. The syndrome progresses through four stages: early onset, regression, plateau, and late motor deterioration, in their respective progression order (Kyle et al., 2018).

During the first phase, which typically occurs between six and eighteen months, there is a developmental delay characterized by the development of microcephaly and slow growth of the brain and head (Sandweiss et al., 2020).

The second stage, spanning from one to four years, is marked by the loss of previously acquired skills, hand stereotypies, and gait abnormalities. Additionally, respiratory dysrhythmias, sleep disturbances, cold hands and feet, and episodes of laughing, crying or screaming may occur.
The third phase, occurring between two and ten years, is characterized by the stabilization of cognitive abilities, and the onset of seizures (Sandweiss et al., 2020).
Finally, the fourth stage, which begins after ten years, is distinguished by a decline in mobility and the development of Parkinsonian features (Collins and Neul, 2022).

2.2 Gut microbiota

The gut microbiota is a complex ecosystem consisting of approximately 3.8×10^{13} microorganisms in the gut, which maintain a symbiotic relationship with the human body (Góralczyk-Bińkowska et al., 2022; Wang et al., 2022). It is considered as an extra organ in the human body (Umu et al., 2017). About 90% of the microorganisms in the body inhabit both the small and large intestines, encompassing bacteria, fungi, viruses and protists (Góralczyk-Bińkowska et al., 2022). The most predominant bacteria belong to the phyla Firmicutes, Bacteroides, Proteus, and Actinomycetes, constituting approximately 99% of the total bacteria in the gut microbiota (Qiu et al., 2022).
Alterations in the gut microbiota diversity and relative abundance may contribute to neurodegenerative disorders, metabolic disorders, and infection susceptibility (Ling et al., 2022). Various factors, including environment, dietary habits, medication usage, lifestyle, age, and stress, influence microbiota diversity and abundance (Wang et al., 2022).
The intestinal microbiota plays pivotal roles in nutrition, infection resistance, immunity, metabolism, enteric nervous system and central nervous system development, mood regulation, cognition, and learning processes (Ling et al., 2022; Margolis et al., 2021).

2.2.1 The Microbiota-Gut-Brain Axis

Evidence has established bidirectional interactions among the microbiota, the gut and the brain, which regulate physiological and homeostasis functions, including food intake, immune regulation, and sleep (Mayer et al., 2022). The interactions are depicted in **Figure 1** below:

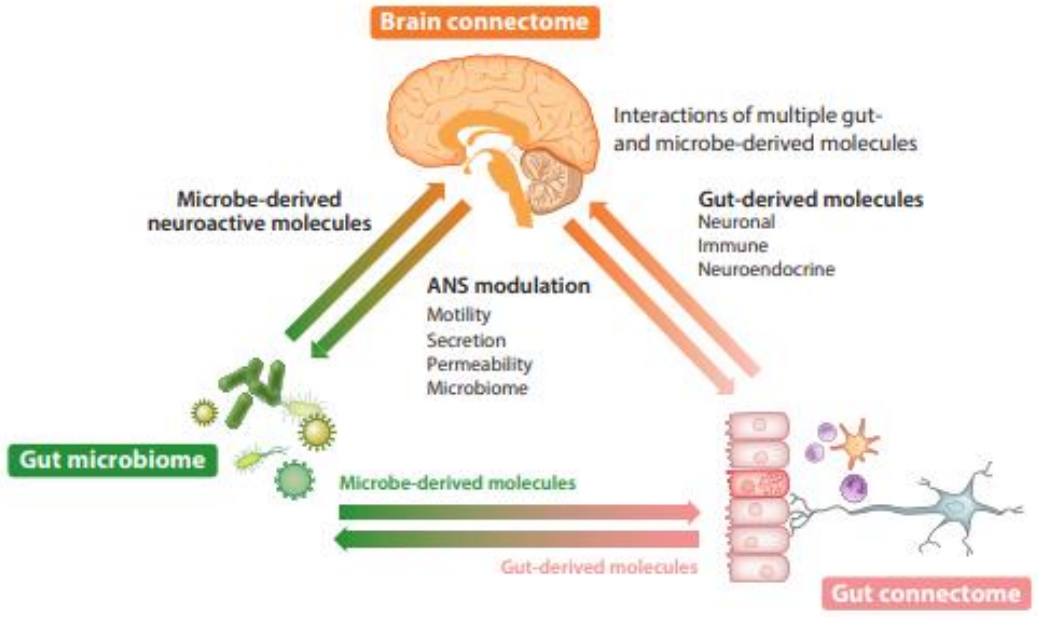

Figure 1. Microbiota-Gut-Brain Axis (Mayer et al., 2022).

The gut microbiome is directly and indirectly involved in the modulation of signaling molecules, which can be classified into three groups: food-related metabolites (such as short-chain fatty acids and certain neurotransmitters), metabolites of endogenously produced molecules (such as secondary bile acids and estrogen), and signals composed of microbial cell wall components (such as interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor α) (Mayer et al., 2022).

2.2.2 Key gut microbiota molecules

The gut microbiota is capable of synthesizing neuroactive molecules, including neurotransmitters and their precursors. Neurotransmitters are chemical messengers that are transported between neurons, affecting motility, mood, memory, and other functions. Bacteria possess genes that encode enzymes capable of converting precursors into neurotransmitters. Additionally, some bacteria synthesize molecules that can trigger the synthesis and/or release of neurotransmitters (Chen et al., 2021). Examples of neurotransmitters synthesized by the gut microbiota include glutamate (*Lactobacillus plantaram*), GABA (*Bifidobacterium*), serotonin (*Staphylococcus*), dopamine (*Staphylococcus*), acetylcholine (*Bacillus subtilis*), Tyramine (*Staphylococcus*),

phenylethylamine (*Staphylococcus*), and Tryptamine (*Ruminococcus gnavus*) (Chen et al., 2021; Góralczyk-Bińkowska et al., 2022).

Short-chain fatty acids are the most abundant product synthesized by the gut microbiota in the colon as a result of fermentation of dietary fibers and complex starch (Silva et al., 2020). The most abundant short-chain fatty acids produced by gut microbiota are acetate (60%), propionate (20%), and butyrate (20%) (Portincasa et al., 2022; Fusco et al., 2023). Short-chain fatty acids have a wide range of functions, including promoting integrity and permeability of the gut barrier, modulation of oxidative stress, modulation of brain-induced gluconeogenesis, modulation of inflammation, modulation of neurotransmitters and their precursors, regulation of appetite, and regulation of immunity (Fusco et al., 2023; Tan et al., 2014). In the early stages of life, the production of acetate is carried out by Bifidobacteria. In adults, most of the short-chain fatty acids are produced by Firmicutes (*Lactobacillaceae*, *Ruminococcaceae*, and *Lachnospiraceae*) and Bacteroidates. In the elderly, there is a decrease in microbiota diversity, which leads to a decrease in levels of production of short-chain fatty acids (Fusco et al., 2023).

2.2.3 Gut microbiota alterations in RETT syndrome

Two independent studies conducted in Italy by Strati et al in 2016 and by Borghi et al in 2017 both concluded that the gut microbiota of Rett syndrome exhibits decreased α-diversity and β-diversity compared to that of healthy subjects. α-Diversity of a microbial sample refers to the number of different microbial taxa and how uniformly these taxa are distributed, while β-diversity refers to dissimilarity between two different samples (i.e RETT syndrome patients versus healthy subjects) (Borghi and Vignoli, 2019). Borghi et al (2017) demonstrated a direct relationship between reduced α-diversity and the severity of RETT syndrome, indicating that patients with lower α-diversity had more severe symptoms. Strati et al. (2016) showed that the β-diversity could distinguish between RETT syndrome patients and healthy subjects.

2.2.4 Modulation of Gut microbiota

Since the gut microbiota plays pivotal roles in the human body, maintaining a favorable equilibrium is crucial. This necessity has driven researchers to investigate various methods, including pre-biotics and post-biotics (Liu et al., 2022). Researchers have demonstrated that

dietary intervention and long-term eating habits can modulate the gut microbiota (Umu et al., 2017).

According to Gibson et al (2017), pre-biotics are substrates utilized by the gut microbiota that, in-turn provide health benefits to the host. They belong to a subgroup of dietary fibers known to selectively stimulate abundance of bacteria associated with good health. Pre-biotics serve as food source for the bacteria, promoting the growth and the abundance of *Bifidobacterium* and *Lactobacillus*. These bacteria are involved in fermentation of pre-biotics. Anaerobic fermentation produces formate, lactate, and succinate, which lower pH levels in the gut, thereby preventing the growth of pathogenic bacteria (Umu et al., 2017). Common examples of pre-biotics include fructooligosaccharides, lactulose, galactooligosaccharides, and inulin (Gibson et al., 2017; Umu et al., 2017).

Post-biotics are metabolic by-products, generated by probiotics (live microorganisms) that confer health benefits to the host. Examples include short-chain fatty acids (SCFAs) (Huang and Huang, 2021). The short-chain fatty acids decrease pH levels, inhibiting the growth and activity of pathogenic bacteria while promoting the growth and abundance of fermenting bacteria (Peluzio et al., 2021).

2.3 RETT syndrome treatment options

To date, RETT syndrome is an incurable disorder and the treatment options available are centered on symptom relief (Panayotis et al., 2023). The trials have been mainly focused on MECP2 function in the development and maintenance of neurons, and specific neurotransmitters (drugs include: Dextromethorphan and Desipramine). However, this has not been the most successful strategy (Vidal et al., 2019). It is difficult to come up with a treatment strategy because RETT syndrome is a complex disorder involving many organ systems and pathways in the body, hence, a combination therapy will most likely be more effective compared to targeting a single organ or pathway (Vashi and Justice, 2019). Below is a figure showing the various treatment options targeting the MECP2 gene and the pathways downstream:

Figure 2. Available treatment options for RETT syndrome (Vashi and Justice, 2019).

2.3.1 Trofinetide

Trofinetide is a tripeptide synthetic analogue of glycine-proline-glutamate, which was approved by the FDA in March 2023 for the treatment of RETT syndrome for patients older than 2 years. Trofenitide belongs to several classes of compounds, including anti-inflammatories, glutamates, neuropeptides, neuroprotectants, oligopeptides, pyrrolidines, and small molecules. The mechanism by which Trofinetide exerts its therapeutic effect is unknown (Keam, 2023).

In a phase three clinical trial, Trofinetide achieved a RETT Syndrome Behavior Questionnaire (RSBQ) score of -5.1, compared to -1.7 for the placebo group. However, the drug has several

disadvantages, as it caused diarrhea (82%), vomiting (29%), fever (9%), seizure (9%), anxiety (8%), decreased appetite (8%), fatigue (8%), and nasopharyngitis (5%). These treatment-emergent adverse effects (TEAEs) were all higher compared to the placebo group (Keam, 2023). A total of 17.2% of the participants withdrew due to TEAEs (Neul et al., 2023).

2.3.2 Pre- and Post-biotics in neurological disorder treatment

Due to the existence of the Microbiota-Gut-Brain axis and the key molecules modulated by the microbiota, it is possible to control or reduce the symptoms of neurological and mental disorders such as anxiety, depression, autism, schizophrenia, and Alzheimer's (Aghamohammad et al., 2023; Ansari et al., 2023). The pre- and post-biotics approach is much more favorable than the current available drugs because there are no specific side effects, since the compounds are natural. Pre- and post-biotics can affect brain function via several ways: 1) corticosteroid and adrenocorticotropic hormone modulation, 2) stimulation of central nervous system through inflammatory cytokines and inflammation, 3) modulation of neurotransmitters and neuropeptides such as GABA, serotonin, dopamine, etc., and 4) modulation of short-chain fatty acids (Ansari et al., 2023).

2.3.3 α-Lactalbumin

α-Lactalbumin is a globular whey protein present in human milk and constitutes 22% of the total proteins. α-Lactalbumin modulates the gut microbiota by acting as a prebiotic and promotes growth of probiotics, e.g., *Bifidobacteria* (Gallo et al., 2024). Research conducted by Boscaini et al. showed higher abundance of *Lactobacillus*, *Parabacteroides*, and *Bifidobacterium* genera in mice fed with α-lactalbumin compared to the control group. It is a source of bioactive compounds and essential amino acids. α-Lactalbumin has the following uses: 1) infant formulas component, 2) supplement to modulate gut microbiota leading to improvement of neurological function (sleep and depression), and 3) therapeutic agent in mood disorders and seizures (Layman et al., 2018).

2.3.4 Butyrate

Butyrate is a postbiotic synthesized by anaerobic fermentation of dietary fibers and functions as an energy source for colonocytes, a histone acetylation regulator, an anti-oxidant, an anti-

inflammatory agent, and a gut microbiota modulator (Guo et al., 2023; Hodgkinson et al., 2023). Sodium butyrate has been shown to improve brain function in neurological disorders by modulating the gut microbiota. In a study conducted in Parkinson's diseased mice by Guo et al. in 2023, sodium butyrate significantly restored gut microbiota symbiosis, improved striatal neurotransmitter levels, improved motor function, and reduced death of dopaminergic neurons.

3. Objective of thesis

The objective of this thesis was to investigate the impact of a pre- and/or post biotics treatment in RETT syndrome patients through neurochemistry and microbiota analyses. The levels of neurotransmitters and short-chain fatty acids in plasma and fecal samples were evaluated using mass spectrometry techniques. Several studies have shown that Rett syndrome patients have a lower α-diversity compared to healthy subjects. Furthermore, Rett syndrome patients exhibit altered neurochemistry. The gut microbiota has been demonstrated to play a key role in the microbiota-gut-brain axis, particularly in the synthesis and modulation of certain neurotransmitters and short-chain fatty acids. Hence, promoting diverse α-diversity by using pre- and/or post biotics may lead to modulation of neurotransmitters and short-chain fatty acids, which in turn may result in improvements in Rett syndrome symptoms.

4. Materials and Methods

4.1 Clinical trial – NCT05420805

The trial was approved by CEASVE local ethical committee. The clinical trial was conducted at Centro di Ricerca e Sperimentazione Sindrome di Rett e U.O.C. Neuropsichiatria Infantile, Policlinico (S. Maria alle Scotte), Azienda Ospedaliera Universitaria Senese – Siena, Italy.

4.1.1 Inclusion criteria

The inclusion criteria for the clinical trial were as follows:

- Diagnosis of classic/typical Rett Syndrome with proven loss-of-function of the MECP2 gene, gastrointestinal dysfunction, and/or positive history of epilepsy.
- Female, aged 3 or older.
- Signed written informed consent form by parent(s)/legal guardian(s).
- Stable medications for at least 4 weeks prior to the baseline visit.

4.1.2 Exclusion criteria

The exclusion criteria for the clinical trial were as follows:

- Diagnosis not fitting into the Rett syndrome consensus guidelines.
- Nonpathogenic MECP2 mutation or mutations in non-MECP2 gene.
- Male.
- Percutaneous endoscopic gastronomy tube.
- Proven hypersensitivity to one or more components of the dietary supplements.
- Unstable concomitant medications less than 4 weeks prior to enrolment visit.
- Concomitant antibiotic therapy at enrolment. In case of antibiotic therapy, a 4-week washout period will be undertaken.
- Rejection of the informed consent form by parent(s)/legal guardian(s) and/or lack of compliance to the study procedures.

4.1.3 Study design

A randomized crossover trial with two treatment arms was conducted. A total of 28 Rett syndrome patients were enrolled in the study. Each of the two arms was treated with both Lalbaay® (α-lactalbumin + inulin + fructo-oligosaccharides (FOS) + sodium butyrate) and Bizetaflox Pediatrico (sodium butyrate + zinc oxide) both from Kolfarma, as shown in **Figure 3** below:

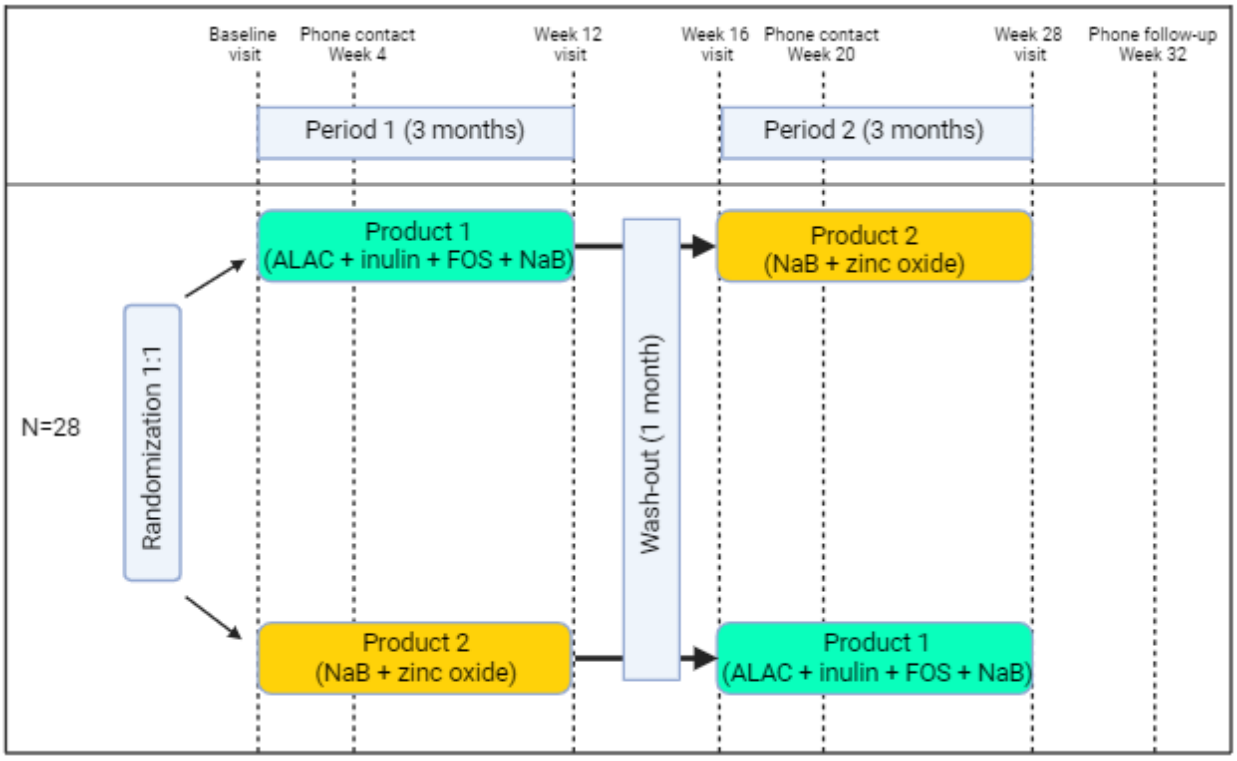

Figure 3. Study design for NCT05420805 clinical trial.

4.1.4 Trial demographics at baseline

Table 1 shows the demographics for the admitted Rett Syndrome patients for the NCT05420805 trial.

	Mean ± SD **median [IQR]** **N (%)**
N	28
Age (years)	20.9 ± 11.7 (range 3.9 – 45.2)
BMI z-score	0.71 ± 1.63
RTT disease stage	
Stage I	–
Stage II	4 (14.3%)
Stage III	15 (53.6 %)
Stage IV	9 (32.1 %)
MECP2 mutation severity	
Mild	9 (32.1%)
Moderate	11 (39.3 %)
Severe	8 (28.6 %)
Clinical severity	
Rett clinical severity scale (RCSS)	23.2 ± 5.7 (range 8 – 32)
Motor Behavior Assessment Scale (MBAS)	51.3 ± 8.7 (range 18 – 62)
Rett Syndrome Behaviour Questionnaire (RSBQ)	41.6 ± 10.7 (range 19 – 61)
IQ – Disability (IQ-Disability)	70.6 ± 9.4 (range 51 – 89)
Sleep Disturbance Scale for Children (SDSC)	42.6 ± 6.4 (range 29 – 57)
Complete count cell and markers of systemic inflammation	
White blood cells (WBC) (10^3 /mmc)	6.87 ± 2.15
Absolute Neutrophil Count (ANC) (10^3 /mmc)	3.62 ± 1.57
Absolute Monocyte Count (AMC) (10^3 /mmc)	0.48 [0.41 – 0.59]
Absolute Eosinophil Count (AEC) (10^3 /mmc)	0.08 [0.05 – 0.12]
Absolute Lymphocyte Count (ALC)(10^3 /mmc)	2.54 ± 0.98
HDL cholesterol (mg/dL)	61.2 ± 16.6
Neutrophil-Lymphocyte Ratio (NLR)	1.37 [0.93 – 1.95]
Monocyte-HDL cholesterol Ratio (MHR)	0.0077 [0.0059-0.0108]

Blood samples and stool samples were collected for all patients at baseline, week 12, week 16, and week 28. The samples were store at -80 °C and transported to UPO-CAAD (Centro di Ricerca Traslazionale sulle Malattie Autoimmuni e Allergiche) for mass spectrometry-based analysis.

4.2 Sample preparation

Samples for all 28 patients were received and stored at -80 °C until the sample preparation was performed. The sample preparation was done differently for each matrix (plasma and stool samples) and for each mass spectrometry instrument (liquid chromatography mass spectrometry and gas chromatography). Each sample preparation method is presented in the following subsections.

4.2.1 Extraction of neurotransmitters in plasma

To extract neurotransmitters from plasma, methanol was used. The frozen samples were thawed, followed by dispensing 40 µl aliquots into 1.5 ml Eppendorf tubes. These samples were spiked with 10 µl of internal standard mix for both C18 column (glutamine: 16 µg/ml, melatonin: 1 µg/ml, kynurenine: 8 µg/ml, and GABA: 12 µg/ml) and HILIC column (serotonin: 6 µg/ml, acetylcholine: 12 µg/ml, trimethylamine n-oxide: 1 µg/ml, glutamine: 32 µg/ml, and GABA: 12 µg/ml). Subsequently, 350 µl of cold methanol was added, and the solution was vortexed for 10 seconds before incubating the tubes at -20 °C for 30 minutes. After incubation, the tubes were centrifuged at 15000×g for 10 minutes at 4 °C, yielding a supernatant which was transferred to new 1.5 ml Eppendorf tubes. For the dilute samples, 70 µl of the supernatant was collected, while for concentrated samples, 140 µl of supernatant was collected. The sample was dried using a SpeedVac vacuum concentrator. For C18 column analysis, samples were reconstituted with water and acetonitrile in a 98:2 ratio, with 140 µl of the solution added for dilute samples and 60 µl concentrated samples. Similarly, for HILIC column analysis, the samples were reconstituted with water and acetonitrile in a 5:95 ratio, with the same volume adjustments for dilute and concentrated samples (Figure 4A).

4.2.2 Extraction of neurotransmitters in stool

To extract neurotransmitters from stool, methanol and water was used. The frozen samples were thawed, followed by transferring 20 mg of stool into 2.0 ml Eppendorf with tubes screw caps. To each sample, 300 µl of cold methanol and 300 µl of water were added. Subsequently, these samples were spiked with 10 µl of internal standard mix for both C18 column (glutamine: 16 µg/ml, melatonin: 1 µg/ml, kynurenine: 8 µg/ml, and GABA: 12 µg/ml) and HILIC column (serotonin: 6 µg/ml, acetylcholine: 12 µg/ml, trimethylamine n-oxide: 1 µg/ml, glutamine: 32 µg/ml, and GABA: 12 µg/ml). The samples were homogenized using a TissueLyzer for 40 seconds at 6500 r.p.m before incubating the tubes at -20 °C for 30 minutes. After incubation, the tubes were centrifuged at 15000×g for 10 minutes at 4 °C, yielding a supernatant which was transferred to new 1.5 ml Eppendorf tubes. For the dilute samples, 70 µl of the supernatant was collected, while for concentrated samples, 140 µl of supernatant was collected. The sample was dried using a SpeedVac vacuum concentrator. For C18 column analysis, samples were reconstituted with water and acetonitrile in a 98:2 ratio, with 140 µl of the solution added for dilute samples and 60 µl concentrated samples. Similarly, for HILIC column analysis, the samples were reconstituted with water and acetonitrile in a 5:95 ratio, with the same volume adjustments for dilute and concentrated samples.

4.2.3 Extraction of short-chain fatty acids in plasma

To extract short-chain fatty acids from plasma, methyl-tert-butyl ether was used. The frozen samples were thawed, followed by dispensing 50 µl aliquots into 0.6 ml Eppendorf tubes. The pH of the plasma was adjusted to ≤ 2 using 1.5 µl of HCL (6 M). These samples were then spiked with 5.0 µl of internal standard mix (propanoic acid: 20.4 ppm and acetic acid: 100 ppm). Subsequently, 140 µl of methyl-tert-butyl ether was added, and the Eppendorf tube cap was sealed with parafilm. The samples were mixed using a rotator for 10 minutes at 40 r.p.m. After mixing, the tubes were centrifuged at 21100×g for 15 minutes at 4 °C, resulting in a solution with two phases. Then, 100 µl of the organic top phase was transferred to 250 µl vial with an insert (Figure 4B).

4.2.4 Extraction of short-chain fatty acids in stool

To extract short-chain fatty acids from stool, methyl-tert-butyl ether was used. The frozen samples were thawed, followed by transferring 20 mg of stool into 2.0 ml Eppendorf with tubes screw caps. To each sample, 300 µl of water was added. The samples were then spiked with 30 µl of internal standard mix (propanoic acid: 20.4 ppm and acetic acid: 100 ppm). The samples were homogenized using a TissueLyzer for 40 seconds at 6500 r.p.m before incubating the tubes in a shaking thermos-block (1000 r.p.m) at 4 °C for 30 minutes. After incubation, the tubes were centrifuged at 21100×g for 30 minutes at 4 °C, and 100 µl of the supernatant were transferred to a 0.6 ml Eppendorf tube. The pH of the plasma was adjusted to ≤ 2 using 1.5 µl of HCL (6 M). Subsequently, 140 µl of methyl-tert-butyl ether was added, and the Eppendorf tube cap was sealed with parafilm. The samples were mixed using a rotator for 10 minutes at 40 r.p.m. After mixing, the tubes were centrifuged at 21100×g for 15 minutes at 4 °C, resulting in a solution with two phases. Then, 100 µl of the organic top phase was transferred to 250 µl vial with an insert.

4.3 LC-MS – Neurotransmitter analysis

The neurotransmitter analysis for both plasma samples and stool samples was conducted using a UHPLC Vanquish system (Thermo Scientific, Rodano,Italy) coupled with an Orbitrap Q-Exactive Plus (Thermo Scientific, Rodano, Italy). Two complimentary chromatographic columns were used, a reverse phase (CSHÔ C18, 2.1 x150 mm, particle size 1.7 µm, Acquity UPLCâ) and a hydrophilic interaction chromatography, (HILIC, particle size 1.7 µm, 2.1 mm × 150 mm, Milford, MA).

For the C18 column, the mobile phases consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in ACN (B), with a 15 min gradient: 0–1 min with 2 % B, 1–3 min from 2 % to 15 % B, 3–6 min from 15 % to 50 % B, 6–7.5 min from 50 % to 98 % B, 7.5–11.5 min B held at 98 %, 11.5–11.6 min from 98 % to 2 % B, and 11.6–15 min 2 % B. For the HILIC column, the mobile phases comprised 50:50 water/MeOH (A) and 15:5:80 (v/v/v) water/MeOH/ACN (B), with 10 mM ammonium formate for both phases, and a 11 min gradient was used: 0 min with 95 % B, 0–3.5 min from 95 % to 50 % B, 3.5–5.5 min from 50–5 % B, 5.5–6.5 min 5 % B, 6.5–6.7 min from 5 % to 95 % B, and 6.7–11 min 95 % B.

The analyses were conducted in electrospray ionization positive mode with sheath gas flow rate set as 60 L/min, aux gas flow rate at 10 L/min, sweep gas flow rate at 1 L/min, spray voltage at 2.75 kV, capillary temperature at 325 °C, and aux gas heater temperature at 400 °C. The mass

spectral data for quantitation were acquired in PRM mode, with AGC target set at 2e5, maximum injection time (IT) as 50 ms, and an isolation window of 1.2 m/z for ion fragmentation. The PRM was performed according to an inclusion list containing the elemental composition, (ESI) species, charge numbers, (ionization) polarity, range of retention time for fragmentation, and normalized collision energy (NCE) of higher-energy C-trap dissociation (HCD) that was optimized for each individual compound.

4.4 GC-MS – Short-chain fatty acids analysis

The SCFA analysis for both plasma samples and stool samples was conducted using a mono-dimensional gas chromatographer coupled with a mass spectrometer (GC-MS Pegasus BT, LECO®, St. Joseph, MI, USA). A 30-meter Stabilwax-DA (Restek) chromatographic column was used. The analysis temperature settings were as follows: starting at 65 °C, the temperature increased to 130 °C with a gradient of 10 °C/min, then it rose to 180 °C with a gradient of 5 °C/min, and finally reached 240 °C with a gradient of 15 °C/min, maintained for two minutes. The following parameters were set for the mass analysis: 200 seconds of delay, acquisition frequency was set at 20 (spectra/sec), and the mass range from 40 to 400 (m/z). The total duration of the analysis was 22 minutes and 30 seconds.

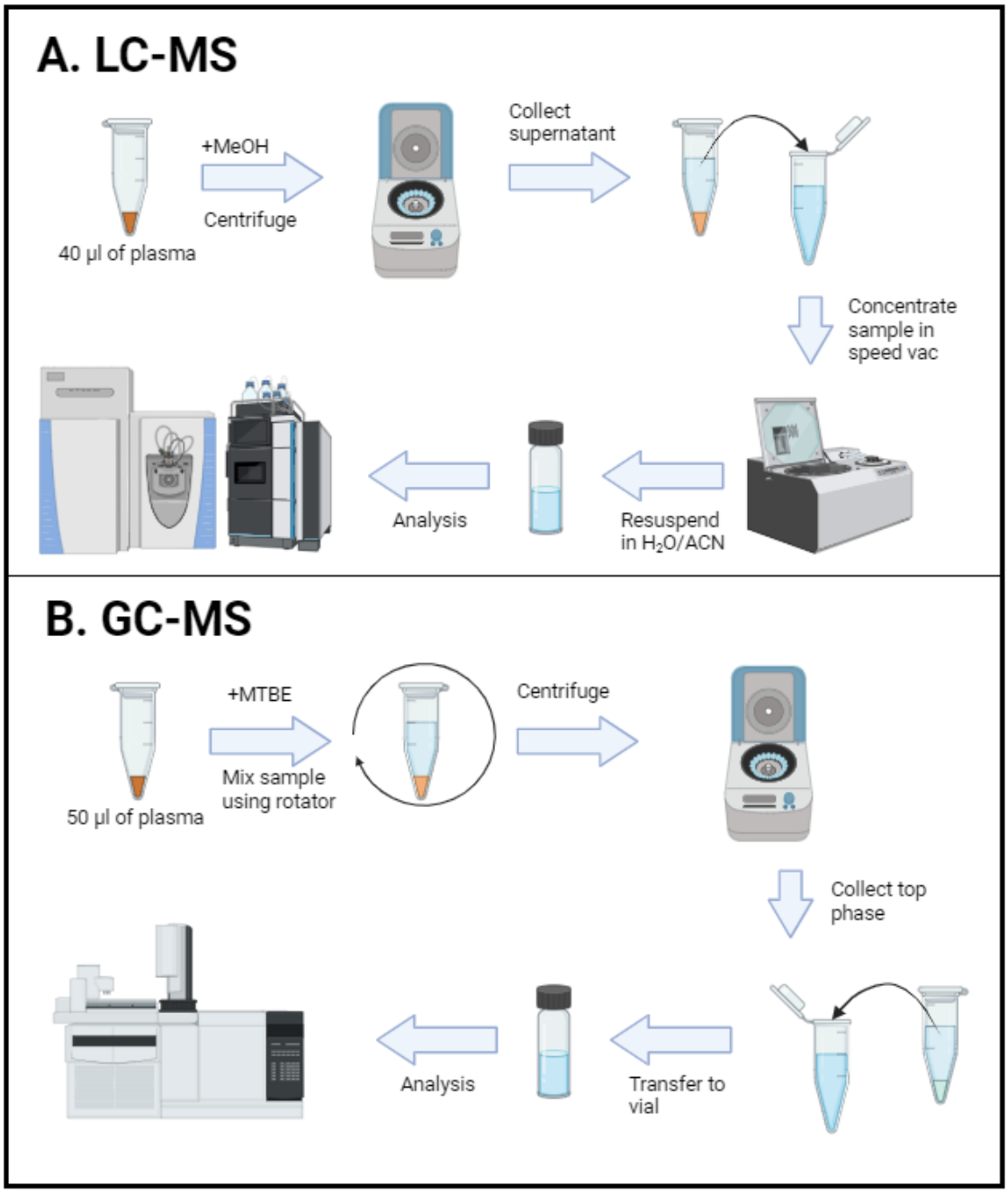

Figure 4. General workflow for LC-MS (A) and GC-MS (B)

5. Results

5.1 Clinical Trial

To investigate the effect of prebiotics and/or postbiotics on levels of neurotransmitters and short-chain fatty acids in Rett syndrome patients, a clinical trial (NCT05420805) was conducted at the Centro di Ricerca e Sperimentazione Sindrome di Rett e U.O.C. Neuropsichiatria Infantile, Policlinico (S. Maria alle Scotte), Azienda Ospedaliera Universitaria Senese – Siena, Italy. The patients were treated with both Lalbaay® and Bizetaflox Pediatrico (both from Kolfarma), with a washout period implemented before switching from one treatment to the other. Stool and plasma samples were collected before and after each treatment and sent for mass spectrometry analysis.

5.1.1 Rett Syndrome Behavior Questionnaire (RSBQ) Score

To assess the behavioral symptoms of Rett syndrome, caregivers filled out a questionnaire that evaluates various behavioral symptoms in patients, including hand movements, stereotypic behaviors, breathing irregularities, mood, social relations, and speech. A comparison of the total RSBQ score was made between Lalbaay®, Bizetaflox Pediatrico, and Trofinetide (which was approved by the FDA in 2023 for the treatment of Rett syndrome) to determine the superior treatment. The total RSBQ score for Trofenitide was -5.1, for Bizetaflox Pediatrico it was -9.0, and for Lalbaay® it was -14.0, as shown in Figure 5 below. When interpreting RSBQ scores, higher scores indicate more severe symptoms.

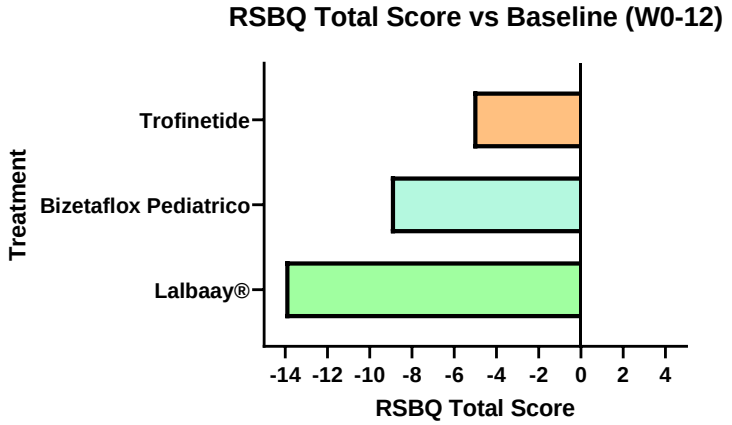

Figure 5. Total RSBQ scores for Trofinetide, Bizetaflox Pediatrico, and Lalbaay®.

5.1.2 Motor Behavioral Assessment Scale (MBAS)

In Rett syndrome, motor behavior is a key element that clinicians monitor as patients typically lose acquired motor skills. During treatment, clinicians assess motor behaviors to determine treatment effectiveness. For the clinical trial, motor behavior was monitored according to MBAS. When interpretating the MBAS, lower scores indicate less severe symptoms. The MBAS score for the clinical trial is shown in Figure 6. For both treatments, there was an improvement in motor behavior from the baseline to week 12, as evidenced by a decrease in the MBAS score. During the washout phase, from week 12 to week 16, the motor behavior became more severe, as indicated by an increase in the MBAS score due to the discontinuation of treatment. The drug crossover occurred at week 16, and the motor behavior improved again, which is evident from the decrease in the MBAS score.

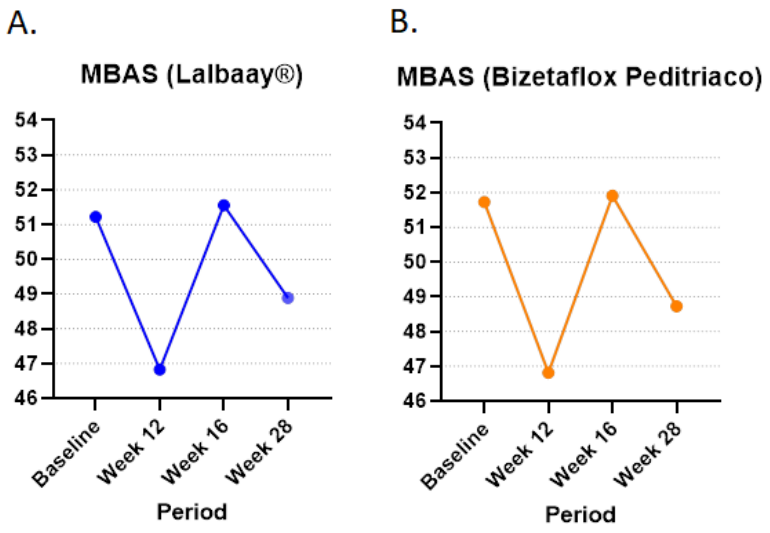

Figure 6. MBAS scores for Lalbaay® (A) and Bizetaflox Pediatrico (B).

5.1.3 Sleep Disturbance Scale for Children (SDSC)

Rett syndrome patients often experience sleep disturbances, which can significantly impact the quality of life for both the patient and the care giver. These disturbances include difficulties in sleeping, breathing irregularities, and sleep-related seizures, among others. Therefore, monitoring sleep quality in Rett syndrome patients is crucial to assess the effectiveness of the treatments. For the clinical trial, sleep disturbance was monitored using SDSC. When interpretating the SDSC,

lower the scores indicate less severe the symptoms. The SDSC scores for the clinical trial are shown in Figure 7. For both treatments, there was an improvement in sleep disturbances, as evidenced by a decrease in the SDSC score during treatment, and an increase in the score during the washout phase.

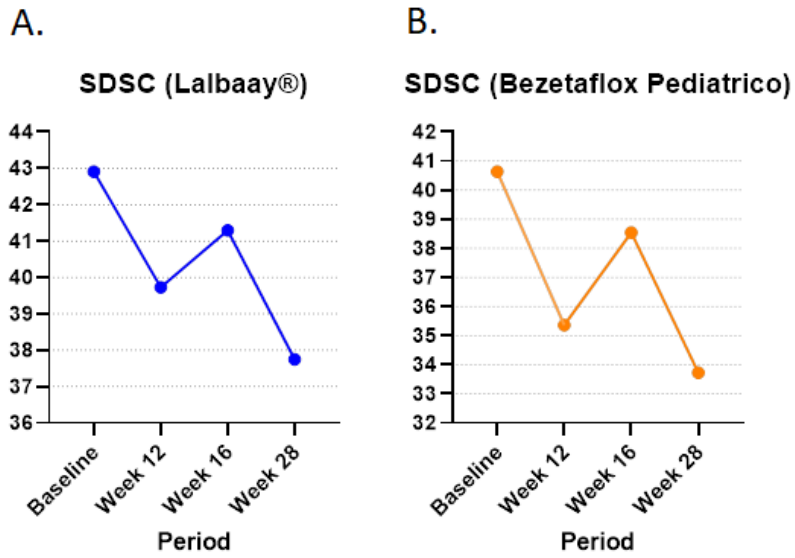

Figure 7. SDSC scores for Lalbaay® (A) and Bizetaflox Pediatrico (B).

5.2 Impact of treatments on short-chain fatty acids

Short-chain fatty acids (SCFAs) are key molecules in the microbiota-gut-brain axis due to their involvement in neurotransmitter modulation, inflammation, and immunity. Neurotransmitter dysregulation and inflammation are common issues in Rett syndrome patients. Therefore, we conducted a targeted GC-MS approach to quantify SCFAs in plasma and fecal samples before and after treatment to determine the modulations caused by prebiotics and/or postbiotics. The SCFAs targeted were acetic acid, propanoic acid, butanoic acid, butanoic acid 3-methyl, pentanoic acid, and propanoic acid 2-methyl. All the six targeted SCFAs were reliably identified and quantified in both matrices, as shown in Table 2.

Table 2. List of detected SCFAs in plasma and fecal samples using GC-MS.

Molecule	Mass to charge ratio (m/z)
Acetic acid	60
Propanoic acid	74
Butanoic acid	60
Butanoic acid 3-methyl	60
Pentanoic acid	60
Propanoic acid 2-methyl	88

5.2.1 Circulating SCFAs - Lalbaay®

For the Lalbaay® treatment in Rett syndrome patients, three of the six identified and quantified circulating SCFAs were modulated. Acetic acid and propanoic acid levels increased after treatment, while propanoic acid 2-methyl levels decreased, as shown in Figure 8. There were no modulations in the levels of butanoic acid, butanoic acid 3-methyl, and pentanoic acid.

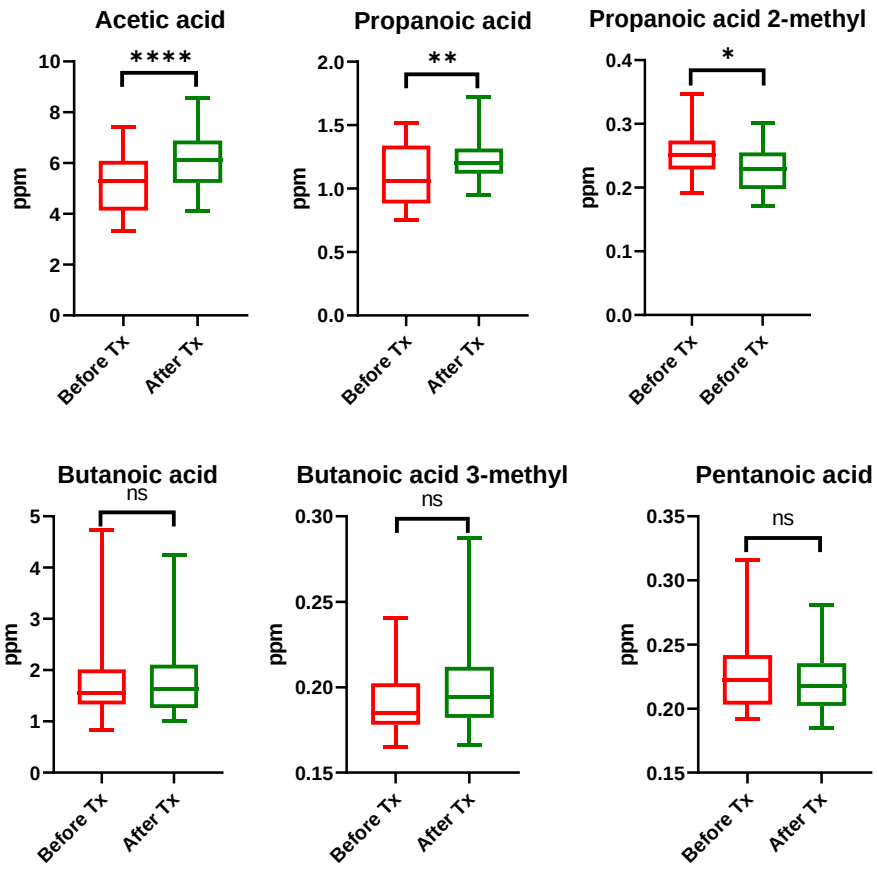

Figure 8. Circulating SCFA levels for the Rett syndrome patients treated with Lalbaay® before and after treatment. A paired t-test was performed using GraphPad Prism 8 software to determine the significant difference between the levels of circulating SCFAs. Key: * - p value < 0.05; ** - p value < 0.01; **** - p value < 0.0001; ns – p value > 0.05.

In Figure 9 and Figure 10, the multivariate classification PLS-DA and VIP score analysis were conducted using Metaboanalyst 6.0. The PLS-DA classifies observations into groups based on predictor variables, while the VIP score measures the importance of each variable.

In the PLS-DA shown in Figure 9, there is no clear-cut separation between the samples collected before treatment and the samples collected after treatment with Lalbaay®, although two groups of samples can be identified. From the VIP score in Figure 10, it can be concluded that acetic acid contributes the most to the separation of the two sample groups, followed by propanoic acid, butanoic acid, propanoic acid 2-methyl, butanoic acid 3-methyl, and pentanoic acid, respectively.

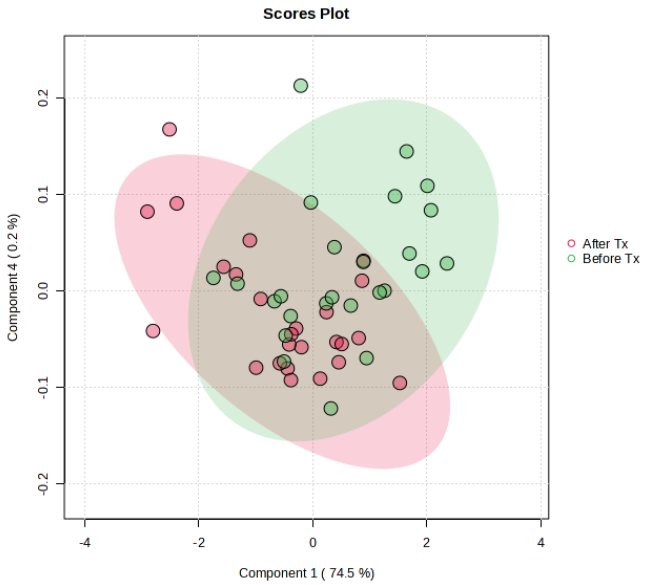

Figure 9. PLS-DA scores plot for circulating SCFAs in patients treated with Lalbaay®.

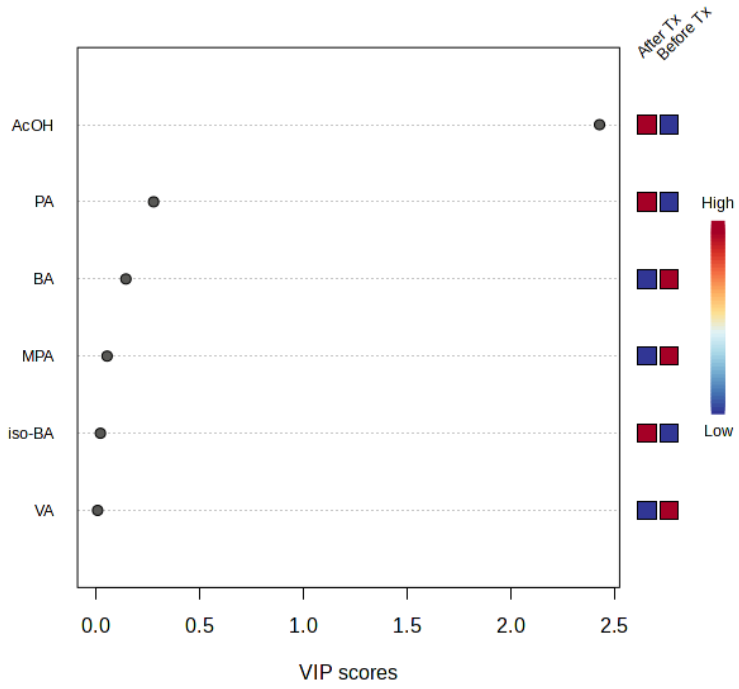

Figure 10. VIP scores plot for circulating SCFAs for patients treated with Lalbaay®. Abbreviations: AcOH – acetic acid, PA – propanoic acid, BA – butanoic acid, MPA – propanoic acid 2-methyl, iso- BA – butanoic acid 3-methyl, and VA – pentanoic acid.

A hierarchical clustering heat map of variables (Figure 11) generated using Metaboanalyst 6.0, illustrates the differences in circulating levels of SCFAs before and after treatment with Lalbaay®. Acetic acid and propanoic acid levels increased while propanoic acid 2-methyl decreased.

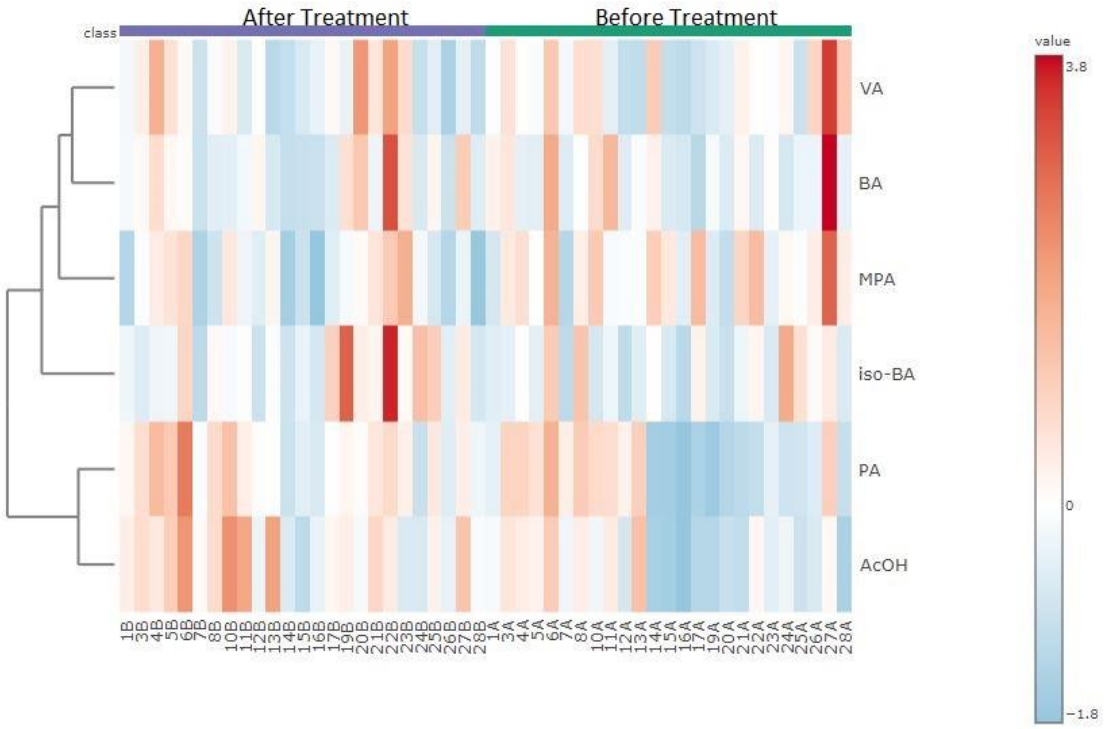

Figure 11. A hierarchical clustering heat map showing circulating levels of SCFAs before and after treatment with Lalbaay®.

A correlation heat map of variables (Figure 12) generated using Metaboanalyst 6.0, illustrates correlations among SCFAs, SDSC, MBAS, and RSBQ in patients treated with Lalbaay®.
SDSC shows a moderate negative correlation with acetic acid and pentanoic acid. RSBQ exhibits a moderate positive correlation with propanoic acid 2-methyl. There is a strong positive correlation between acetic acid and propanoic acid, and a moderate correlation between acetic acid and butanoic acid 2-methyl. Propanoic acid shows a moderate positive relationship with butanoic acid 2-methyl, butanoic acid, and pentanoic acid. There is a moderate positive correlation between butanoic acid 3-methyl and propanoic acid 2-methyl, and between butanoic acid 3-methyl and pentanoic acid, while a strong positive correlation exists between butanoic acid 3-methyl and butanoic acid. Propanoic acid 2-methyl exhibits a strong positive relationship with butanoic acid and pentanoic acid. Butanoic acid and pentanoic acid demonstrate a strong positive correlation

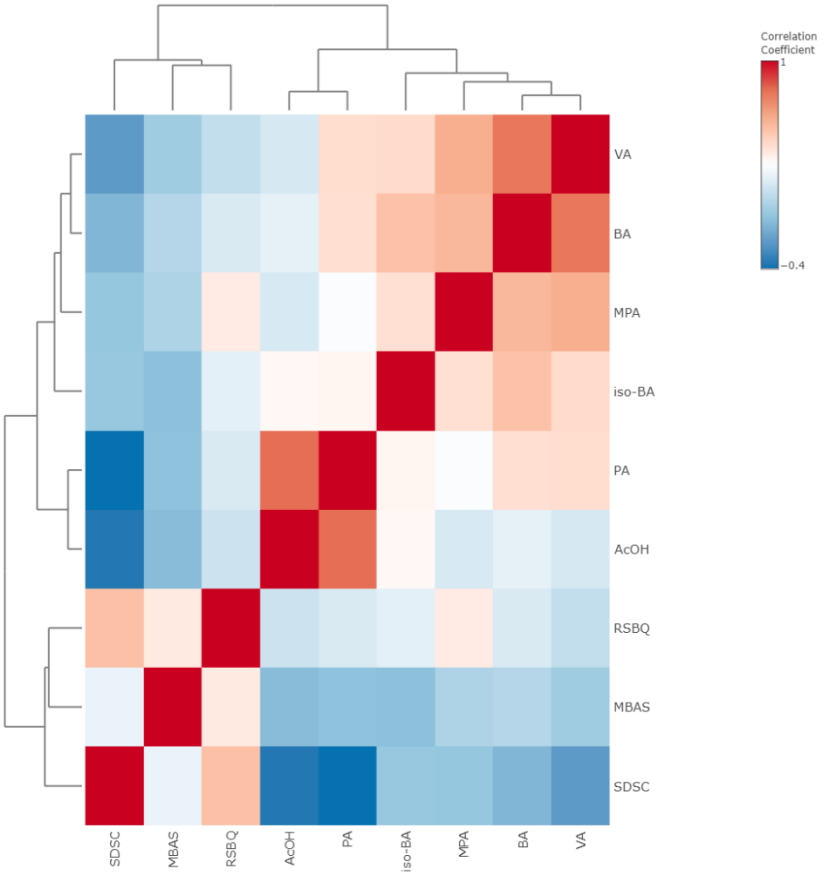

Figure 12. A correlation heat map showing SCFAs, SDSC, MBAS, and RSBQ in patients treated with Lalbaay®.

After the Lalbaay® treatment, a 4-week washout period was conducted before the drug crossover. Plasma and fecal samples were collected after the first treatment and before the second treatment to assess whether the improvement in symptoms was due to the treatment. During the washout period, clinicians observed a worsening of symptoms. The results for circulating SCFAs during the washout period for the Lalbaay® treatment are shown in Figure 13. Propanoic acid levels increased, while butanoic acid 3-methyl decreased.

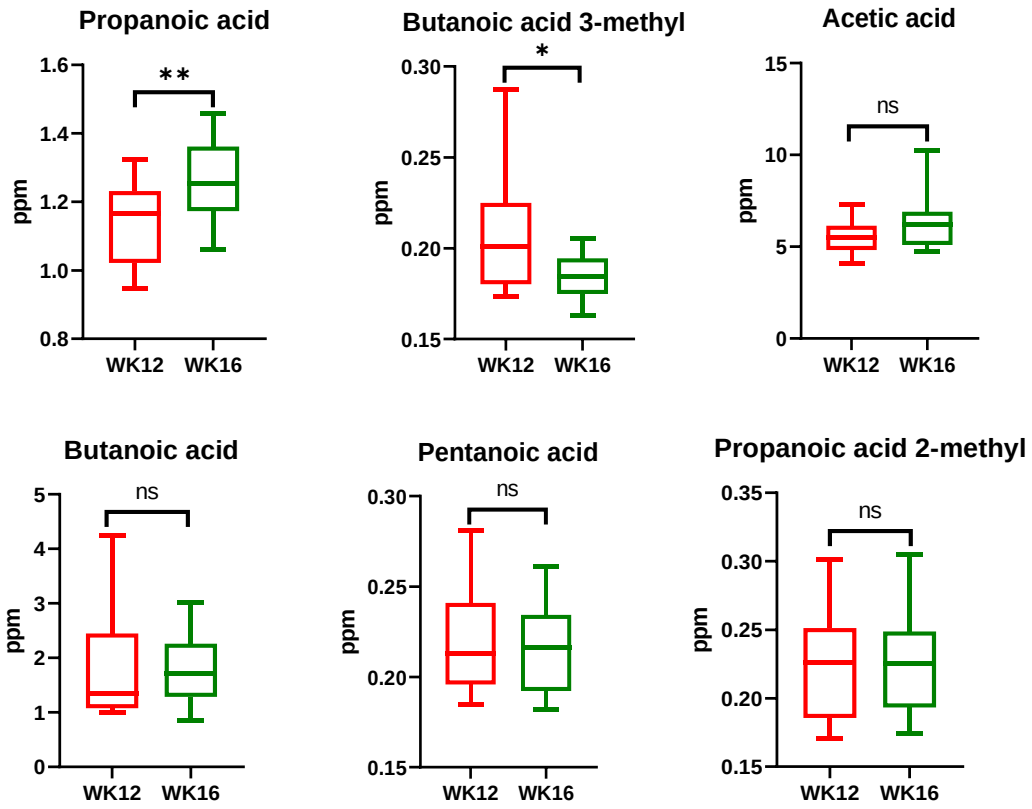

Figure 13. Circulating SCFA levels during the washout period for the Rett syndrome patients treated with Lalbaay®. A paired t-test was used to determine the significant difference between the two sample groups. Key: * - p value < 0.05; ** - p value < 0.01; ns – p value > 0.05.

5.2.2 Circulating SCFAs - Bizetaflox Pediatrico

For the Bizetaflox Pediatrico treatment, two of the six circulating SCFAs identified and quantified were modulated. Acetic acid and propanoic acid levels increased as shown in Figure 14.

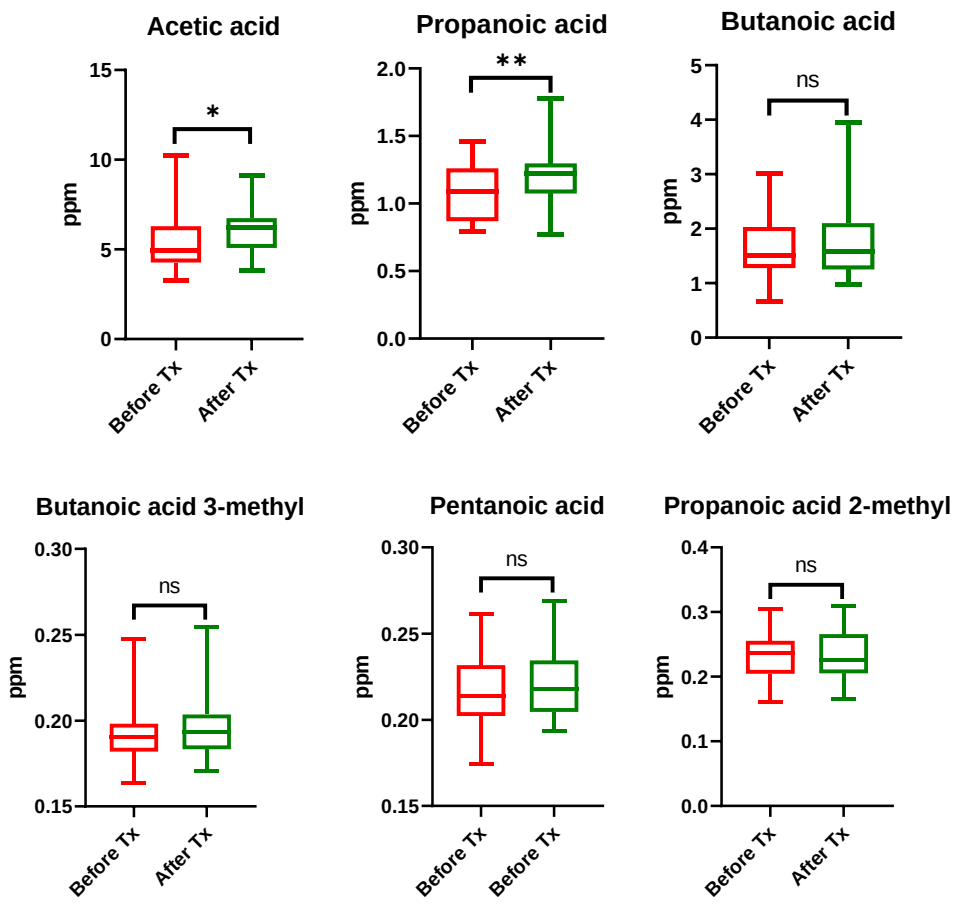

Figure 14. Circulating SCFA levels for the Rett syndrome patients treated with Bizetaflox Pediatrico before and after treatment. A paired t-test was used to determine the significant difference between the two sample groups. Key: * - p value < 0.05; ** - p value < 0.01; ns – p value > 0.05.

In Figure 15 and Figure 16, multivariate classification PLS-DA and VIP score were conducted in Metaboanalyst 6.0. For the PLS-DA in Figure 15, there is no clear-cut separation between the samples collected before treatment and those collected after treatment with Bizetaflox Pediatrico, although some clusters of samples with similar behavior are present. From the VIP score in Figure 16, it can be concluded that acetic acid contributes the largest part to the separation of the two sample groups, followed by butanoic acid, propanoic acid, pentanoic acid, butanoic acid 3-methyl, and propanoic acid 2-methyl, respectively.

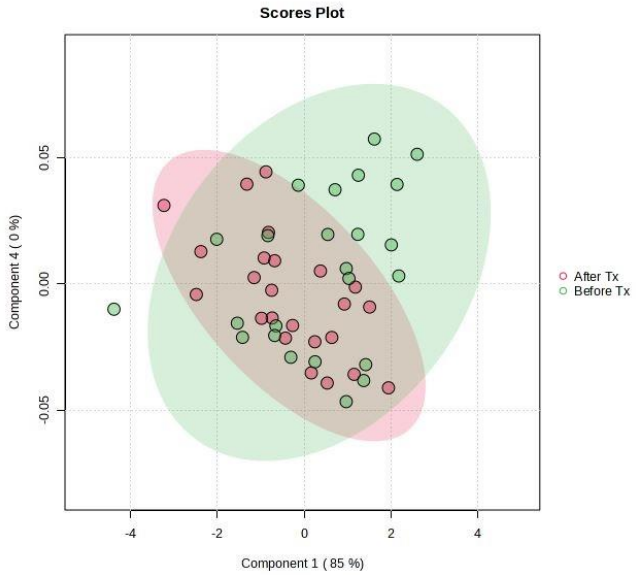

Figure 15. PLS-DA scores plot for circulating SCFAs in patients treated with Bizetaflox Pediatrico.

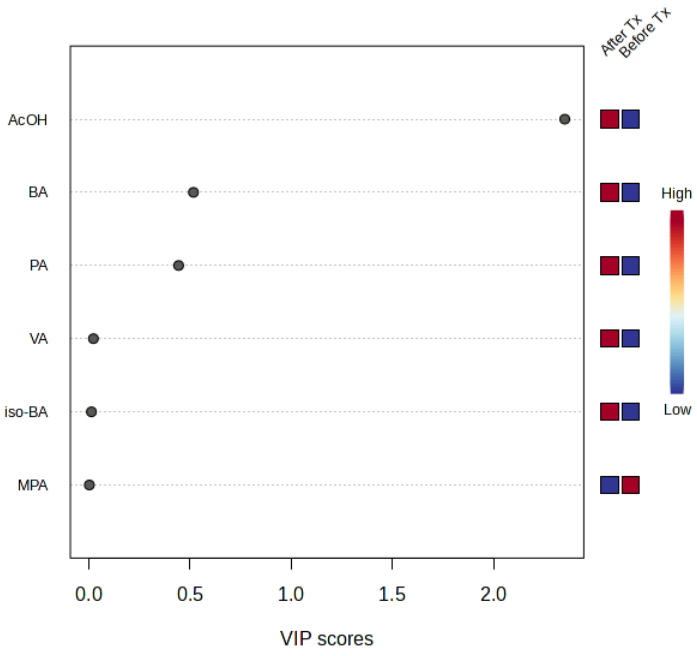

Figure 16. VIP scores plot for circulating SCFAs for patients treated with Bizetaflox Pediatrico. Abbreviations: AcOH – acetic acid, PA – propanoic acid, BA – butanoic acid, MPA – propanoic acid 2-methyl, iso- BA – butanoic acid 3-methyl, and VA – pentanoic acid.

The hierarchical clustering heat map (Figure 17) generated using Metaboanalyst 6.0 shows the difference between circulating levels of SCFAs before and after treatment for all the patients. Acetic acid and propanoic acid levels increased.

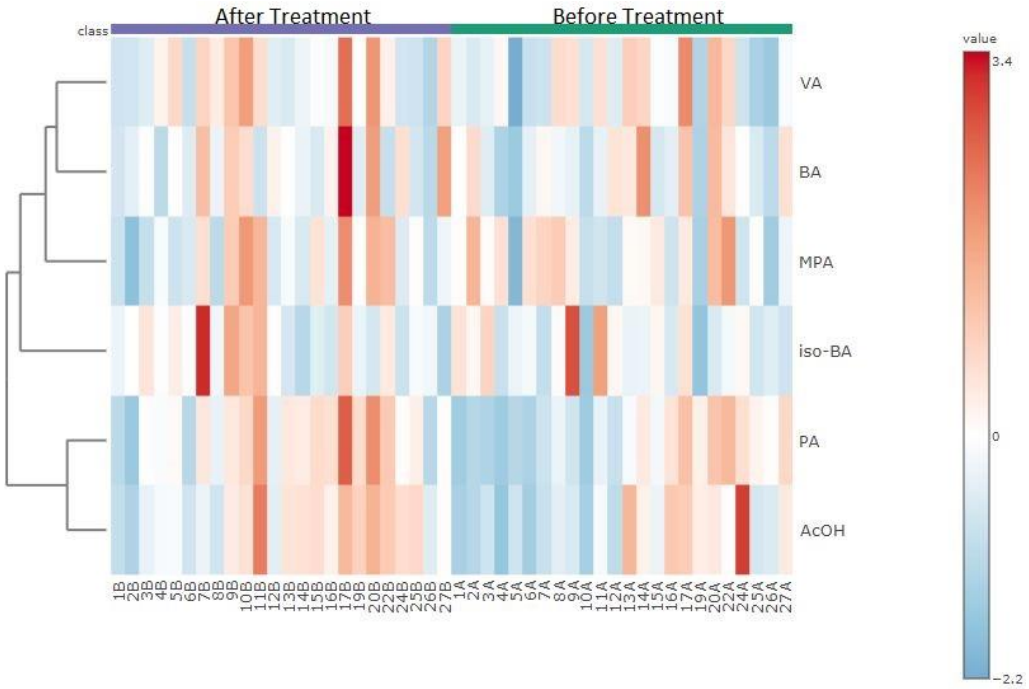

Figure 17. A hierarchical clustering heat map showing circulating levels of SCFAs before and after treatment with Bizetaflox Pediatrico.

A correlation heat map (Figure 18) showing correlations among SCFAs, SDSC, MBAS and RSBQ in patients treated with Bizetaflox Pediatrico. RSBQ moderately positively correlates with propanoic acid 2-methyl. MBAS shows a moderate correlation with butanoic acid, while SDSC exhibits a moderate negative correlation with butanoic acid. Butanoic acid 3-methyl is positively correlated with propanoic acid 2-methyl and pentanoic acid. Acetic acid strongly correlates with pentanoic acid, and moderately correlates with butanoic acid and pentanoic acid. Pentanoic acid shows strong positive correlations with acetic acid, butanoic acid, and pentanoic acid, and a moderate correlation with propanoic acid 2-methyl. Butanoic acid demonstrates strong positive correlations with pentanoic acid, propanoic acid, and propanoic acid 2-methyl. Propanoic acid 2-methyl strongly correlates positively with pentanoic acid, butanoic acid, and propanoic acid.

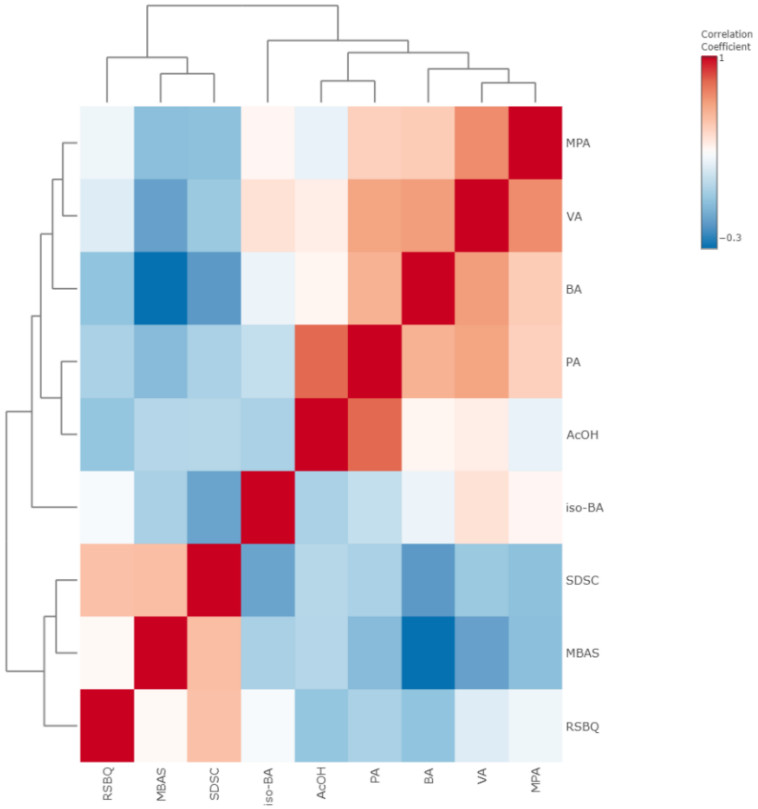

Figure 18. A correlation heat map showing SCFAs, SDSC, MBAS and RSBQ in patients treated with Bizetaflox Pediatrico.

After the Bizetaflox Pediatrico treatment, a 4-week washout period was conducted before the drug crossover. The results for the circulating SCFA levels during the washout period are shown in Figure 19 below. Propanoic acid levels increased, while butanoic acid 3-methyl levels decreased.

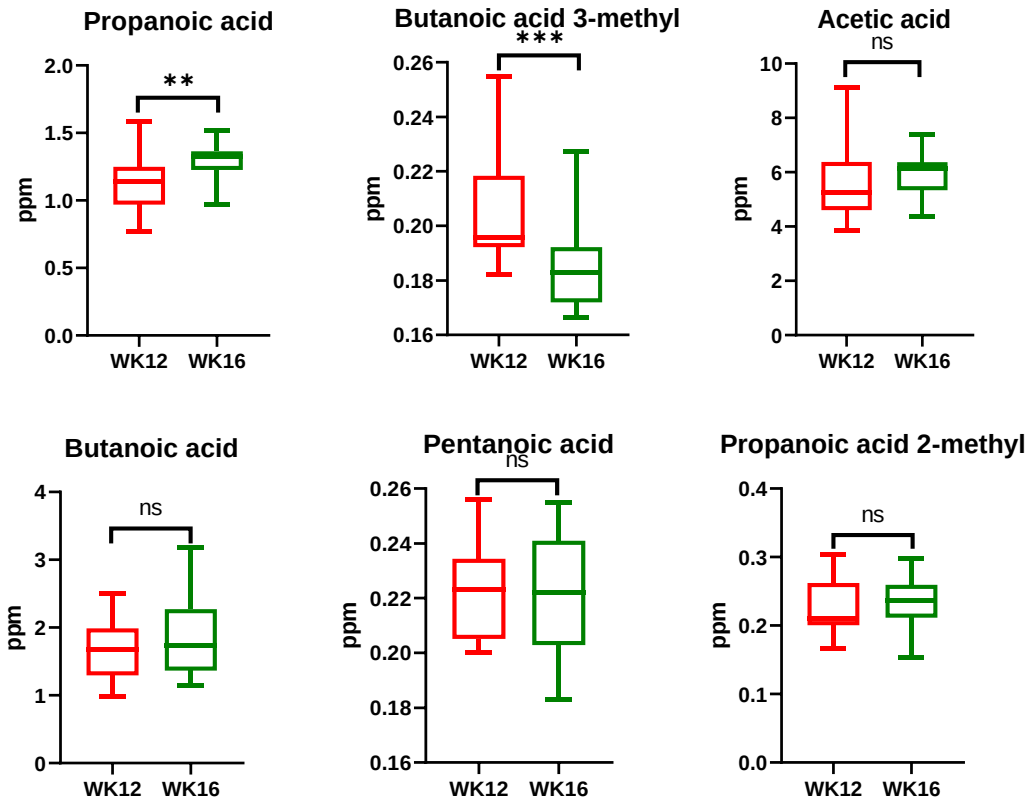

Figure 19. Circulating SCFA levels during the washout period for the Rett syndrome patients treated with Bizetaflox Pediatrico. A paired t-test was used to determine the significant difference between the two sample groups. Key: ** - p value < 0.01; *** - p value < 0.001; ns – p value > 0.05.

5.2.3 Fecal SCFAs - Lalbaay®

For the Lalbaay® treatment, there were no significant changes in any of the six SCFAs in the fecal samples (Figure 20**)**.

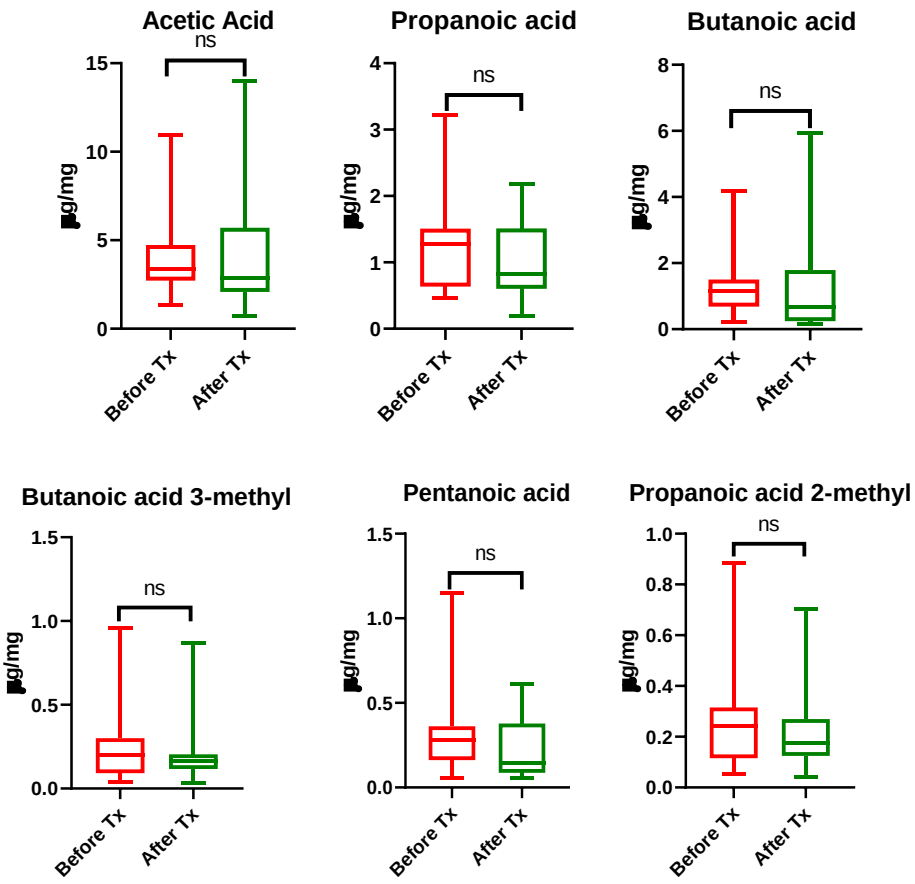

Figure 20. Fecal SCFA levels for the Rett syndrome patients treated with Lalbaay® before and after treatment. A paired t-test was used to determine the significant difference between the two sample groups. Key: ns – p value > 0.05.

5.2.4 Fecal SCFAs - Bizetaflox Pediatrico

For the Bizetaflox Pediatrico treatment, there were no significant changes in the levels of fecal SCFAs (Figure 21**)**.

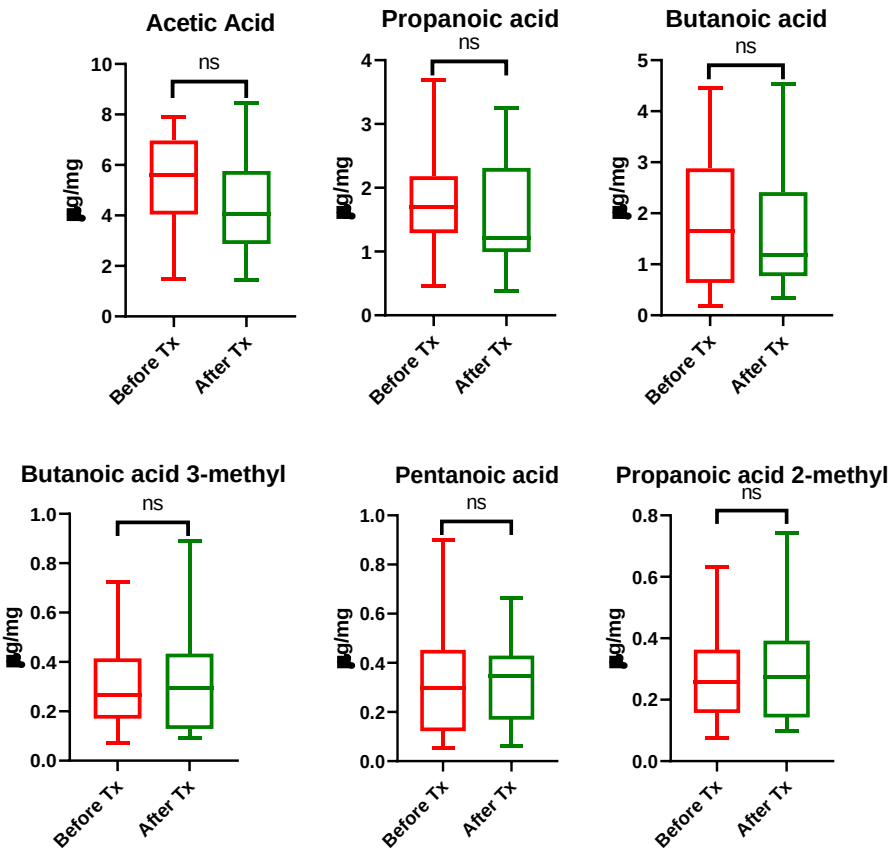

Figure 21. Fecal SCFA levels for the Rett syndrome patients treated with Bizetaflox Pediatrico before and after treatment. A paired t-test was used to determine the significant difference between the two sample groups. Key: ns – p value > 0.05.

5.3 Impact of treatments on neurotransmitters and precursors

Neurotransmitter dysregulation in RETT syndrome patients has been identified by several studies. Other studies have shown that gut microbiota is involved in the synthesis and/or modulation of certain neurotransmitters. RETT syndrome patients have reduced gut-microbiota α-diversity, which affects the synthesis and/or modulation of neurotransmitters. Therefore, we conducted a targeted LC-MS approach for the quantification of neurotransmitters, neuromodulators, and their precursors in plasma and fecal samples before and after treatment to observe what modulations had occurred due to the prebiotics and/or postbiotics. A total of 50 molecules were targeted, and 37 molecules were identified and quantified (Table 3).

Table 3. List of detected molecules in plasma and fecal samples using LC-MS.

Molecule	Abbreviation	Column	Retention Time (min)	Mass to charge ratio (m/z)
L-arginine	Arg	C18	1.0	175.1190
L-lysine	Lys	C18	1.0	147.1133
L-carnitine	LC	C18	1.1	162.113
Creatinine		C18	1.1	114.0667
L-acetyl carnitine	ALC	C18	1.3	204.1236
L-glutamic acid	Glu	C18	1.4	148.0604
2-pyrrolidinone		C18	2.2	86.0600
Uridine		C18	2.3	245.0774
Nudifloramide		C18	2.9	153.0659
Kynurenine	Kyn	C18	3.4	209.0921
L-phenylalanine	Phe	C18	3.7	166.0863
2-phenethylamine	PEA	C18	3.7	122.0963
L-tryptophan	Trp	C18	4.7	205.0970
Indole-3-acrylic acid		C18	4.7	188.0708
Indole-3-acetonitrile	IAN	C18	4.8	157.0760
Phenylacetyl L-glutamine	PAG	C18	6.2	265.1183
N-[3-[2-(formylamino)-5-methoxyphenyl]-3-oxypropyl]-acetamide	AFMK	C18	6.2	265.1183
Hippuric acid		C18	6.3	180.0655
Indole-3-lactic acid		C18	7.1	206.0812
Indole-3-carboxaldehyde	I3A	C18	7.3	146.0596
Indole-3-propionic acid	IPA	C18	7.9	190.0863
Methyl indole-3-acetic acid	meIAA	C18	7.9	190.0862
Cortisol		C18	7.9	363.2166
Serotonin	5-HT	HILIC	3.0	177.1022
Tyramine		HILIC	3.0	138.0913
Kynurenic acid	Kyna	HILIC	3.2	190.0499
Choline		HILIC	3.4	104.1070
Nα-acetyl-L-glutamine	GlcNAc	HILIC	3.6	189.0870
Nicotinic acid	niacin	HILIC	3.7	124.0393
L-methionine	Met	HILIC	4.2	150.0589
L-proline	Pro	HILIC	4.5	116.0706
L-tyrosine	Tyr	HILIC	4.5	182.0812
Histamine		HILIC	4.6	112.0869
L-pyroglutamic acid	PCA	HILIC	4.9	130.0497
3-aminopiperidine-2,6-dione		HILIC	5.3	129.0659
γ-aminobutyric acid	GABA	HILIC	5.4	104.0703
Trimethylamine N-oxide	TMAO	HILIC	5.4	76.0757

5.3.1 Circulating neurotransmitters, neuromodulators and precursors - Lalbaay®

For the Lalbaay® treatment, 10 of the 37 circulating molecules identified and quantified were modulated. Levels of circulating choline, L-arginine, 2-pyrrolidinone, L-phenylalanine, 2-phenethylamine, indole-3-acrylic acid, L-lysine, L-carnitine, and L-tryptophan decreased, while Nα-acetyl-L-glutamine increased (Figure 22).

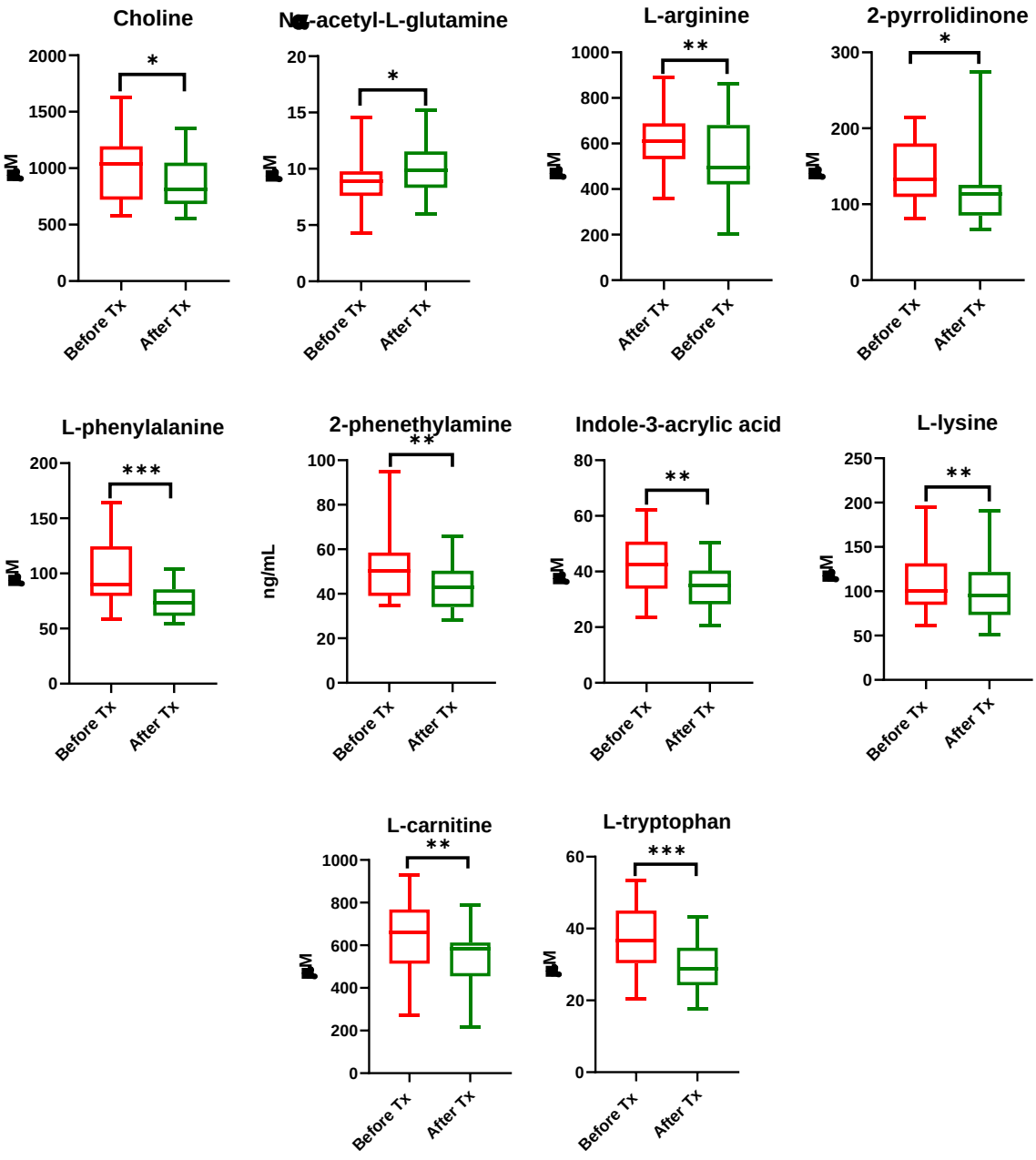

Figure 22. Circulating molecules before and after treatment with Lalbaay®. Key: * - p value < 0.05; ** - p value < 0.01; *** - p value < 0.001.

In Figures 23 and 24, the multivariate classification PLS-DA and VIP score were conducted in Metaboanalyst 6.0. For the PLS-DA in Figure 23, there is no clear-cut separation between the groups, although two groups of samples can be identified. From the VIP score in Figure 24, it can be concluded that choline contributes the most towards the separation of the two sample groups, followed by L-carnitine, L-arginine, nudifloramide, and L-phenylalanine as the top five, respectively.

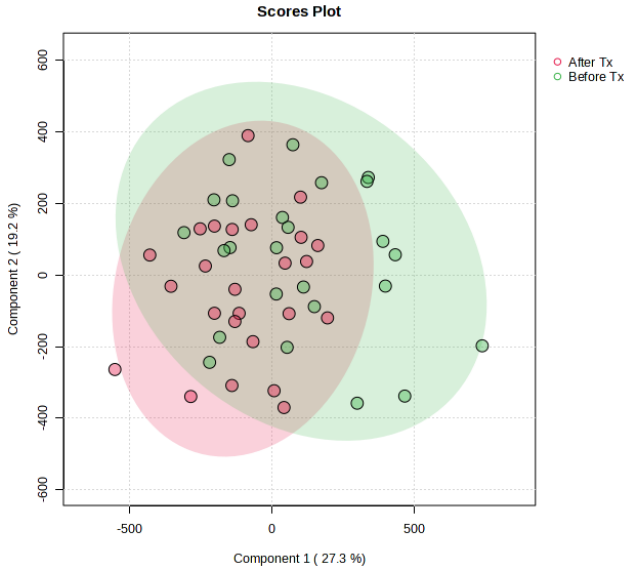

Figure 23. PLS-DA scores plot for circulating molecules in patients treated with Lalbaay®.

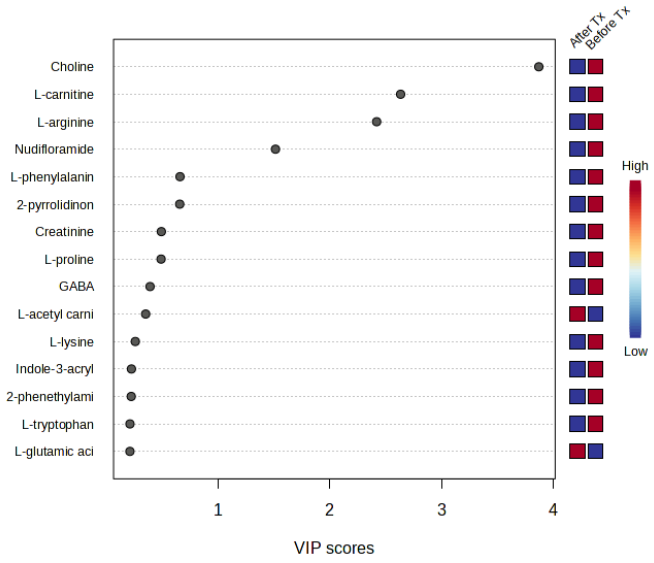

Figure 24. Top 15 VIP scores plot for circulating molecules for patients treated with Lalbaay®.

A hierarchical clustering heat map (Figure 25), generated using Metaboanalyst 6.0, illustrating the differences in circulating molecules before and after treatment with Lalbaay®. Levels of circulating choline, L-arginine, 2-pyrrolidinone, L-phenylalanine, 2-phenethylamine, indole-3-acrylic acid, L-lysine, L-carnitine, and L-tryptophan decreased, while Nα-acetyl-L-glutamine increased.

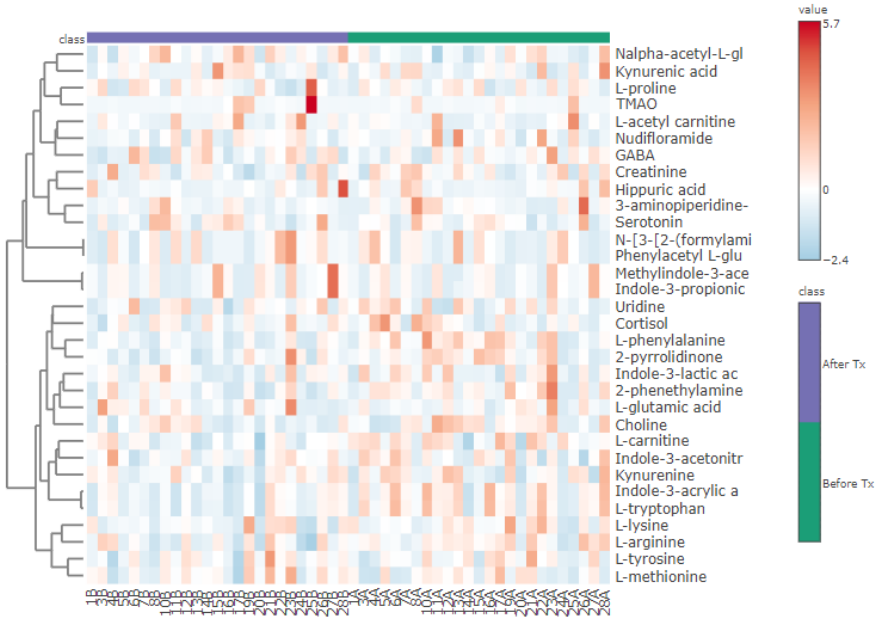

Figure 25. A hierarchical clustering heat map showing levels of circulating molecules before and after treatment with Lalbaay®.

A correlation heat map of variables (Figure 26) generated using Metaboanalyst 6.0, illustrates correlations among neurotransmitters, neuromodulators, precursors, SDSC, MBAS, and RSBQ in patients treated with Lalbaay®.

MBAS shows a moderate positive correlation with L-lysine, L-arginine, 2-pyrrolidinone, RSBQ, and SDSC. RSBQ exhibits a moderate positive correlation with indole-3propionic acid and methylindole-3-acetate. SDSC shows a moderate positive relationship with L-carnitine and 3-aminopiperidine, while it has a negative correlation with cortisol, serotonin, indole-3-lactic acid, and L-glutamic acid.

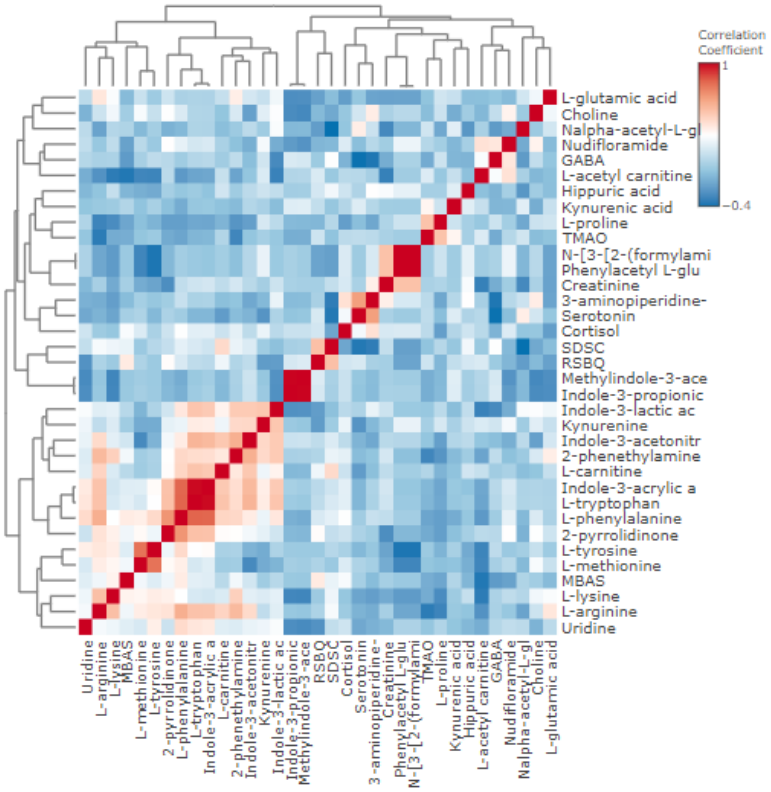

Figure 26. A hierarchical clustering heat map showing circulating molecules, MBAS, RSBQ, and SDSC in patients treated with Lalbaay®.

During the washout period, levels of 3-aminopiperidine-2,6-dione, L-methionine, Choline, L-tyrosine, and nudifloramide increased while GABA levels decreased for patients treated with Lalbaay® (Figure 27).

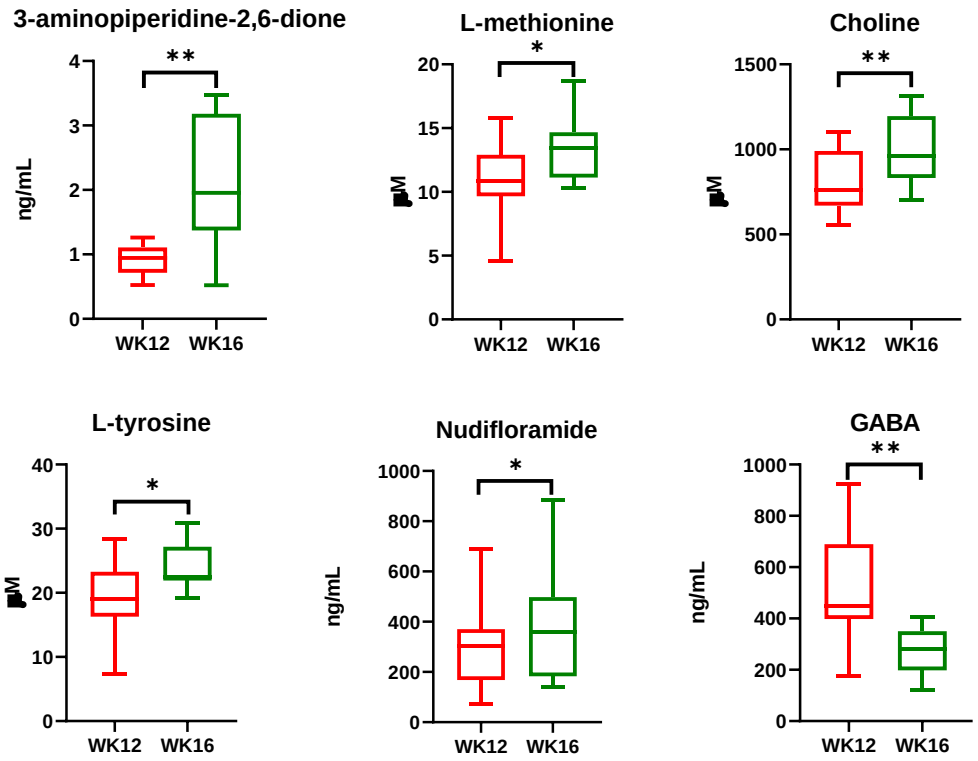

Figure 27. Modulated molecules during the washout period for patients treated with Lalbaay®. Key: * - p value < 0.05; ** - p value < 0.01.

5.3.2 Circulating neurotransmitters, neuromodulators and precursors – Bizetaflox Pediatrico

For the Bizetaflox Pediatrico treatment, 5 of the 37 circulating molecules identified and quantified were modulated. Levels of circulating 2-pyrrolidinone, nudifloramide, L-phenylalanine, 2-phenethylamine, and L-tryptophan decreased (Figure 28).

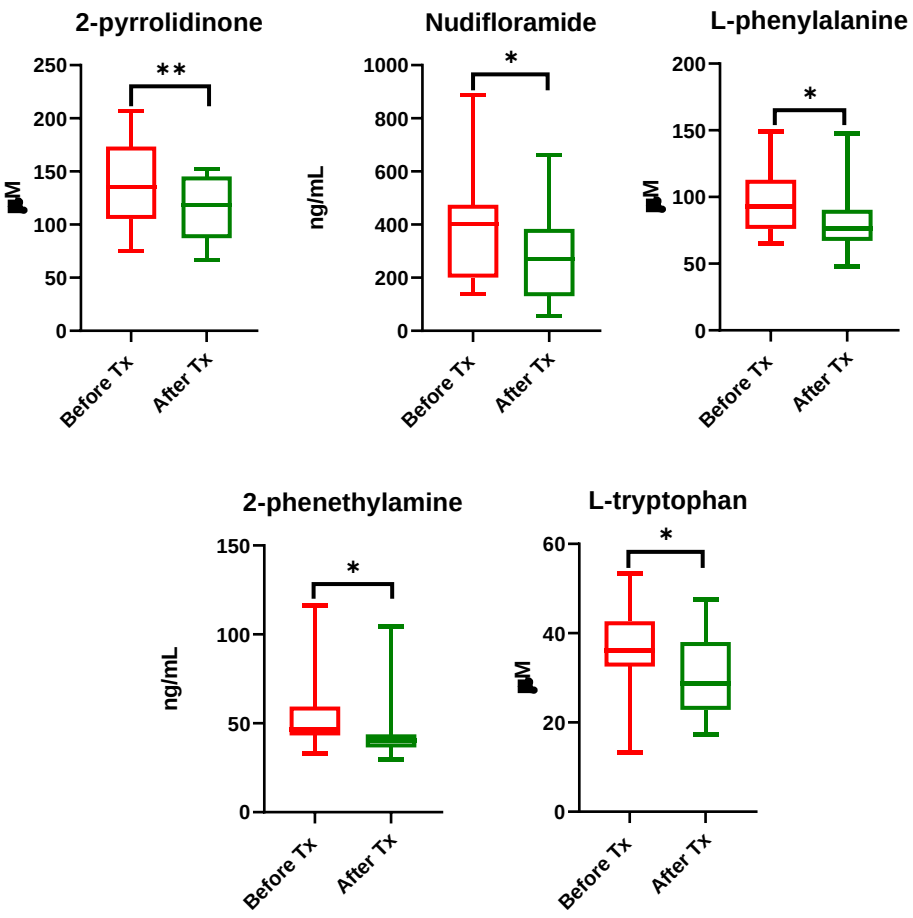

Figure 28. Circulating molecules modulated after treatment with Bizetaflox Pediatrico. Key: * - p value < 0.05; ** - p value < 0.01.

In Figures 29 and 30, the multivariate classification PLS-DA and VIP score were conducted in Metaboanalyst 6.0. For the PLS-DA in Figure 29, there is no clear-cut separation between the groups, although two groups of samples can be identified. From the VIP score in Figure 30, it can be concluded that nudifloramide, choline, L-arginine, GABA, L-carnitine, and L-proline contribute the most towards the separation of the two sample groups, as the top five, respectively.

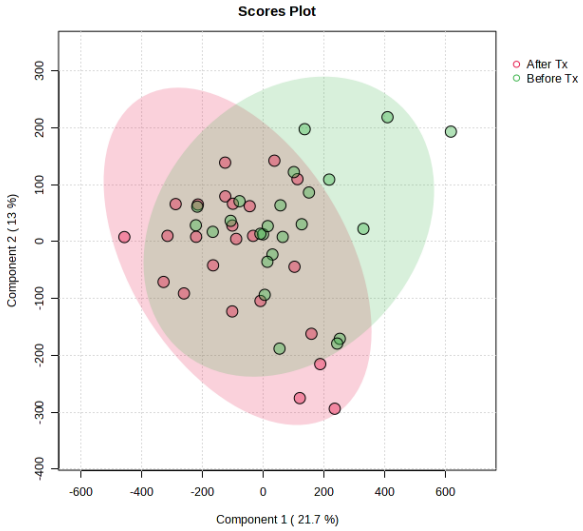

Figure 29. PLS-DA scores plot for circulating molecules in patients treated with Bizetaflox Pediatrico.

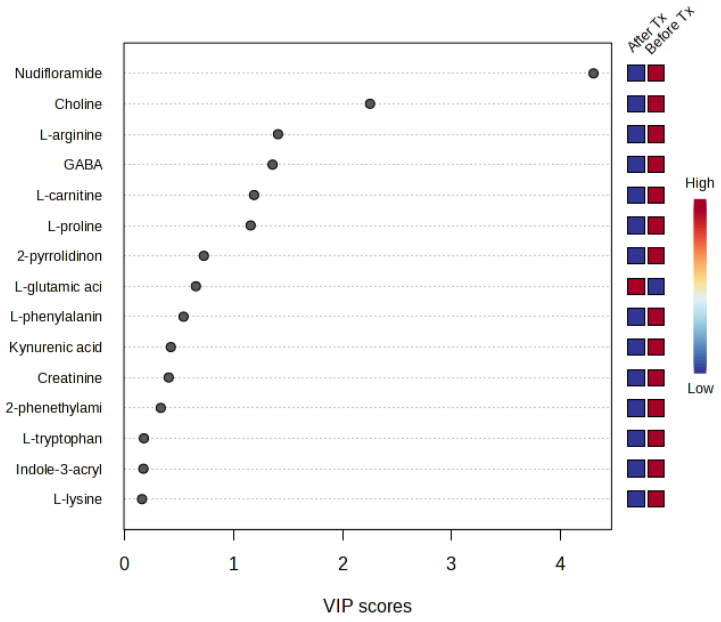

Figure 30. Top 15 VIP scores plot for circulating molecules for patients treated with Bizetaflox Pediatrico.

A hierarchical clustering heat map (Figure 31), illustrating the differences in circulating molecules before and after treatment with Bizetaflox Pediatrico. Levels of circulating of 2-pyrrolidinone, nudifloramide, L-phenylalanine, 2-phenethylamine, and L-tryptophan decreased.

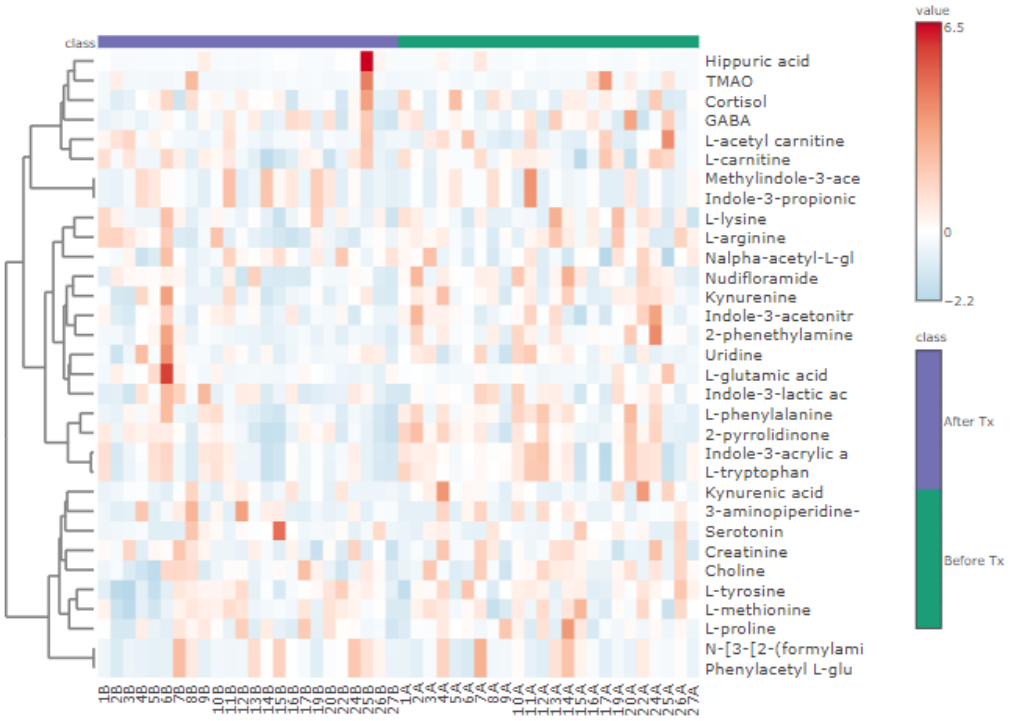

Figure 31. A hierarchical clustering heat map showing levels of circulating molecules before and after treatment with Bizetaflox Pediatrico.

Figure 32 shows a correlation heat map illustrating correlations among neurotransmitters, neuromodulators, precursors, SDSC, MBAS, and RSBQ in patients treated with Bizetaflox Pediatrico.

MBAS shows a moderate positive correlation with L-arginine, and Nα-acetyl-L-glutamine. SDSC shows a moderate positive relationship with kynurenine, indole-3-acrylic acid, L-glutamic acid, uridine, 2-phenethylamine, GABA, cortisol, L-arginine, and L-lysine.

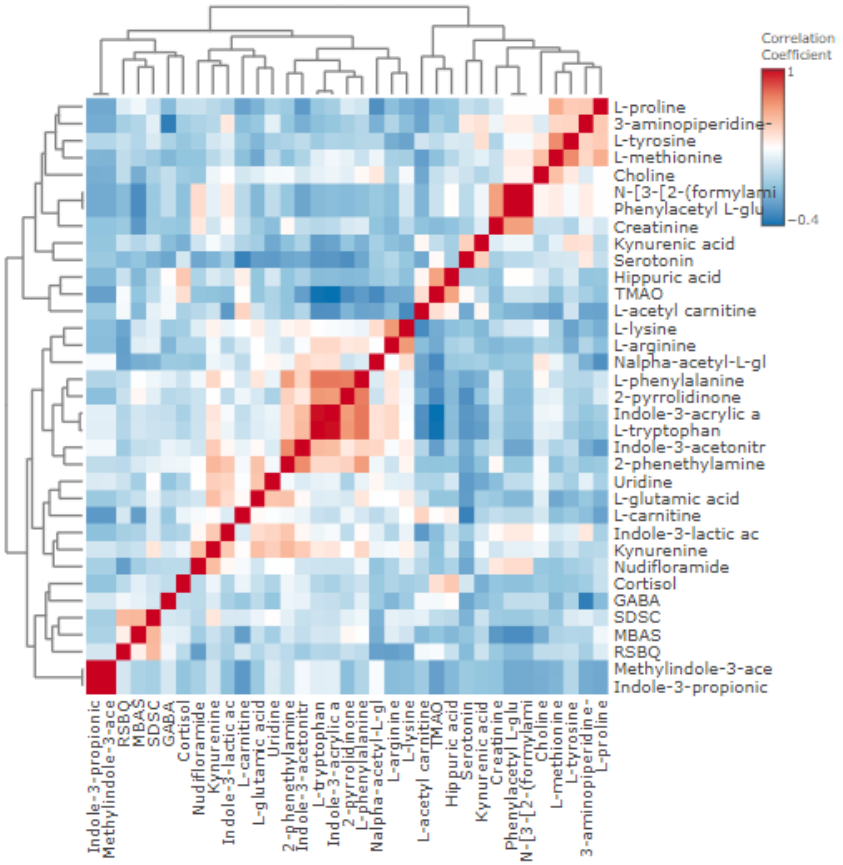

Figure 32. A hierarchical clustering heat map showing circulating molecules, MBAS, RSBQ, and SDSC before and after treatment with Bizetaflox Pediatrico.

During the washout period for patients treated with Bizetaflox Pediatrico, indole-3-propionic acid and indole-3-acetonitrile levels decreased, while 3-amino-piperidine-2,6-dione and nudifloramide levels increased (Figure 33).

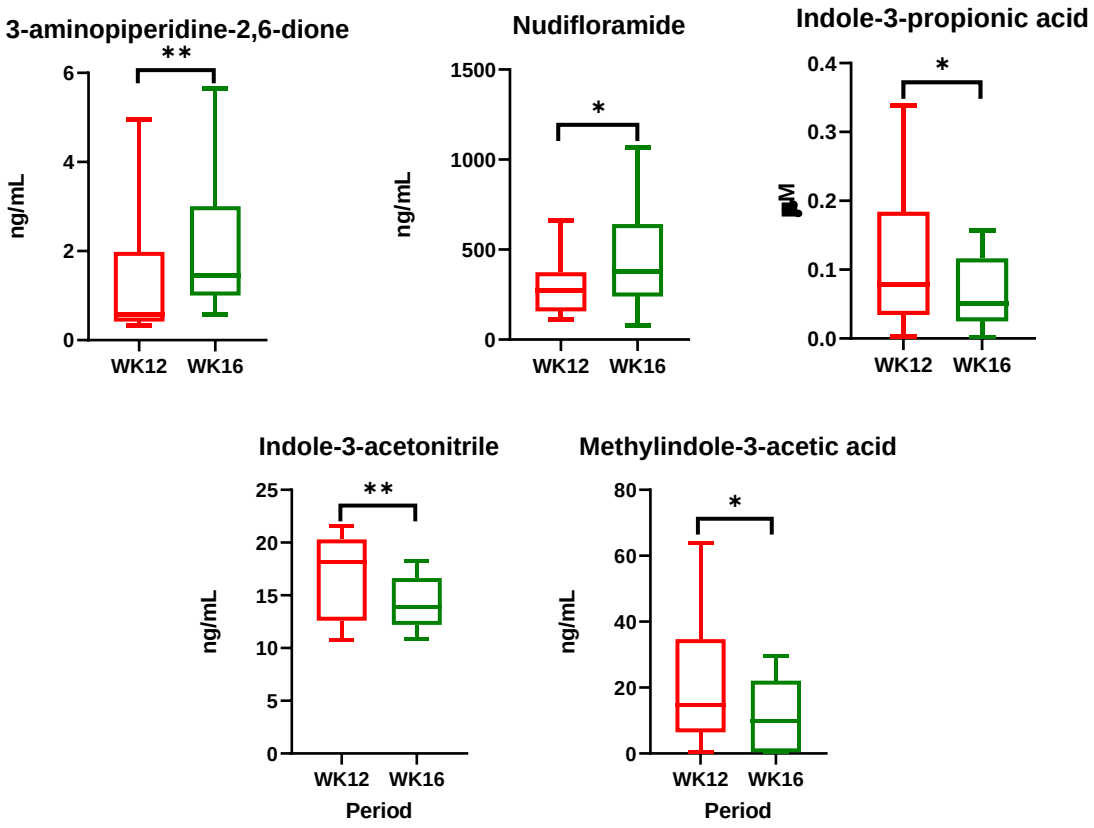

Figure 33. Modulated molecules during the washout period for patients treated with Bizetaflox Pediatrico. Key: * - p value < 0.05; ** - p value < 0.01.

5.3.3 Neurotransmitters, neuromodulators and precursors in fecal samples – Lalbaay®

For the Lalbaay® treatment, 2 of the 37 molecules identified and quantified were modulated in the fecal samples. Levels of indole-3-acrylic acid and L-phenylalanine decreased (Figure 34).

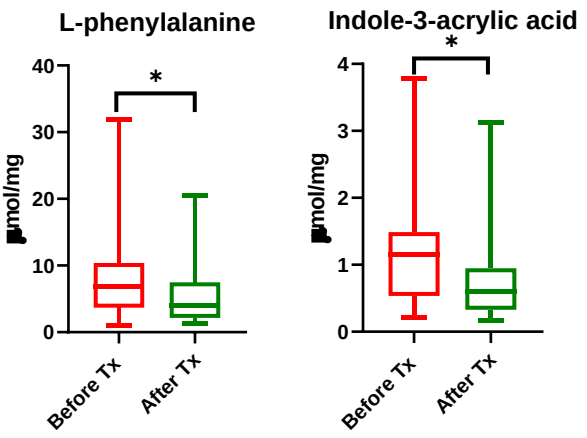

Figure 34. Modulated molecules in fecal samples before and after treatment with Lalbaay®. Key: * - p value < 0.05.

Figures 35 and 36 show the multivariate classification PLS-DA and VIP score. For the PLS-DA in Figure 35, there is no clear-cut separation between the groups, although two groups of samples can be identified. The VIP score in Figure 36 shows that histamine, L-proline, choline, L-glutamic acid, and GABA contribute the most towards the separation of the two sample groups, as the top five, respectively.

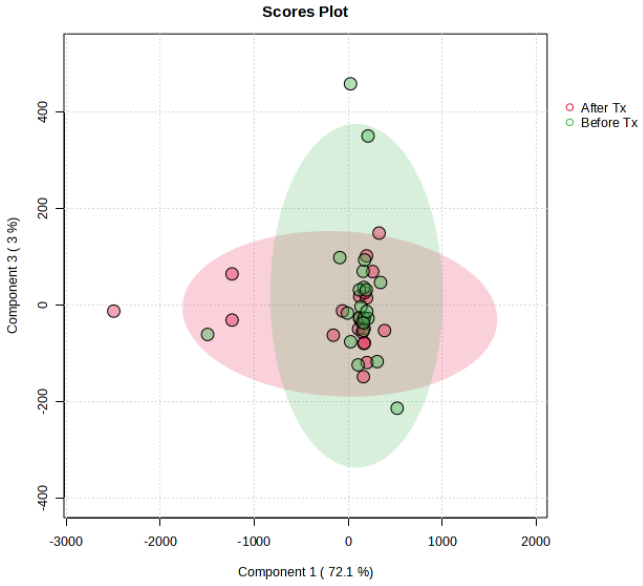

Figure 35. PLS-DA scores plot for molecules in the fecal samples for patients treated with Lalbaay®.

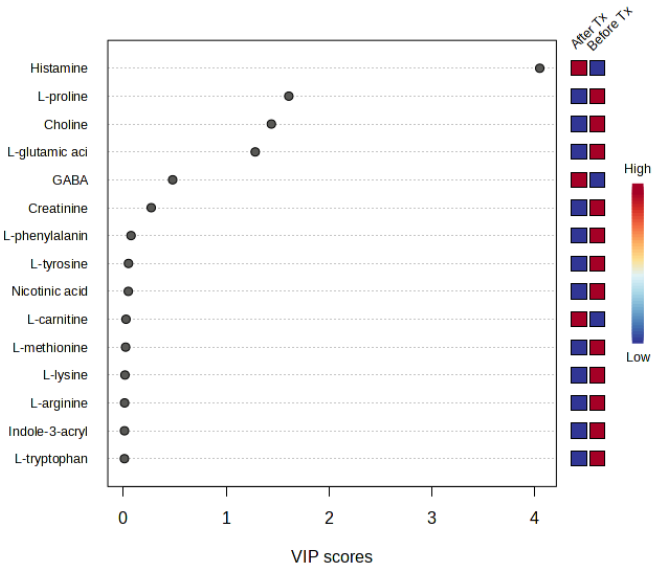

Figure 36. Top 15 VIP scores plot for molecules in fecal samples from patients treated with Lalbaay®.

Figure 37 shows a hierarchical clustering heat map illustrating levels of molecules in fecal samples before and after treatment with Lalbaay®. Levels of indole-3-acrylic acid and L-phenylalanine decreased.

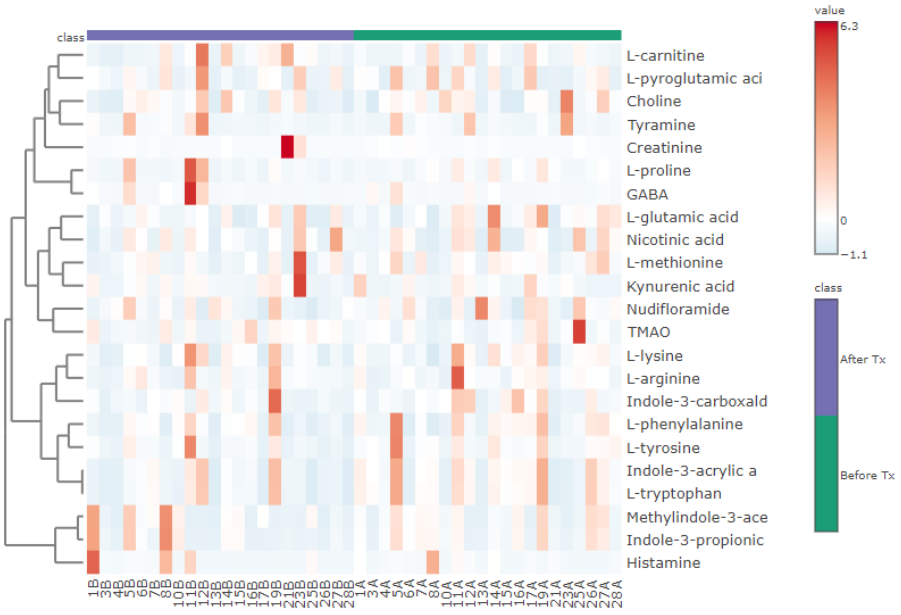

Figure 37. A hierarchical clustering heat map showing levels of molecules in fecal samples before and after treatment with Lalbaay®.

Figure 38 shows a correlation heat map illustrating correlations among neurotransmitters, neuromodulators, precursors, SDSC, MBAS, and RSBQ in patients treated with Lalbaay®.
MBAS shows a strong positive correlation with L-glutamic acid, GABA, L-proline, Choline, and L-carnitine. RSBQ exhibits strong positive correlation with histamine, indole-3-propionic acid, creatinine, L-glutamic acid, L-tyrosine, GABA, L-proline, and choline. SDSC shows a strong positive relationship with indole-3-propionic acid, histamine, methylindole-3-acetic acid, nudifloramide, nicotinic acid, L-lysine, L-phenylalanine, L-tryptophan, indole-3-acrylic acid, and choline.

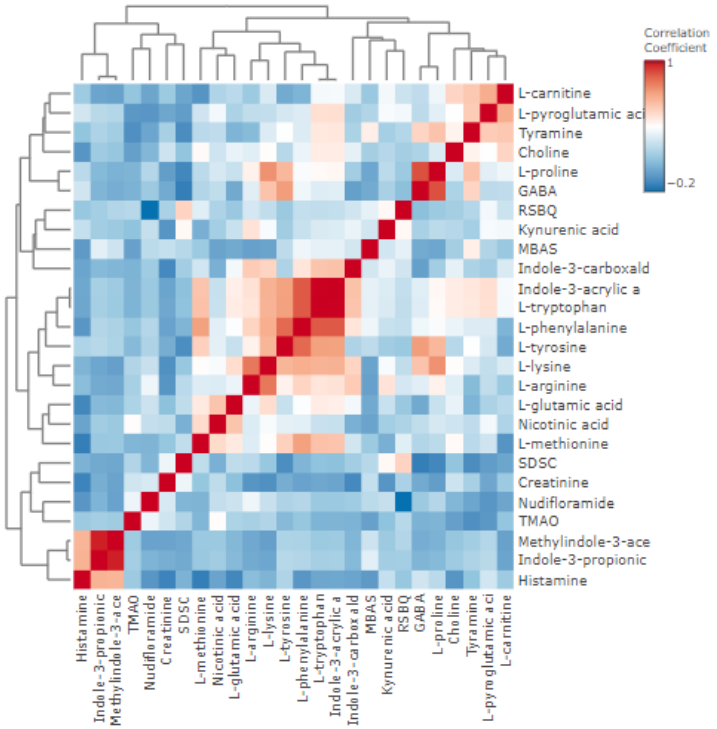

Figure 38. A hierarchical clustering heat map showing molecules in fecal samples, MBAS, RSBQ, and SDSC in patients treated with Lalbaay®.

For the washout period for patients treated with Lalbaay®, there were no modulations in the fecal samples.

5.3.3 Neurotransmitters, neuromodulators and precursors in fecal samples – Bizetaflox Pediatrico

For the Bizetaflox Pediatrico treatment, 5 of the 37 molecules identified and quantified were modulated in the fecal samples. Levels of indole-3-acrylic acid, L-methionine, L-glutamic acid, nudifloramide, and indole-3-carboxyaldehyde decreased (Figure 39).

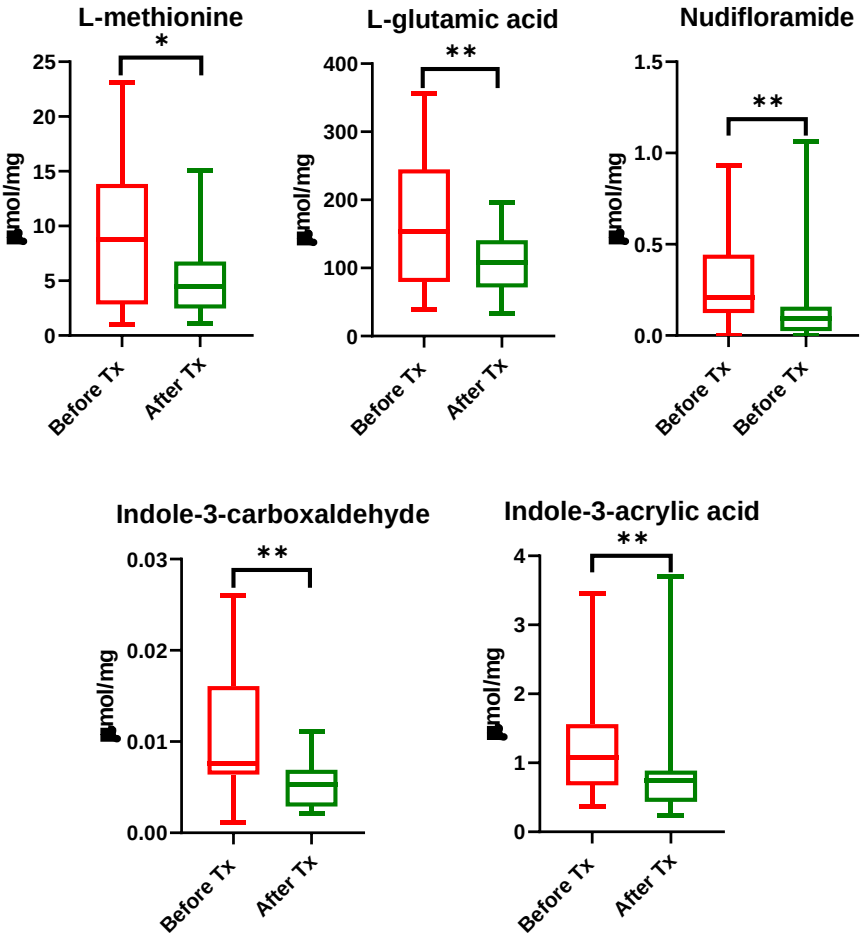

Figure 39. Molecules in fecal samples modulated after treatment with Bizetaflox Pediatrico. Key: * - p value < 0.05; ** - p value < 0.01.

Figures 40 and 41 show the multivariate classification PLS-DA and VIP score. For the PLS-DA in Figure 40, there is no clear-cut separation between the groups, although two groups of samples can be identified. From the VIP score in Figure 41, it can be concluded that L-proline, L-glutamic acid,

histamine, choline, and nicotinic acid contribute the most towards the separation of the two sample groups, as the top five, respectively.

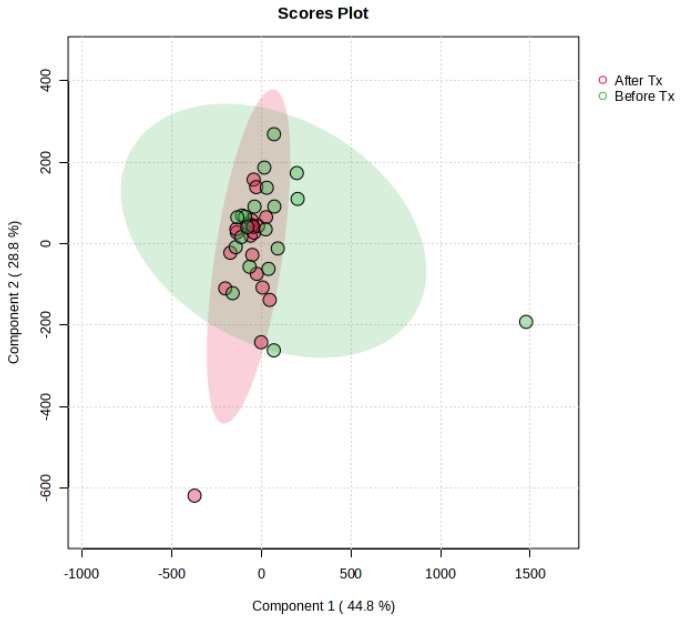

Figure 40. PLS-DA scores plot for molecules in the fecal samples for patients treated with Bizetaflox Pediatico.

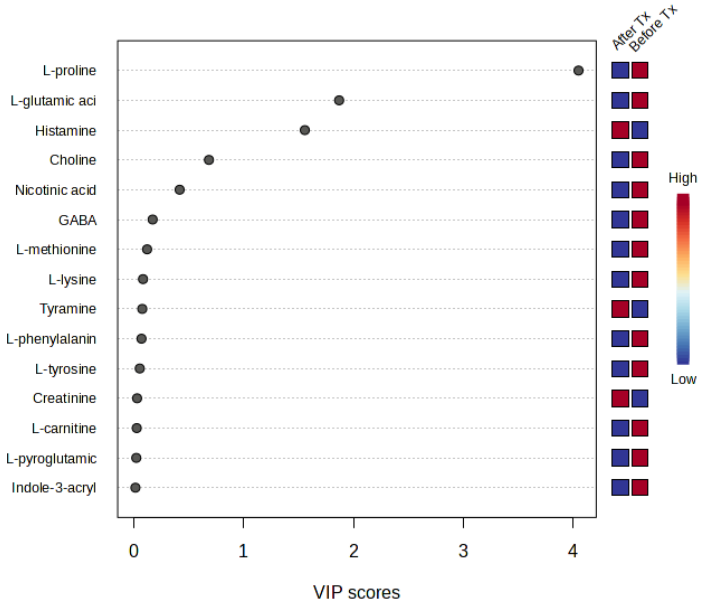

Figure 41. Top 15 VIP scores plot for molecules in fecal samples from patients treated with Bizetaflox Pediatrico.

A hierarchical clustering heat map (Figure 42), illustrating the differences in molecules in fecal samples before and after treatment with Bizetaflox Pediatico. Levels of indole-3-acrylic acid, L-methionine, L-glutamic acid, nudifloramide, and indole-3-carboxyaldehyde decreased.

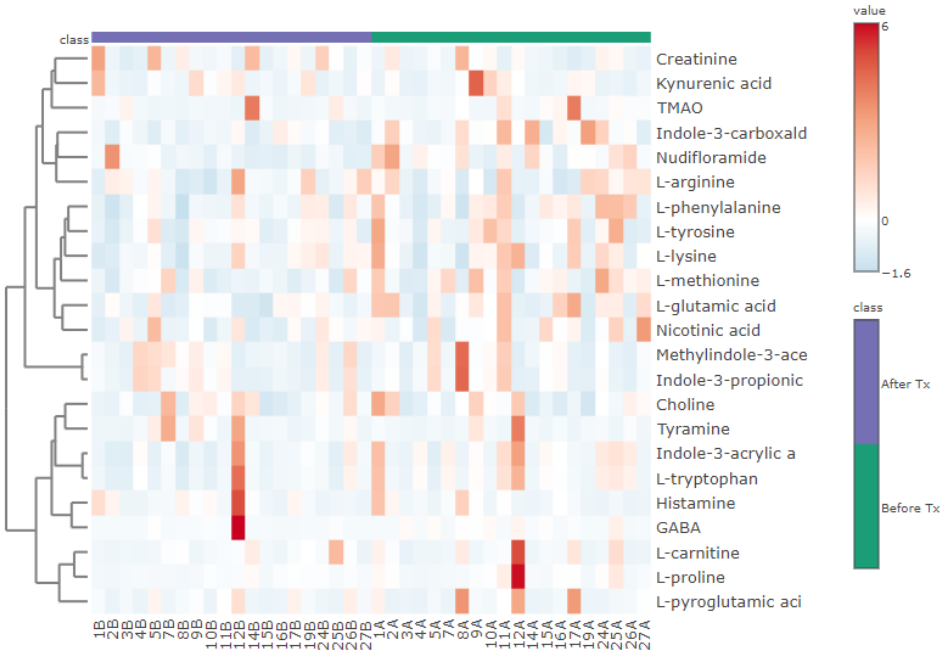

Figure 42. A hierarchical clustering heat map showing levels of molecules in fecal samples from patients treated with Bizetaflox Pediatico.

A correlation heat map (Figure 43) illustrating correlations among neurotransmitters, neuromodulators, precursors, SDSC, MBAS, and RSBQ in patients treated with Bizetaflox Pediatrico.

MBAS shows a strong positive correlation with L-lysine, L-tryptophan, and indole-3-acrylic acid. RSBQ exhibits strong positive correlation with L-pyroglutamic acid, L-carnitine, indole-3-acrylic acid, and L-tryptophan. SDSC shows a strong positive relationship with nudifloramide, L-tryptophan, indole-3-acrylic acid, L-arginine, GABA, and histamine.

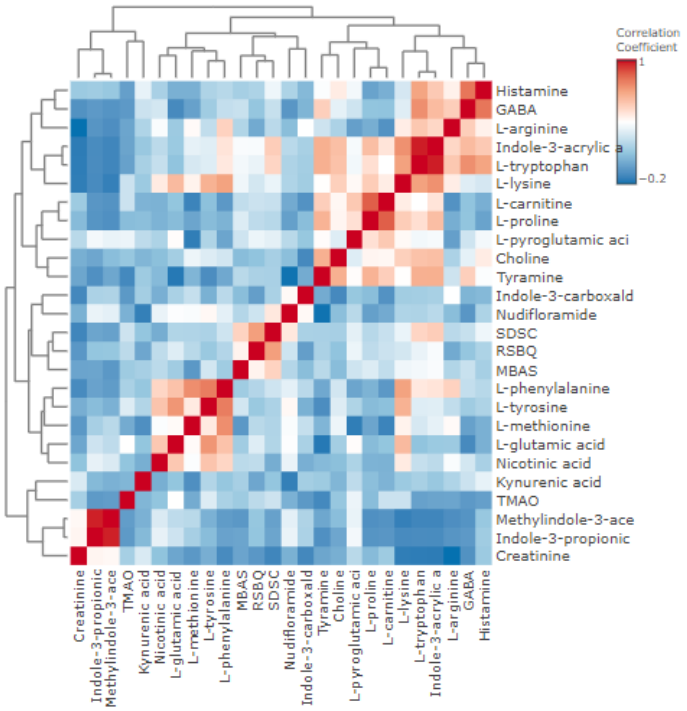

Figure 43. A hierarchical clustering heat map showing molecules in fecal samples, MBAS, RSBQ, and SDSC in patients treated with Bizetaflox Pediatrico.

During the washout period for patients treated with Bizetaflox Pediatrico, levels of choline, L-methionine, indole-3-carboxaldehyde, and indole-3-acrylic acid levels increased (Figure 44).

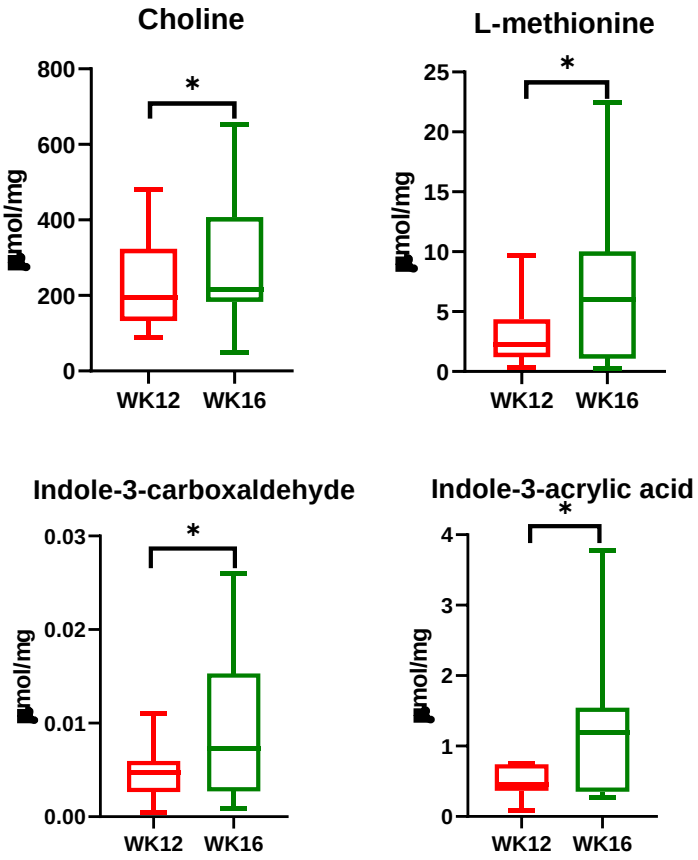

Figure 44. Modulated molecules in fecal samples during washout period in patients treated with Bizetaflox Pediatrico. Key: * - p value < 0.05.

5.4 Plasma and fecal correlations

Plasma and fecal sample correlations are able to shed light on the current metabolomic state of the body. Specifically, they can provide insights into the gut microbiota status in response to different treatments. Correlations between matrices were performed using R software version 4.3.

5.4.1 SCFA correlations - Lalbaay®

Figure 45 shows correlations of molecules between plasma and fecal matrices before treatment with Lalbaay®. Circulating acetic acid has a moderate negative correlation with fecal butanoic acid, butanoic acid 3-methyl, pentanoic acid, and propanoic acid 2-methyl. There is weak positive correlation between circulating butanoic acid 3-methyl and fecal propanoic acid 3-methyl. Plasma butanoic acid has a negative correlation with fecal butanoic acid. Circulating pentanoic acid has a

weak negative correlation with fecal butanoic acid, and a weak positive correlation with fecal propanoic acid 2-methyl. Circulating propanoic acid 2-methyl has a moderate negative correlation with fecal butanoic acid. There is a moderate negative correlation between circulating propanoic acid and fecal butanoic acid.

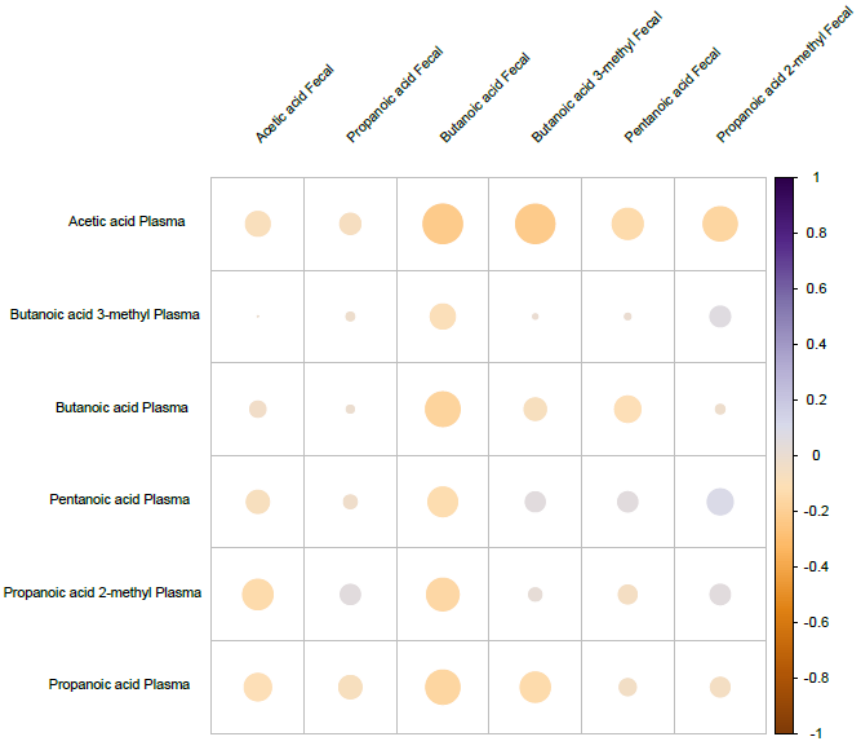

Figure 45. Correlations between plasma and fecal matrices before treatment with Lalbaay®.

Figure 46 shows correlations of molecules between plasma and fecal matrices after treatment with Lalbaay®. Circulating acetic acid has a negative correlation with fecal butanoic acid, pentanoic acid and propanoic acid 2-methyl. There is a weak positive correlation between circulating butanoic acid 3-methyl and fecal propanoic acid 2-methyl, while there is a weak negative correlation between circulating butanoic acid 3-methyl and fecal butanoic acid. Circulating butanoic acid has a negative correlation with fecal butanoic acid. Circulating pentanoic acid has a moderate negative correlation with fecal butanoic acid, and a moderate positive correlation with propanoic acid 2-methyl. Circulating propanoic acid 2-methyl is negatively correlated with fecal acetic acid and butanoic acid. There is a moderate negative correlation between circulating

propanoic acid and fecal butanoic acid, and between circulating propanoic acid and butanoic acid 3-methyl.

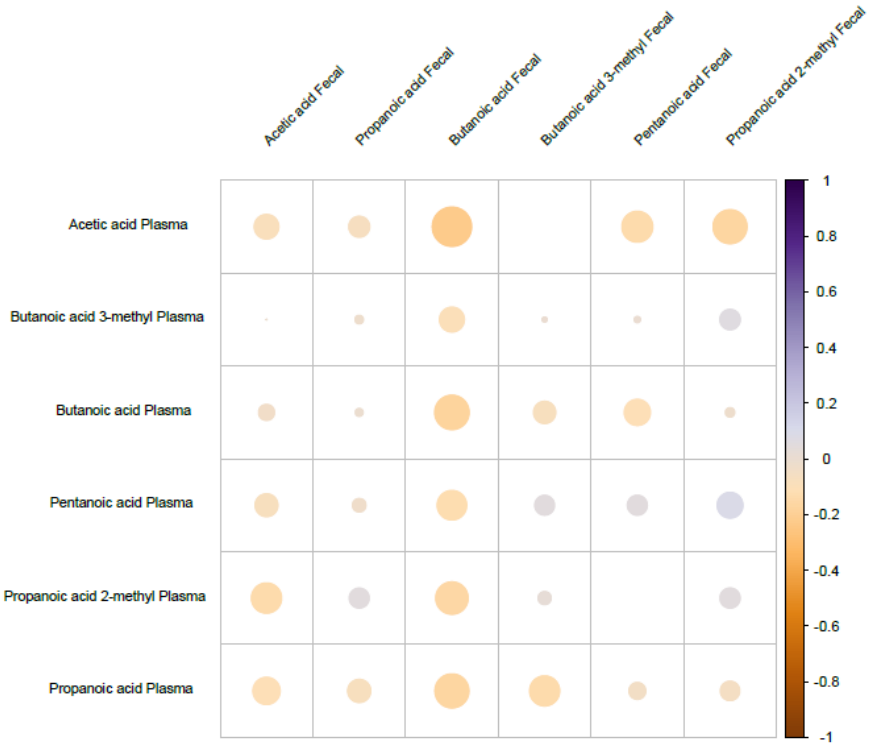

Figure 46. Correlations between plasma and fecal matrices after treatment with Lalbaay®.

5.4.2 SCFA correlations - Bizetaflox Pediatrico

Figure 47 shows correlations of molecules between plasma and fecal matrices before treatment with Bizetaflox Pediatrico. Circulating acetic acid has a negative correlation with fecal acetic acid and pentanoic acid. Circulating butanoic acid 3-methyl has a moderate correlation with fecal butanoic acid 3-methyl, pentanoic acid and propanoic acid 3-methyl, and a moderate correlation with fecal acetic acid. There is a negative correlation between circulating butanoic acid and fecal acetic acid. Circulating butanoic acid and fecal propanoic acid are negatively correlated. Circulating pentanoic acid is negatively correlated with fecal acetic acid and propanoic acid. There is a positive correlation between propanoic acid 2-methyl and fecal butanoic acid. Circulating propanoic acid is negatively correlated with fecal propanoic acid.

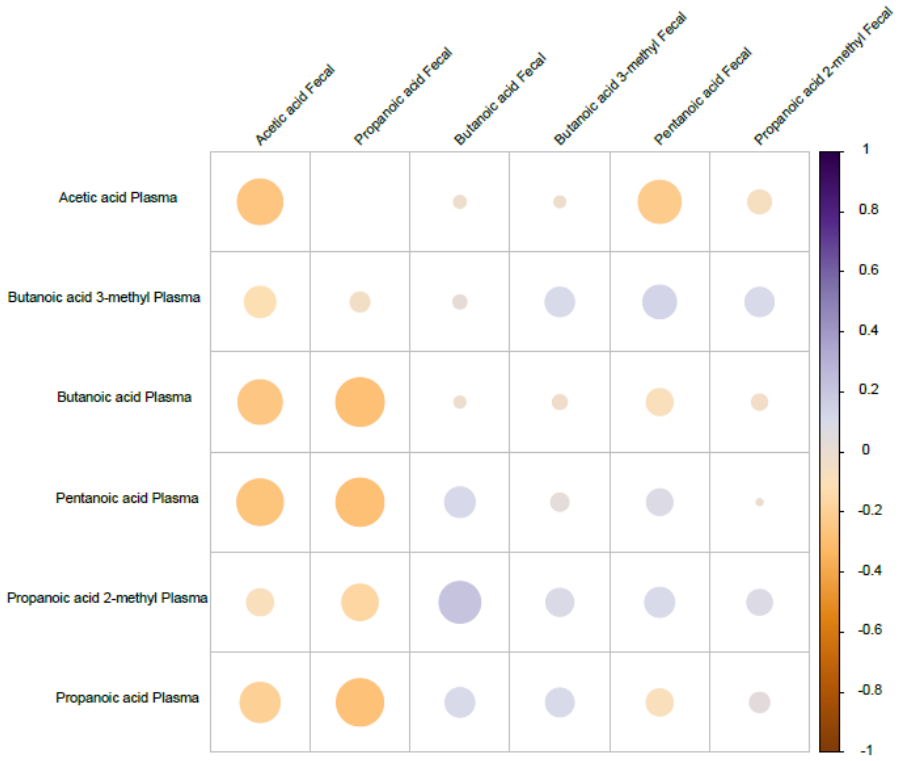

Figure 47. Correlations between plasma and fecal matrices before treatment with Bizetaflox Pediatrico.

Figure 48 shows correlations of molecules between plasma and fecal matrices after treatment with Bizetaflox Pediatrico. Circulating acetic acid has a negative correlation with fecal acetic acid, propanoic acid, and pentanoic acid. There is a moderate positive correlation between circulating butanoic acid 3-methyl and fecal butanoic acid 3-methyl, circulating butanoic acid 3-methyl and fecal pentanoic acid, and circulating butanoic acid 3-methyl and fecal propanoic acid 2-methyl. Circulating butanoic acid has a negative correlation with fecal acetic acid and propanoic acid. Circulating pentanoic acid has a negative correlation with fecal acetic acid and propanoic acid. There is a positive correlation between circulating propanoic acid 2-methyl and fecal butanoic acid. Circulating propanoic acid has a positive correlation with fecal acetic acid and propanoic acid.

Figure 48. Correlations between plasma and fecal matrices before treatment with Bizetaflox Pediatrico.

5.4.3 Neurotransmitter correlations - Lalbaay®

Figure 49 shows correlations of molecules between plasma and fecal matrices before treatment with Lalbaay®. Circulating 2-pyrrolidinone has a positive correlation with fecal kynurenic acid and L-methionine. Circulating creatinine has a negative correlation with fecal L-lysine. There is a positive correlation between circulating indole-3-propionic and fecal nicotinic acid. Plasma L-glutamic acid has a positive correlation with fecal kynurenic acid and L-methionine. Circulating L-lysine has a positive correlation with fecal L-tryptophan, indole-3-acrylic acid, and indole-3-carboxyaldehyde. There is a positive correlation between circulating L-methionine and fecal kynurenic acid. Circulating L-tyrosine has a positive correlation with fecal creatinine, while circulating methoxyindole-3-acetic acid and fecal nicotinic acid.

Figure 49. Correlations between plasma and fecal matrices before treatment with Lalbaay®.

Figure 50 shows correlations of molecules between plasma and fecal matrices after treatment with Lalbaay®. There is a positive correlation between circulating 2-phenethylamine and fecal choline. Circulating L-acetyl carnitine has a positive correlation with fecal TMAO, L-arginine, L-lysine, and nicotinic acid. There is a strong negative correlation between circulating creatinine and fecal TMAO. Circulating nudifloramide has a strong positive correlation with fecal L-arginine and nudifloramide. Circulating GABA has a negative correlation with fecal indole-3-propionic acid and methylindole-3-acetic acid.

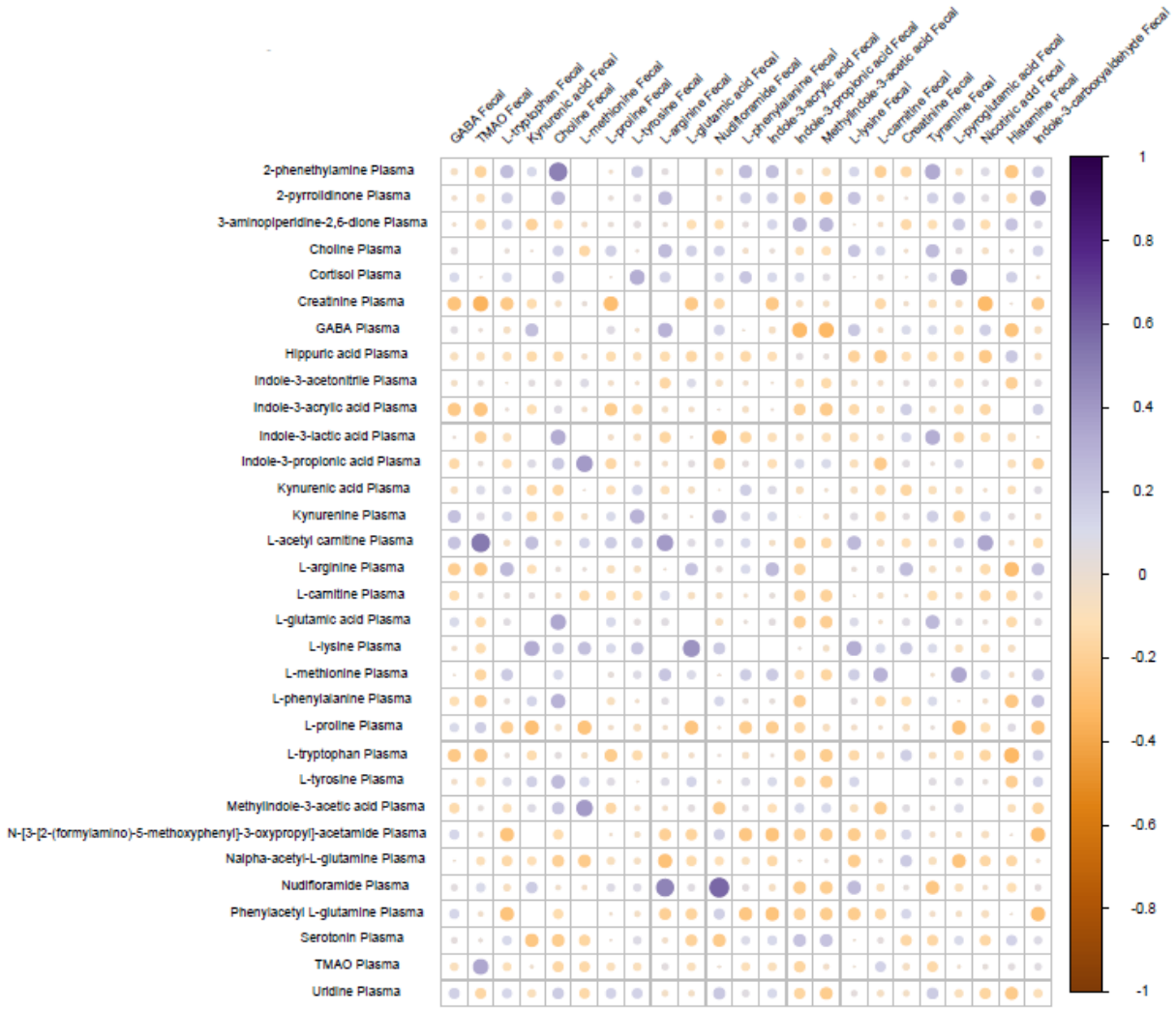

Figure 50. Correlations between plasma and fecal matrices after treatment with Lalbaay®.

5.4.4 Neurotransmitter correlations - Bizetaflox Pediatrico

Figure 51 shows correlations of molecules between plasma and fecal matrices before treatment with Bizetaflox Pediatrico. Circulating 3-aminopiperidine-2,6-dione has a positive correlation with fecal GABA, L-tryptophan, indole-3-acrylic acid, tyramine, and histamine. Circulating indole-3-lactic acid has a positive correlation with fecal indole-3-propionic acid and methoxyindole-3-acetic acid. There is a strong negative correlation between circulating L-tryptophan and fecal TMAO. Circulating serotonin has a negative correlation with fecal L-glutamic acid, L-tyrosine, and choline.

Figure 51. Correlations between plasma and fecal matrices before treatment with Bizetaflox Pediatrico.

Figure 52 shows correlations of molecules between plasma and fecal matrices after treatment with Bizetaflox Pediatrico. Circulating 2-phenethylamine has a positive correlation with fecal L-methionine, L-phenylalanine, and indole-3-carboxyaldehyde. Circulating GABA has a positive correlation with fecal L-tyrosine and L-glutamic acid. There is a negative correlation between circulating indole-3-lactic acid and fecal TMAO, while circulating indole-3-lactic acid has a positive correlation with fecal L-proline and indole-3-carboxyaldehyde. Circulating choline has a negative correlation with fecal L-tyrosine, L-glutamic acid, and kynurenic acid.

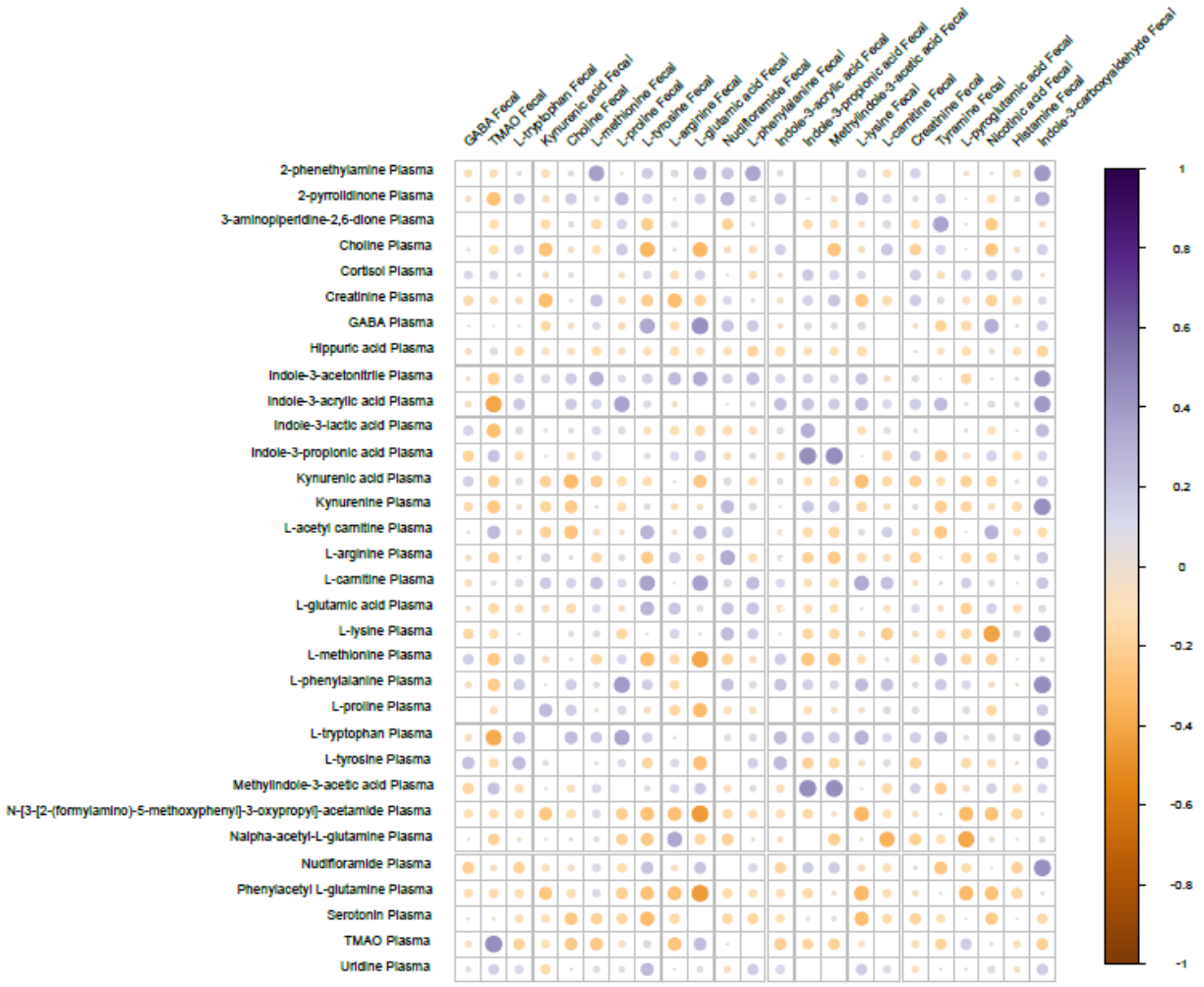

Figure 52. Correlations between plasma and fecal matrices after treatment with Bizetaflox Pediatrico.

6. Discussion

Recent studies have demonstrated the existence of a microbiota-gut-brain axis and the crucial roles of the gut microbiota, which include digestion, immune system regulation, synthesis of essential nutrients, and neuromodulation, among other functions (Jandhyala, 2015; Strandwitz, 2018). Gut microbiota dysbiosis has been linked to several diseases, including brain disorders, respiratory disease, cancer, inflammatory bowel disease, and many others (Hou et al., 2022). Borghi et al. (2017) demonstrated that individuals affected by RETT syndrome have a lower α-diversity compared to healthy individuals, which affects the normal function of the nervous system. Several strategies are being used to promote and modify gut microbiota abundance and diversity. These strategies include diet, prebiotics, postbiotics, probiotics, live biotherapeutic products, fecal microbiota transplantation, engineered bacteria, and phage therapy (Hou et al., 2022).

In this study, we investigated the effects of prebiotics and/or postbiotics on levels of neurotransmitters and SCFAs in RETT syndrome patients. The rationale of the study is that prebiotics and postbiotics can increase both the α-diversity and β-diversity of the gut microbiota, which in turn will modulate key molecules involved in the microbiota-gut-brain axis that can alleviate RETT syndrome symptoms. A clinical trial (NCT05420805) was conducted under the supervision of Prof. Claudio De Felice at the Policlinico Le Scotte – Siena, Italy. The 28 admitted patients were treated with both Lalbaay® and Bizetaflox Pediatrico (both from Kolfarma), with a washout period between drug crossovers.

During the trial, key RETT syndrome symptom assessments were investigated, including the RSBQ, MBAS, and SDSC. From total RSBQ score in Figure 5 we can conclude that both Lalbaay® and Bizetaflox Pediatrico are superior compared to Trofenetide in terms of reducing the severity of RETT syndrome symptoms. In addition, there were no adverse events with Lalbaay® and Bizetaflox Pediatrico, while Trofenitide caused diarrhea in 82% of the enrolled patients according to Keam (2023).
For the two different treatments, both MBAS and SDSC scores decreased during treatment, which indicates a reduction of RETT syndrome symptoms. During the washout period, both the MBAS

and SDSC scores increased, explained by the worsening of symptoms due to the discontinuation of the treatment.
From a clinical point of view, the trial was a success for both Lalbaay® and Bizetaflox Pediatrico, and the next task was to investigate the modulation of molecules in the body that led to the improvement of the symptoms using mass spectrometry techniques.

We evaluated the effect of prebiotics and/or postbiotics on SCFAs in plasma and fecal samples using GC-MS. SCFAs are the main metabolites produced by the gut microbiota from the fermentation of dietary fibers and complex carbohydrates. They play a key role in neuro-immunoendocrine regulation and are currently being explored as a possible target for the treatment of disorders involving the central nervous system (Silva et al., 2020).

Following the Lalbaay® treatment, there was a significant increase in circulating levels of acetic acid and propanoic acid, while levels of propanoic acid 2-methyl decreased (Figure 8). Erny et al. (2015) and Sampson et al. (2016) showed that acetic acid and propanoic acid are essential for microglia maturation and function. Microglia are the main macrophages in the central nervous system, and Derecki et al. (2012) used murine models to show that microglia are involved in the pathophysiology of RETT syndrome. The increase in the levels of circulating acetic acid and propanoic acid suggests that the activity of the microglia improved, lessening neuroinflammation, which is evidenced by the improvement in the RSBQ, SDSC, and MBAS scores. Furthermore, acetic acid can modulate the levels of neurotransmitters, namely glutamate, glutamine, and GABA (Frost et al., 2014). The decrease in propanoic acid 2-methyl circulating levels is possibly due to microbial competition, whereby acetic acid and propanoic acid-producing bacteria thrived over propanoic acid 2-methyl-producing bacteria. Under normal conditions, acetic acid, propanoic acid, and butanoic acid are the most abundant SCFAs produced by the gut microbiota (Portincasa et al., 2022).
During the washout period for patients treated with Lalbaay®, circulating propanoic acid levels continued to increase, while levels of butanoic acid 3-methyl decreased (Figure 13). Propanoic acid levels continued to increase even after discontinuation of the treatment because the effect of the prebiotics had a longer-lasting impact on the microbial community. The decrease in butanoic

acid 3-methyl levels during washout is possibly due to the shift in microbial ecology caused by the discontinued treatment.

In the fecal samples of the patients treated with Lalbaay®, there were no significant modulations in SCFAs (Figure 20). The reason for the lack of significant modulations in the fecal samples is that approximately 95% of the SCFAs are rapidly absorbed in the large intestines, leaving just about 5% to be excreted in the feces (Den Besten et al., 2013).
Following the Bizetaflox Pediatrico treatment, there was a significant increase in circulating levels of acetic acid and propanoic acid (Figure 14), which are beneficial for RETT syndrome patients as discussed above in the Lalbaay® treatment.
During the washout period for Bizetaflox Pediatrico treatment, circulating levels of propanoic acid increased, while levels of butanoic acid 3-methyl decreased (Figure 19), similar to the treatment with Lalbaay®.
In the fecal samples of patients treated with Bizetaflox Pediatrico, there were no significant modulations in SCFAs (Figure 21), similar to the patients treated with Lalbaay®.

We evaluated the effect of prebiotics and/or postbiotics on neurotransmitters, neuromodulators, and precursors in plasma and fecal samples using LC-MS. Samaco et al. (2009) found a reduction in dopamine metabolites and serotonin metabolites in the cerebrospinal fluid of RETT syndrome patients compared to healthy subjects, confirming dysregulated levels of neurotransmitters, neuromodulators and precursors in this neurological disorder.

For the Lalbaay® treatment in Rett syndrome patients, levels of circulating choline, L-arginine, 2-pyrrolidinone, L-phenylalanine, 2-phenethylamine, indole-3-acrylic acid, L-lysine, L-carnitine, and L-tryptophan decreased after treatment with Lalbaay®, while Nα-acetyl-L-glutamine increased (Figure 22). Several bacteria, *Escherichia coli* and *Staphylococcus aureus* are able to produce acetylcholine from choline. In addition, acetylcholine cannot cross the blood-brain barrier, but choline can (Chen et al., 2021). Therefore, an increase in acetylcholine can lead to a decrease in choline levels. L-arginine is a precursor of glutamate, GABA, and nitric oxide. In the study, levels of GABA did not change significantly during treatment, suggesting that the decrease in L-arginine levels is due to its conversion to nitric oxide, which is a retrograde neurotransmitter and

also plays an important role in immunity and inflammation (Picón-Pagès et al., 2019; Coleman, 2001). The product 2-pyrrolidinone can be synthesized from GABA by Lactobacillus brevis (Wang et al., 2018). Since GABA levels did not increase during the treatment, the decrease in 2-pyrrolidinone may be due to the decrease of Lactobacillus brevis species in the gut microbiota. Having less 2-pyrrolidinone is beneficial to patients because GABA levels will not be affected.

L-phenylalanine is a precursor for dopamine, which is a neurotransmitter. Sodium butyrate, one of the constituents of Lalbaay®, has been found to reduce the degeneration of dopaminergic neurons and increase dopamine levels in murine models (Kakoty et al., 2021). This suggests that L-phenylalanine was converted to dopamine. 2-Phenethylamine is formed through the decarboxylation of L-phenylalanine (Irsfeld et al., 2014). In this study, levels of L-phenylalanine decreased after treatment, which may explain the decreases in 2-phenethylamine. Another possible explanation for the decrease is the positive correlation between 2-phenethylamine and Ruminococcus gnavus, which may have decreased during treatment, leading to less production of 2-phenethylamine (Sugiyama et al., 2022). Indole-3-acrylic acid is a metabolite of tryptophan (Pan et al., 2023). Levels of indole-3-acrylic acid decreased after treatment, and we also observed a decrease in tryptophan levels, which explains the decrease in indole-3-acrylic acid. L-lysine is a precursor of glutamate (Papes et al., 2001). An increase in glutamate is a possible explanation for the decrease in L-lysine.

The tryptophan pathway branches off into three main pathways: the kynurenine pathway, serotonin pathway, and indole pathway (Roth et al., 2021). In the study, levels of tryptophan decreased, while those of kynurenine and serotonin showed no significant difference, and indole levels decreased. The decrease can be explained by the increase in molecules that were below the detection threshold for the instrument, such as melatonin, which is synthesized from serotonin. Nα-acetyl-L-glutamine is a central nervous system stimulant that can pass through the blood-brain barrier and function as a substitute for glutamine (Zhang et al., 2017). Glutamine is a precursor of glutamate, aspartate, and GABA (Albrecht et al., 2010), suggesting that increased levels of Nα-acetyl-L-glutamine can result in more glutamate and aspartate, which is beneficial to the body.

During the washout period for patients treated with Lalbaay®, levels of circulating 3-aminopiperidine-2,6-dione, L-methionine, choline, L-tyrosine, and nudifloramide increased, while GABA levels decreased. In this same period, the clinicians noticed a worsening of symptoms. Piperidine derivatives can function as inhibitors for both acetylcholine esterase and butyrylcholine

esterase (Frolov and Vereshchagin, 2023). Increased levels of 3-aminopiperidine-2,6-dione reduce the production of acetylcholine from choline, corresponding to the worsening of symptoms. Disruptions in L-methionine have been linked to neurological and psychiatric disorders. In particular, Alachkar et al. (2022) concluded that injection of L-methionine into mice caused neuroinflammation and neurogenesis. The increase in L-methionine is in line with the worsening of symptoms. Choline levels increased when treatment was discontinued, meaning acetylcholine production decreased in the body, worsening the symptoms. Tyrosine is a precursor for dopamine, and Lieberman et al. (2015) showed that an increase in tyrosine during exposure to psychological stress increases anger in humans. Therefore, an increase in tyrosine during the washout period may lead to negative mood changes. Nudifloramide is a metabolite of nicotinamide. A study by Yan-Jie et al. (2013) showed that excess nicotinamide increases serotonin and histamine levels. The increase in levels of nudifloramide means a reduction in nicotinamide, therefore there is no increase in levels of serotonin and histamine, which is not beneficial to the body. GABA levels decreased during the washout period, and lower levels of GABA are associated with autism spectrum disorder, as Clostridium species can consume GABA (Zuffa et al., 2023). The decrease in GABA causes some autism spectrum disorder symptoms.

For the Bizetaflox Pediatrico treatment in Rett syndrome patients, circulating levels of 2-pyrrolidinone, nudifloramide, L-phenylalanine, 2-phenethylamine, and L-tryptophan decreased (Figure 28). For all these molecules, we observed a similar trend to that of the Lalbaay® treatment. The decrease in these molecules leads to the synthesis of key neurotransmitters and neuromodulators, as discussed above.

During the washout period for patients treated with Bizetaflox Pediatrico, circulating levels of indole-3-propionic acid and indole-3-acetonitrile decreased, while levels of 3-amino-piperidine-2,6-dione and nudifloramide increased (Figure 33). A similar trend was observed during the washout period for the Lalbaay® treatment, with an increase in 3-amino-piperidine-2,6-dione and nudifloramide. This increase may lead to worsened symptoms, as discussed above. Indoles are tryptophan metabolites and have been shown to play a key role in the progression of neurological and psychiatric diseases. They are known to reduce inflammation in the body, including in the central nervous system (Zhou et al., 2023). During the washout period, the levels of circulating

indoles decreased, which may lead to the progression of the neurological disorder as symptoms worsened.

Neurotransmitters were also measured in fecal samples. For the Lalbaay® treatment, levels of indole-3-acrylic acid and L-phenylalanine decreased after treatment (Figure 34). This was also observed in the circulating molecules for the same treatment. A decrease suggests these molecules were rapidly absorbed and used as precursors to synthesize neurotransmitters. During the washout period for Lalbaay® treatment, there were no modulated molecules in the fecal samples.

For the Bizetaflox Pediatrico treatment in Rett syndrome patients, levels of indole-3-acrylic acid, L-methionine, L-glutamic acid, nudifloramide, and indole-3-carboxyaldehyde decreased (Figure 39). The decrease in these molecules in the fecal samples suggests fast absorption and conversion to neurotransmitters that improve symptoms. During the washout period for patients treated with Bizetaflox Pediatrico, levels of choline, L-methionine, indole-3-carboxaldehyde, and indole-3-acrylic acid increased (Figure 44). This is the opposite of what was observed during the treatment, leading to the conclusion that the treatment is capable of modulating neurotransmitters, modulators, and precursors.

7. Conclusion

In conclusion, this thesis has investigated the impact of prebiotics and/or postbiotics on neurotransmitter and SCFA levels in Rett syndrome patients. Mass spectrometry techniques were used to evaluate levels of neurotransmitter and SCFA levels before and after treatment with Lalbaay® and Bizetaflox Pediatrico. Both treatments were able to modulate neurotransmitters and SCFAs, suggesting an improvement in both the α and β-diversity of the gut microbiota of Rett syndrome patients. These modulations resulted in a reduction in the severity of the symptoms without any adverse effects.

8. References

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