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In Vivo Analysis of the Effects of Emerging Enniatin Mycotoxins Using a Murine Model of Human Intestinal Microbiota

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Ce mémoire

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Présenté par

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Résumé

Cette étude visait à évaluer l'impact d'une exposition à des doses faible et élevée d'un mélange de mycotoxines émergentes du groupe des enniatines sur le microbiote intestinal à l'aide d'un modèle murin humanisé Sprague Dawley. L'hypothèse était que l'utilisation de ce modèle humanisé permettrait de caractériser la dysbiose induite par les enniatines. L'étude comprenait trois parties. Dans la phase initiale, une déplétion du microbiote a été induite chez 24 rats Sprague Dawley par un traitement par un cocktail d'antibiotiques mélangé à du sirop de cassis pendant 14 jours. Par la suite, les rats ont été répartis de façon aléatoire en deux groupes, recevant chacun des matières fécales fraîches par transferts fécaux bi-hebdomadaires de deux donneurs humains adultes (un mâle et une femelle) en bonne santé, pendant 3 semaines. Dans la phase finale, les 24 rongeurs humanisés ont été divisés en trois groupes en fonction de leur dose d'exposition orale aux enniatines pendant quatre semaines: un groupe contrôle (0 µg/kg de poids corporel d'enniatine), un groupe recevant 2 µg/kg de poids corporel d'enniatines par jour, correspondant au niveau d'exposition moyen aux enniatines dans la population humaine, et un groupe recevant 200 µg/kg de poids corporel d'enniatines par jour. Les doses d'enniatines étaient administrées mélangées à du syrop de cassis. Des échantillons fécaux ont été collectés au début, et chaque semaine tout au long de cette phase. La structure et la diversité du microbiote ont été évaluées à l'aide du kit d'extraction d'ADN de matières fécales QIAamp DNA stool micro kit et de la méthode du séquençage du gène codant pour l'ARNr 16S.

Les résultats de la phase de déplétion ont montré des différences significatives avant et après le traitement antibiotique, confirmant l'épuisement de la flore intestinale initiale des rats par le cocktail d'antibiotiques. De plus, nous avons réussi à générer un modèle de rat humanisé à partir du microbiote intestinal humain. L'établissement réussi d'un modèle animal humanisé a été confirmé par le transfert de divers phylums bactériens, notamment *Bacteroidaceae*, *Bacteroidales*, *Bacteroides*, *Barnesiella*, *Bifidobacterium*, *Desulfovibriionales*, *Eggerthella*, *Enterococcaceae*, *Hungatella*, *Lachnospiraceae*, *Lactobacillaceae*, *Limosilactobacillus*, *Mycobacteriales*, *Peptostreptococcaceae*, *Romboutsia* et *Streptococcus*. Dans la phase finale de l'étude, l'abondance relative de certains genres bactériens a été modifiée de manière dose-dépendante par l'exposition aux enniatines, bien que l'impact global des mycotoxines ait été limité. Cet impact global limité peut être dû au fort effet homogénéisant du sirop de cassis sur le microbiote dans tous les groupes expérimentaux, masquant potentiellement les effets des enniatines sur la structure du microbiote. Ces résultats indiquent que l'exposition aux enniatines, même aux doses moyennes observées dans la population humaine pourraient modifier significativement la composition du microbiote intestinal humain.

Mots clés : Enniatine, mycotoxine, microbiote, rats, modèle animal humanisé, analyse du microbiote, structure du microbiote.

ABSTRACT

This study aimed to evaluate the impact of exposure to low and high doses of a mixture of emerging mycotoxins from the enniatin group on the gut microbiota using a humanized Sprague Dawley mouse model. The hypothesis was that using this humanized model would allow for the characterization of dysbiosis induced by enniatins. The study consisted of three parts. In the initial phase, microbiota depletion was induced in 24 Sprague Dawley rats through a treatment with an antibiotic cocktail mixed with blackcurrant syrup for 14 days. Subsequently, the rats were randomly divided into two groups, each receiving fresh fecal matter through bi-weekly fecal transplants from two healthy adult human donors (one male and one female) for three weeks. In the final phase, the 24 humanized rodents were divided into three groups based on their oral exposure dose to enniatins over four weeks: a control group (0 µg/kg body weight of enniatin), a group receiving 2 µg/kg body weight of enniatins mixed with blackcurrant syrup daily , and a group with 200 µg/kg body weight of enniatin daily. The daily 2 µg/kg body weight of enniatins represents the mean chronic exposure calculated for humans in Europe. Fecal samples were collected at the beginning and every week throughout this phase. The structure and diversity of the microbiota were evaluated using the QIAamp DNA Stool Mini Kit and 16S rRNA sequencing method.

The results indicated significant differences before and after antibiotic treatment, confirming the initial depletion of the gut flora by the antibiotic cocktail. Additionally, we successfully generated a humanized rat model from human gut microbiota. The successful establishment of a humanized animal model was confirmed by the transfer of various bacterial genus, including Bacteroidaceae, *Bacteroidales, Bacteroides, Barnesiella, Bifidobacterium, Desulfovibriionales, Eggerthella,* Enterococcaceae*, Hungatella,* Lachnospiraceae*,* Lactobacillaceae*, Limosilactobacillus, Mycobacteriales,* Peptostreptococcaceae*, Romboutsia, and Streptococcus*. In the final phase of the study, the relative abundance of certain bacterial genera was dose-dependently modified by exposure to enniatins, although the global impact of the mycotoxins was limited. This limited global impact may be due to the strong homogenizing effect of the blackcurrant syrup on the microbiota across all experimental groups, potentially masking the effects of enniatins on the microbiota structure. Altogether, these results indicate that exposure to enniatins, even at the mean level observed in the human population, could significantly modify the composition of the human intestinal microbiota.

Keywords: Enniatin, mycotoxin, microbiota, rats, humanized animal model, microbiota analysis, microbiota structure.

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LIST OF ABBREVIATIONS

ABCB1: Adenosine triphosphate-binding cassette transporters P-glycoprotein
ABCG2: Mixture of enniatins to inhibit breast cancer resistance protein
ACAT: Against Cholesterol acyl transferase
AFB1: Aflatoxin
PERMANOVA: Permutational Multivariate Analysis of Variance
AOH: Alternariol
ATB: Microbiota depletion
BEA: Beauvericin
BW: Body weight
CIT: Citrinin
C_q: Quantification cycle
CUL: Culmorin
DMSO: Dimethyl sulfoxide
DON: Denoxynivalenol
EFSA: European Food Safety Authority
ENNs: Enniatins
F: Fusarium
FMT: Fecal microbiota transplantation
FUS: Fusaproliferin
FX: Fusarenone-X
GF: Germ-free mice
GI: Gastrointestinal systems
GM: Gut microbiome
HPRT locus: Gene mutation assay
HPRT: Hypoxanthine-guanine phosphoribosyltransferase
IL-1β: Interleukin-1beta
KJA: Kojic Acid
LC-HRAM: Liquid chromatography high-resolution accurate mass spectrometry analysis
LOQ: Limit of quantitation
LPS: Lipopolysaccharide
M13: Monohydroxylated metabolite
MDCK: Madin-Darby canine kidney
MON: Moniliformin
NGS: Next-generation sequencing
NIV: Nivalenol
NMDS: Non-metric multidimensional scaling
OTA: Ochratoxin A
OTUs: Operational Taxonomic Units
PCoA: Principal coordinate analysis
qPCR: Quantitative PCR
STG: Sterigmatocystin
TeA: Tenuazonic Acid
TEN: Tentoxin
ZEN: Zearalenone

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Mycotoxins are toxic compounds produced by certain fungi that can cause harm to humans and animals when ingested (Prosperini et al., 2017). *Fusarium* species are a type of fungi commonly found in cereal grains, animal feed, and food commodities worldwide. These microorganisms can contaminate food products and pose a risk to human as well as animal health (Fraeyman et al., 2017). Enniatins are a specific type of mycotoxin that are produced by *Fusarium* species through secondary metabolism. They are classified as hexadepsipeptidic mycotoxins.

Enniatins are considered "emerging mycotoxins" because they have gained attention in recent years due to their presence in various food products (Ivanova et al., 2017). They are commonly found in grains and their derived products, as well as in fish, dried fruits, nuts, spices, cocoa, and coffee (Prosperini et al., 2017). Enniatins were found in 59% of cereal-based food samples in Spain (Serrano et al., 2012), 73% of grain samples (Malachova et al., 2015), 45% of fish and seafood samples (Tolosa et al., 2014), 35% of dried fruit and nut samples (EFSA, 2014), 28% of spice samples (Jestoi et al., 2009), and 20% of cocoa and coffee samples in European countries (Habschied et al., 2021). These mycotoxins can be present in various food items at levels measured in milligrams per kilogram. It is important to note that cooking methods such as baking, frying, and roasting do not affect the chemical structure of enniatins. After these food items are cooked or processed, the enniatins can remain intact and potentially pose a risk if consumed (de Nijs et al., 2016). The mean chronic exposure to the sum of enniatins has been estimated to be between 0.42-1.82 µg/kg of body weight per day. The 95th percentile dietary exposure in adults has been estimated to be between 0.91-3.28 µg/kg of body weight per day. This indicates that some individuals may have higher levels of exposure to enniatins, potentially reaching the upper range of these estimates (EFSA, 2014) Unlike other mycotoxins produced by the *Fusarium* fungi, enniatins have not been regulated by authorities until now. There are eleven known analogues of enniatins, with enniatin A, A1, B, and B1 being the most commonly found forms in natural contaminants (Yoshinari et al., 2016). Enniatins have been found to possess various properties, including insecticidal, antifungal, antibacterial, and anthelmintic anti-parasitic effects (Jestoi, 2008). *In vitro* studies have shown that enniatins have a potent cytotoxic effect, meaning they can cause damage to cells in both human and animal cell lines at very low concentrations. Such cytotoxicity raises concerns for human and animal health (Prosperini et al., 2017). However, *in vivo* studies (studies conducted in living organisms) have not been able to replicate the same level of cytotoxicity observed *in vitro* (Rodríguez et al., 2016). The European Food Safety Authority (EFSA) has concluded that acute exposure to enniatins does not indicate a concern for human health. However, the effects of chronic exposure to enniatins and the associated risks require further investigation and toxicity data (Chain, 2014).

In summary, enniatins are emerging mycotoxins that have been found in various food sources. They possess various properties and have shown cytotoxic effects *in vitro*. However, the level of cytotoxicity observed *in vitro* has not been replicated *in vivo* studies. Acute exposure to enniatins is not considered a concern for human health, but the effects of chronic exposure and associated risks require further investigation.

Gut microbiota refers to the community of microorganisms that reside in the gastrointestinal tract. These microorganisms play a crucial role in maintaining the health of the host and regulating various physiological functions (Thursby et al., 2017). They not only impact the gastrointestinal tract but also other organs and the systemic immune system (Sekirov et al., 2010). The relationship between gut microbiota and the host is symbiotic, meaning they depend on each other for mutual benefit. An imbalance in the gut microbiota, known as dysbiosis, has been implicated in the development of multiple diseases. Dysbiosis of gut microbiota has been associated with conditions such as autism, asthma, cardiovascular disease, colon cancer, Crohn's disease, diabetes, eczema, food allergies, irritable bowel syndrome, obesity, hepatic encephalopathy, and mental disorders (Schnabl et al., 2014 ; Fung et al., 2017). The composition of gut microbiota can be influenced by various external factors. These factors include diet, medications, and environmental toxins. Diet refers to the food and nutrients consumed, which can impact the types and abundance of microorganisms in the gut. Medications, such as antibiotics, can disrupt the balance of gut microbiota by killing off certain types of bacteria. Environmental toxins, such as pollutants or chemicals, can also have an impact on the composition of gut microbiota. It is essential to maintain or regulate the balance of intestinal bacteria for the health of animals and humans. This balance is crucial for the proper functioning of the gastrointestinal tract and overall well-being. Imbalances in gut microbiota can lead to various health problems and diseases. Therefore, understanding the factors that influence gut microbiota composition and finding ways to maintain a healthy balance is important for promoting good health (Kamada et al., 2013 ; Sommer et al., 2013 ; Korem et al., 2015).

The field of gut microbiology and associated health outcomes has advanced greatly with “humanized” mouse models. Gut microbiology refers to the study of microorganisms that reside in the gastrointestinal tract, particularly the intestines. Humanized mouse models are experimental models in which mice are colonized with human gut bacteria to mimic the microbial composition found in humans. These models have contributed significantly to our understanding of the gut microbiota and its impact on health. Traditionally, these models require seeding germ-free mice with bacteria derived from human donors, thus providing a useful system to study the interactions between human microbiota and chronic disease in studies where using human subjects is not appropriate. Germ-free mice are mice that are completely devoid of any microorganisms, including bacteria. In humanized mouse models, germ-free mice are colonized with bacteria obtained from human donors, which allows researchers to study the interactions between human gut bacteria and chronic diseases. This is particularly useful when studying chronic diseases where using human subjects is not feasible or ethical. However, maintenance of germ-free mice colonies is expensive, requiring substantial institutional investment in infrastructure and specialized personnel. Maintaining germ-free mice colonies involves creating and maintaining a controlled environment that is completely free of microorganisms. This requires specialized facilities, equipment, and trained personnel to ensure the mice remain germ-free. The cost of establishing and maintaining such colonies can be significant, making it a resource-intensive process. Moreover, germ-free rodents are not readily available for the most common and/or most important strains used in chronic disease research, including many inbred and genetic mouse

models. Inbred and genetic rodent models are strains of mice that have specific genetic characteristics or traits that make them valuable for studying certain diseases. Germ-free rodents with these specific genetic characteristics may not be readily available, making it difficult to study the interactions between human gut bacteria and chronic diseases using these models. This limitation can hinder research efforts focused on understanding the role of gut microbiota in specific disease models (Turnbaugh et al., 2009); Goodman et al., 2011 ; Gootenberg et al., 2011).

This project was conducted using a murine model to characterize the human microbiota alteration treated with 2 µg/kg and 200 µg/kg body weight of enniatin administered orally. The findings provide new insights into the effect of enniatin-induced composition change in the gut microbial community of the humanized rodent model. The findings demonstrated significant different of microbiota structure and community compare before antibiotic and after antibiotic experiments. Additionally, we generate the humanized rat model using human gut microbiota. There was impact from enniatin on the structure of the humanized microbiota. We found some genera was significant difference. The syrup may have standardized the microbiota, canceling the effect of enniatin on its structure. However, the relative proportions of certain bacterial genera suggest the dosage-dependent impact of enniatin exposures.

CHAPTER 2

LITERATURE REVIEW

2.1 Mycotoxins

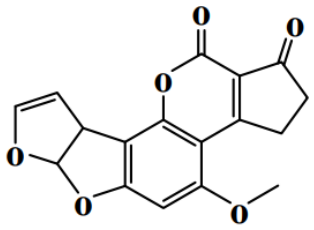
Mycotoxin is a secondary fungi metabolite (Pleadin et al., 2019). The definition of mycotoxin originates from the combination of two words, including “mykes” and “toxicum”. The Greek word “Mykes” means fungus, and the Latin word “toxicum” means poison. The name “mycotoxin” is used for a toxic chemical of fungal origin (Turner et al., 2009). Mycotoxins could be generated from several fungal species. Aflatoxin, trichotecene fumonisins, zearalenone, and ochratoxins are the common mycotoxins created by *Aspergillus, Penicillium* and *Fusarium* species. The pre/post-harvest, storage and transportation stages could induce foodstuffs to be contaminated with mycotoxin (Abia et al., 2013; López et al., 2016; Oueslati et al., 2014). Moreover, high humidity and high temperature may induce mycotoxin generation. The toxicity of mycotoxin includes immunotoxicity, hepatotoxicity, teratogenicity, carcinogenicity, etc. (Bondy et al., 2000; Kensler et al., 2011; Turner et al., 2003).

2.1.1 Regulated Mycotoxins

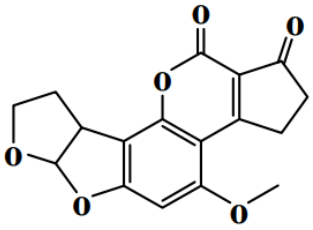
Under Commission Regulation (EC) No. 915/2023, the European Commission (EC) has established specific upper limits for mycotoxin exposure in both adults and children in Europe. Despite the identification of hundreds of mycotoxins in scientific literature, regulation is applied to only a limited number due to their prevalent use and well-documented toxicological effects. These regulated mycotoxins include aflatoxins (AFB1, B2, G1, G2, and M1), deoxynivalenol (DON), fumonisins (FB1 and FB2), ochratoxin A (OTA), zearalenone (ZEN), patulin (PAT), as well as T-2 and H-2 toxins found in cereals and cereal-based products. Additionally, alteriol (AOH) is also strictly regulated and closely monitored by the European Union.

Aflatoxins

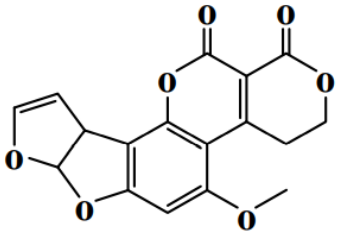
Aspergillus flavus and *Aspergillus parasiticus* are the fungi that produce these mycotoxins. Aflatoxins B1, B2, G1, and G2 (Figure 1) are the most found. They are distinct by their fluorescence under UV light (blue or green) and correspond with chromatographic mobility (Squire, 1981). Normally, these toxins can be found in contaminated corn, cereal, grain, fruits, peanuts, and nuts**.** These toxins can cause various diseases such as liver disease, carcinogen, mutagenic and immunosuppressive in animals and humans (Sá et al., 2021)**.**



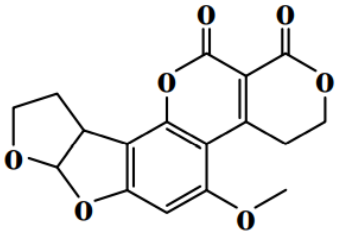
Alfatoxin B1 (AFB1)



Alfatoxin B2 (AFB2)



Alfatoxin G1 (AFG1)

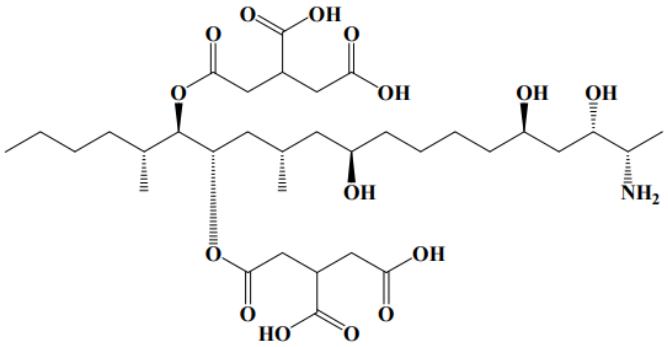


Alfatoxin G2 (AFG2)

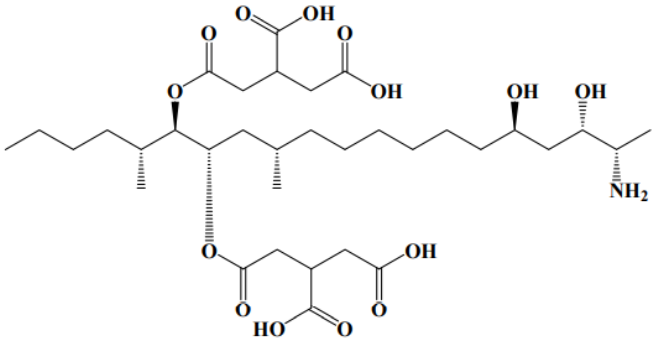
Figure 1. Chemical structures of aflatoxins

Fumonisins

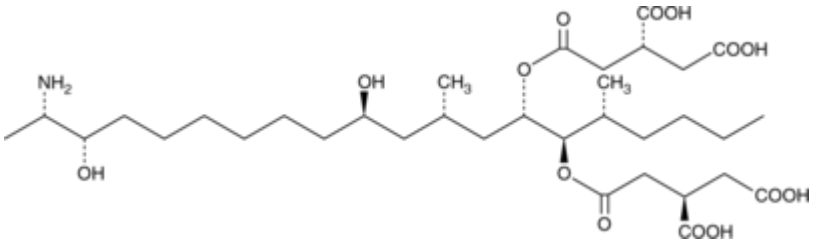
Fumonisins were produced by *F. moniliforme), F. proliferatum, F. oxysporum, F. globosum, several other Fusarium spp., and Alternaria alternata fusarium species. Lycopersici*. Mostly, these mycotoxins have been found to contaminate corn and corn products during pre-harvest (Weidenbörner et al., 2013). The hazards of these mycotoxins include immune suppression, hepatotoxicity, pulmonary edema and nephrotoxicity (Scott, 2012). Fumonisin B2 (FB2) and fumonisin B3 (FB3) could co-occur in lower concentrations than fumonisin B1 (FB1) (Figure 2) (Scott, 2012).



Fumonisin B1 (FB1)



Fumonisin B2 (FB2)



Fumonisin B3 (FB3)

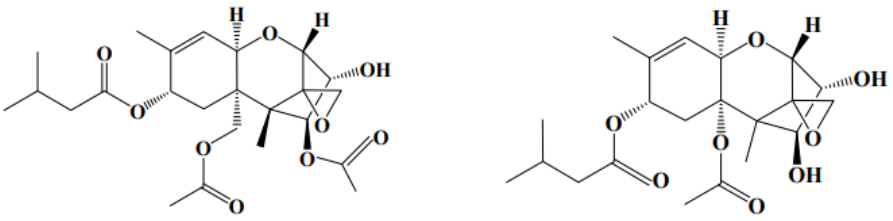
Figure 2. Chemical structures of fumonisins

Trichothecenes

This group of mycotoxins is produced by the *Fusarium* species. There are two types, as described below.

Type A trichothecenes

Mostly found in contaminated rice, soya beans, beans, oats, maize and wheat, these mycotoxins, especially T2 toxin and HT-2 toxin (Figure 3), are mainly produced from *F.sporotrichioides, F. poae, F. equiseti* and *F. acuminatum.* These toxins can cause bloody diarrhea, vomiting, dermal necrosis, immunoglobulins and weight loss (Yu et al., 2023).

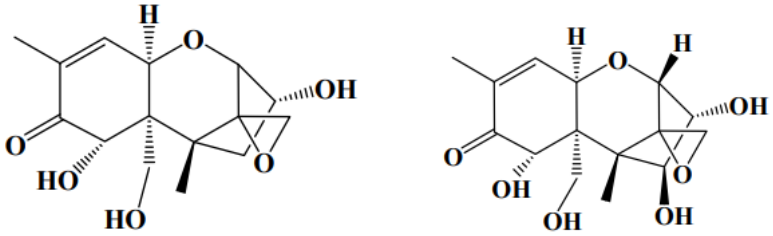


T-2 toxin (T-2) HT-2 toxin (HT-2)

Figure 3. Chemical structures of T-2 toxin and HT-2 toxin

Type B trichothecenes

Type B group has a ketone and includes fusarenon-X (FUS-X), nivalenol (NIV), deoxynivalenol (DON), and its 3-acetyl and 15-acetyl derivatives (3- and 15-ADON). While, type A includes T-2 toxin, HT-2 toxin, neosolaniol, and diacetoxyscirpenol (DAS). Deoxynivalenol (DON) and nivalnol (NIV) (Figure 4) are two of these mycotoxins. *F. graminearum* and *F. culmorum* are the main fungi that produce these mycotoxins. Pre-harvest could be the source of contamination in wheat, oat, maize, rice, rye, and sorghum**.** Anorexia and vomiting are the symptoms in humans who consume food contaminated with these mycotoxins (Yu et al., 2023).

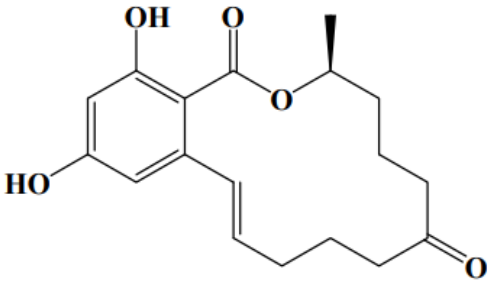


Deoxynivalenol (DON) Nivalenol (NIV)

Figure 4. Chemical structures of DON and NIV toxin

Zearalenone

F. graminearum is a mold that produces zearalenone mycotoxin (Figure 5). This toxin is mainly found in corn, rice, millet, wheat, oats, barley and sorghum (Prelusky et al., 1990) and can induce the development of cancer cell (Zhang et al., 2018), genotoxicity, (Lioi et al., 2004) and immunotoxicity as well as DNA fragmentation (Islam et al., 2017).

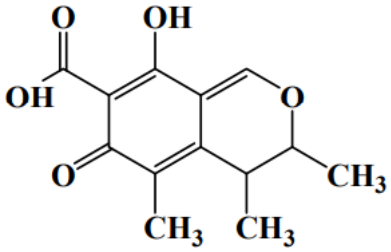


Zearalenone (ZEA)

Figure 5. Chemical structure of zearalenone toxin

Citrinin

Citrinin (Figure 6) is produced by *Penicillium species (P.) Penicillium verrucosum* and *Penicillium citrinum***.** Rice, rye, oats, barley, wheat, and flour are sources of food contamination for humans. This mycotoxin could contaminate with ochratoxin A (OTA). Hepatotoxicity**,** nephrotoxicity, and teratogenicity are the forms of toxicity that could be caused by consuming contaminated foods.



Citrinin (CIT)

Figure 6. Chemical structure of citrinin toxin

Ochratoxins

Ochratoxin A, B and C (Figure 7) are the 3 types of mycotoxins in this family. The most found and highly toxic is Ochratoxin A (OTA). *Aspergillus species (A.) chraceus, A. carbonarius, P. verrucosum, P. viridicatum* and *P.nordicum* are secreted by OTA during pre/post-harvest (Moss, 1996). Cocoa, coffee, soy products, grapes, barley, wheat, cereal and grain are commonly found to be contaminated (Joint, 2001). Nephrotoxicity, carcinogenicity and immunosuppression are included in the forms of toxicity that could be caused by these mycotoxins. (O'Brien at al., 2005).

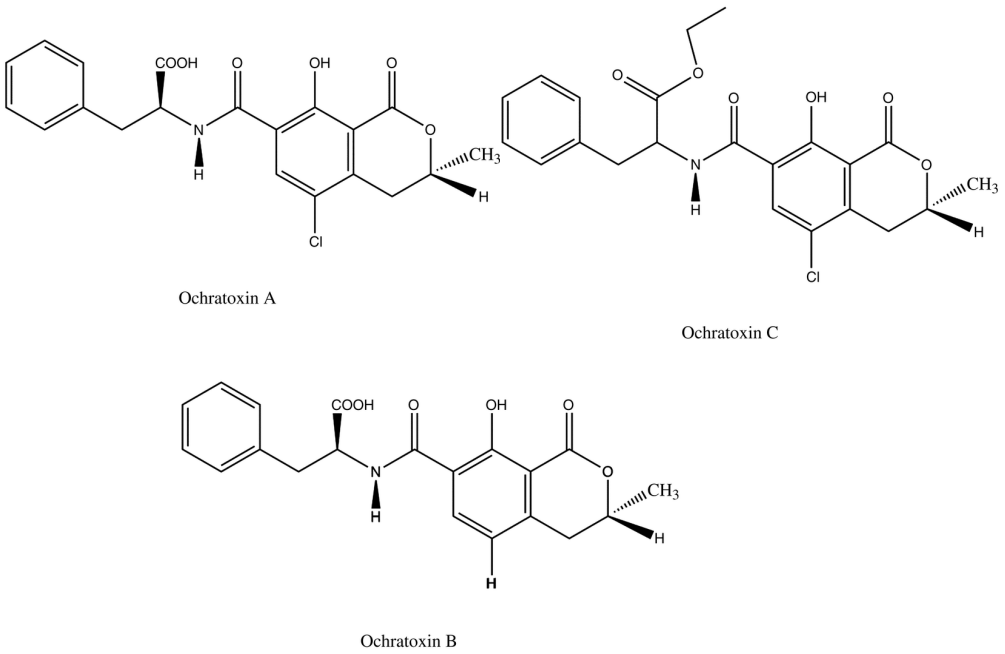


Figure 7. Chemical structures of ochratoxins

2.1.2 Emerging Mycotoxins

The definition of "emerging mycotoxins" was the mycotoxins that contaminated in cereals and in other food and feedstuffs often. The evidence of emerging mycotoxin incidence is rapidly increasing (Vaclavikova et al., 2013). Moreover, these type of mycotoxin are not regulated (Chain, 2014).

Alternariol (AOH)

The structure of Alternariol is a dibenzo-α-pyranone (6H-benzo chromen-6-one) containing methyl group and hydroxy groups. *Alternaria alternata* is a fungi species that produces these mycotoxins (Bottalico et al., 1998; Pose et al., 2004). Small-grain cereals, mushrooms, vegetables and fruits are the most commonly contaminated foodstuffs (Brugger et al., 2006).

Beauvericin (BEA)

The structure of Beauvericin is a cyclic hexadepsipeptide that contains three D-hydroxy-isovaleryl and three N-methyl-phenylalanyl. *Beauveria bassiana, F. sporotrichioides, F. poae, F. langsethiae, F. avenaceum, and F. section Liseola* are the species that produce this mycotoxin (Logrieco et al., 1998).

Citrinin (CIT)

The structure of Citrinin is a polyketide mycotoxin [(3R, 4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benz opyran-7-carboxylic acid]. This mycotoxin is

produced by *Penicillium citrinum*, *Aspergillus* and *Monascus* species (Doughari, 2015) This mycotoxin can be found in contaminated grains, fruit, vegetables, spices, cereals, herbs, nuts, dairy products (Ostry et al., 2013), and red yeast rice in Japan (Doughari, 2015).

Culmorin (CUL)

The structure of Culmorin is a longifolene sesquiterpene with a tri-cyclo-[6.3.0.0]-undecane skeleton. Fusarium species such as *F. culmorum, F. graminearum, F. crookwellense, F. venenatum, and F. praegraminearum a F. graminearum* species are the species that produce this mycotoxin (Weber et al., 2018). The most contaminated foodstuff is wheat crops (Spanic et al., 2020). Moreover, this mycotoxin can be found in contaminated barley, oats, and nuts (Abia et al., 2013). This mycotoxic metabolite includes 15-hydroxy-CUL and 5-hydroxy-CUL, which could contaminate malt, beer (Mastanjević et al., 2018), maize (Hajnal et al., 2020) and wheat (Spanic et al., 2020).

Fusaproliferin (FUS)

The structure of Fusaproliferin has a four C = C double ring and four chiral atoms. Fusarium species such as *F. proliferatum* and *F. subglutinans* produced these mycotoxins. (Meca et al., 2009). Moreover, other Fusarium species also produce these mycotoxins such as *F. proliferatum* (Randazzo et al., 1993), *F. poae, F. langsethiae, F. sporotrichioides* (Marin et al., 2013), *F. antophilum, F. begoniae, F. bulbicola, F. circinatum, F. concentricum, F. succisae, and F. udum* (Moretti et al., 2007). The contamination of these mycotoxins were reported in variable kinds of foodstuffs such as wheat, rice (Meca et al., 2009) and maize kernels(Desjardins et al., 2006).

Kojic Acid (KJA)

The structure of Kojic acid contains a hydroxy group, a hydroxymethyl group and an oxo group. *Aspergillus oryzae* produces this mycotoxin. Moreover, this mycotoxin could be an NF-kappaB inhibitor, a skin-lightening agent, and an inhibitor effect of enzymes such as catechol oxidase, laccase, quercetin 2,3-dioxygenase, tyrosinase, and D-amino-acid oxidase (PubChem, 2020). KJA could be contaminated in miso, soy sauce, sake (Chib et al., 2019) and rice husk (Fujimoto et al., 1999).

Moniliformin (MON)

The structure of Moniliformin is considered a strong acid due to it containing sodium or potassium salt of the cyclobutane 1-hydroxycyclobut-3, 4-dione. Fusarium species such as *F. avenaceum, F. tricinctum, F. proliferatum, F. subglutinans, F. verticillioides,* and *F. fujikuroi* (Fotso et al., 2002). Moreover, MON could be produced by *Penicillium melanoconidium* (Hallas et al. 2016). Previous studies revealed that MON was not melted by water or MeOH (Scott et al., 1987). The most common foodstuffs found to be contaminated were oats (Fredlund et al., 2013), maize (Logrieco et al., 2002), groundnuts and powdered milk (Ojuri et al., 2018).

Sterigmatocystin (STG)

The structure of STG is an organic heteropentacyclic compound. Aspergillus species, such as *A. versicolor, A. nidulans* (Battilani et al., 2020) and *Bipolaris* species produce this mycotoxin (ChEBI, 2020). STG could be soluble in water, DMSO, CF, and pyridine. The most common foodstuffs that have been found to be contaminated included wheat, maize, animal feed, hard cheese, pecan nuts, and green coffee beans (Ayalew, 2010; Tancinova et al., 2009; Viegas et al., 2020).

Tenuazonic Acid (TeA)

Alternaria spp., such as *A. alternata, A. longipes and A. tenuissima* are the species that produce this mycotoxin. TeA has the capacity to be antibiotic, antiviral, and antineoplastic (PubChem 2020). Contaminated foodstuffs include wheat, gain and grain-based products (EFSA Panel on Contaminants in the Food Chain 2011).

Tentoxin (TEN)

Alternaria alternata is the fungus species that produces this mycotoxin. Cereals, fruits, wheat, barley, and sorghum are the foodstuffs that are mostly found contaminated (Rodríguez-Carrasco et al., 2016).

2.2 Enniatins (ENNs)

The structure of enniatins includes cyclic hexadepsipeptides that contain three N-methyl amino acids. Fusarium spp. such as *F. avenaceum*, *F. tricinctum, F. poae, F. sporotrichioides,* and *F. langsethiae* are the fungi species which produced these mycotoxins (Thrane, 2001). Usually, rice or liquid media like yeast extract sucrose (YES) medium or potato dextrose broth (PDB) are used to cultivate this fungus. The cultivation is done in the dark at temperatures between 20°C and 25°C. Depending on the fungus strain and growing conditions, the incubation period might range from one to four weeks (Ivanova et al., 2017). Enniatins are extracted from fungal biomass or culture medium using organic solvents like methanol, acetonitrile, or ethyl acetate. Initial purification involves liquid-liquid extraction, solid-phase extraction (SPE), and high-performance liquid chromatography (HPLC). HPLC is the most common method for final purification, using a C18 reverse-phase column with a gradient of water and acetonitrile or methanol as the mobile phase. Enniatins are detected using UV or mass spectrometry (MS) detectors, with UV detection typically set at 210-220 nm. (Prosperini et al., 2017; Suchfort et al., 2016; Uhlig et al., 2007). The most impactful enniatins are enniatin A, A1, B and B1 (Firakova et al., 2007) Enniatins could dissolve in EtOH, MeOH, dimethylsulfoxide (DMSO), dimethylformamide (DMF) and other organic solvents (Fraeyman et al., 2017). They could contaminate molds and multi-cereal grains (Blesa et al., 2012; Juan et al., 2013; Svingen et al., 2017). Moreover, these mycotoxins can be found in fruit and vegetables, fruit juices, compotes, and purees as well as dairy products (Juan et al., 2014; Pallares et al., 2019; Sun et al., 2019).

2.2.1 Occurrence and regulation

Previous studies found 7 types of enniatins (A, A1, B, B1, B2, B3 and B4). However, enniatins A, A1, B and B1 have been the most prevalent identified in food and feed (Firakova et al., 2007). The various *Fusarium* species (*F. Avenaceum, F. Tricinctum, F. Poae, F. Sporotrichioides and F. Langsethiae*) could produce enniatin (Thrane et al., 2004). Enniatins are one of the mycotoxins that risk human and animal health related to the high incidence of contamination in food (cereal gain, food products and food of animal origin) and feed (Chain, 2014). Finland, Italy, Germany, and Norway have previously reported enniatins A, A1, B and B1 contamination. In Finland, 38 gain batches were found to be contaminated with enniatins in 2001 (n = 22) and 2002 (n = 16) (Jestoi* et al., 2004). Moreover, previous results show that the concentrations of contamination found were significantly increased in 2005 (Uhlig et al., 2007). Enniatins A, A1, B and B1 were present in 25-94% of grain samples from Norway, with enniatin B being the most detected toxin with maximum concentrations of 190-5800 µg/kg in oats, barley and wheat (Uhlig et al., 2006). In Sweden, Enniatins A, A1, B, and B1 were found in high concentrations in wheat and oats, with the highest levels of enniatins B and B1 found in spring wheat; individual data for enniatins B2 and B3 were not available (Fredlund et al., 2013; Lindblad et al., 2013). In Germany, Enniatin B was found in 41% of maize samples in 2006 and 30% in 2007, with maximum concentrations of 1,210 µg/kg and 2,960 µg/kg, respectively (Goertz et al., 2010). In Italy, (Juan et al., 2013) found that enniatin B4 was the most frequently detected enniatin in 39% of organically grown grains samples, with levels ranging from 5.7 to 284.2 µg/kg, while enniatin B had the highest incidence and concentration of 97 µg/kg in a sample of wheat.

From a repeated-dose ENNB study, the No Observed Adverse Effect Level (NOAEL) is determined to be 0.18 mg/kg body weight per day for female or male mice, the NOAEL is identified as 1.8 mg/kg body weight per day, with observed effects including vacuolization of enterocytes in the duodenum and changes in Reactive Oxygen Species and Glutathione levels in the brain. The maternal NOAEL stands at 1.8 mg/kg body weight per day, characterized by a decrease in the white pulp area and an increase in the red to white pulp area ratio in the spleen. Additionally, the developmental NOAEL is set at 18 mg/kg body weight per day (Maranghi et al., 2018).

In conclusion, enniatins A, A1, B, and B1 were found in grains investigated, with the highest concentrations found in enniatins B and B1, and the lowest in enniatin A. Nevertheless, the regulatory of enniatins as the food contaminated remain undefined because of insufficient comprehensive toxicity information.

Occurrence of enniatins in food products

The prevalence of positive samples of sixty-one samples of Portuguese cereal-based foods included 16, 53, 49 and 44 % for enniatins A, A1, B and B1, respectively (Blesa et al., 2012). Enniatin A was the most found contaminant in food products. Moreover, Enniatin B1 had the highest concentration in conventional dry pasta in Spain, and enniatins were found in all types of pasta investigated (Serrano et al., 2013). found high levels of enniatin B in organic cereals, with concentrations up to 12.2 µg/kg. In a later study, found enniatin B in

28% of cereal-based baby foods, with concentrations up to 832 µg/kg. Beauvericin and enniatins were only found in cereal-based baby foods (Juan et al.,2013).

Occurrence of enniatins in foods of animal origin

In Belgian research, no enniatins were detected in 30 samples of commercial eggs from different farming methods in a Belgian study (CODA-CERVA, 2011/2012) using LC-MS/MS methodology (Capriotti et al., 2012). Enniatins were found in 26 chicken meat samples from conventional and bio farms. However, the levels of contamination were below LOQ (2.0-2.7 µg/kg). The study found no detectable amounts of enniatins in pig meat samples, but detected enniatin B in 4 out of 16 pig liver samples. In Finland, beauvericin and enniatins A, A1, B and B1 were studied in 112 whole egg samples. The frequency of contaminated whole egg samples was 79% in 2004 and 56% in 2005 (Jestoi et al., 2009). Beauvericin and enniatins may bioaccumulate into egg yolks via the transportation of lipoproteins (Jestoi et al., 2009). Another, study found no mycotoxins in 27 egg samples from organic, free-range, and intensive farms using a multiclass screening method based on LC-MS/MS (Capriotti et al., 2012).

Occurrence of enniatins in feed

Previous study found high levels of enniatin and beauvericin in fresh whole-crop maize, with enniatin B being the most prevalent(Sørensen et al., 2008). Processing contaminated cereal grains increases the concentrations of beauvericin and enniatins in by-products, which poses a potential risk to livestock. Over 70% of these toxins are found in by-products after food processing, (Hu et al., 2014) explaining the higher proportions of poultry feed samples contaminated with these toxins. (Marin et al., 2013).

2.2.2 Toxicity identification and characterization

***In vitro* studies using human and experimental animal models**

Enniatins A, A1, B, and B1 have varying bio accessibility in different foods (Meca et al., 2012), with the lowest in foods with high dietary fiber content (Alessandra et al.,2013). The duodenal bio accessibility ranges from 0.4-22% in infant formula and increases to 1.6-29% in duodenal and colonic digestion (Serrano et al., 2014). Enniatins A, A1, B, and B1 can be transported across Caco-2 cell monolayers, with transport reaching 20-42% after two hours and 55-70% after four hours (Meca et al., 2012). Enniatin B is extensively metabolized in liver microsomes, with 12 identified metabolites. Further, species differences in metabolite formation rates were observed between rats, dogs, and humans (Fæste et al., 2011).

Chicken liver microsomes produce eight metabolites of enniatin B, including a novel monohydroxylated metabolite (M13), while human liver microsomes produce demethylated metabolites (M6 and M7), indicating species differences in biotransformation pathways (Ivanova et al., 2014).

***In vivo* studies on humans and experimental animals**

Enniatin B was administered to rats and its radioactivity levels in urine and feces were examined, with only 5% of the administered radioactivity being recovered in urine within 48 hours. The label incorporated into the N-Me-Val moiety may potentially be lost due to oxygenation and dealkylation reactions (Faeste et al., 2011, Ivanova et al., 2011) Enniatin A was administered to rats at a concentration of 465 mg/kg feed for six weeks, resulting in increased plasma concentrations, but no detection in urine or feces; no metabolites were examined (Juan et al., 2014). Rats fed with *Fusarium tricinctum* contaminated feed containing 465 mg enniatin A/kg feed had a daily intake of 20.9 mg/kg b.w. per day and showed accumulation of enniatin A in serum, liver, and intestinal tract (Manyes et al., 2014).

There is a lack of *in vivo* studies on the toxicokinetics of enniatins in farm animals, with no studies identified for ruminants and companion animals. The only available data for poultry is based on occurrence studies in eggs of laying hens, and in turkey and broiler tissues, with one old study available for rabbits and guinea pigs. Enniatins were found in Finnish eggs at trace concentrations in 2004-2005, possibly due to their lipophilic properties and transportation by lipoproteins. The carry-over rate of enniatins from feed to eggs of laying hens could not be calculated (Jestoi et al., 2009). Two feeding experiments were conducted on broilers and laying hens to study the effects of enniatin B contamination in their diet. Enniatin B was found in all egg samples, 89% of serum samples, and 63% of liver samples, indicating bioaccumulation in eggs due to their high level of lipoproteins. LC-MS/MS analysis revealed several metabolites from the biotransformation of enniatin B, including a novel metabolite M13, which was the major compound detected in all analyzed liver, serum, and egg samples (Ivanova et al., 2014). A pilot study on the toxicokinetics of enniatins in pigs showed significant differences in the time that different enniatins used to reach the maximum plasma concentrations (Devreese et al., 2013). The bioavailability of Enniatin B in pigs was found to be 90.9% when given orally, with rapid distribution and elimination (Devreese et al., 2014). Two poultry experiments were conducted to study the carry-over of mycotoxins to eggs, liver, and skin. Broilers and hens were fed diets with varying concentrations of mycotoxins, including deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin, enniatins, and beauvericin. In meat, enniatins A and A1 were detected below the limit of quantitation (LOQ). In the liver, enniatin B1 was detected at a low level. In the skin, enniatin B, enniatin B1 and beauvericin were found at low levels close to or below the LOQ. In eggs, enniatin B, enniatin B1 and beauvericin were detected after three days of the repletion period. After 10 days of the depletion period, eggs were free of enniatins and beauvericin (CODA-CERVA, 2011/2012).

Acute, subacute, subchronic and chronic toxicity

The LD_{50} of 350 mg/kg was reported from fusafungine in mice after oral administration (Jestoi, 2008). Enniatin mixture (B, B1 and A1) was tested for anti-HIV activity in an *in vivo* hollow-fiber assay model using SCID mice. Previous study show after 28-day repeated dose assay in rats, using enniatin A (ENA) naturally contaminated feed (20.91 mg/kg bw/day), the concentration found in liver was 23 mg/kg (Okano et al., 2021). Another study showed in the rat livers, enniatin damage the liver by the modification of the mitochondrial activity and

interferer acetylation processes and in consequence, in metabolic disturbance (Cimbalo et al., 2021).

2.2.3 Biochemical modes of action

Ionophoric properties

Enniatins can transport cations through membranes, potentially leading to toxic effects, and can act as ion carriers (Kouri et al., 2003). Previous study show 1 to 100 μM of enniatin could change the pH and physiological ionic balance (Kirst et al., 2001).

Antimicrobial properties

Enniatins have antibiotic, antihelmintic, insecticidal, and phytotoxic properties. They can inhibit the growth of certain bacteria, including *Bacillus pumilus* (Uhlig et al., 2007). Enniatin B inhibits the growth of pathogenic bacteria in the human GI tract and intestinal microflora, with the most active strains being Staphylococcus aureus and Clostridium perfringens (Meca et al., 2011). Enniatin B1 is the most effective enniatin against probiotic microorganisms, with a MIC of 0.02 μg per disc for *Bifidobacterium adolescentis* (Roig et al., 2014). Enniatins have antibiotic effects and are used in the drug fusafungine, which has shown bacteriostatic activity against respiratory tract infections and oral cavity saprophytes (German et al., 2004). Previous study found the antibacterial capacity strongly in the 17 different secondary metabolites from Fusarium against *Lactobacilli,* which could effect to the gut microbiota (Sondergaard et al., 2016). Enniatin A1 show the bactericidal capacity against *Mycobacterium tuberculosis*. This study showed *M. tuberculosis* 's membrane potential might had been interfere by the Ionophobic properties of Enniatin A1 (Wang et al., 2019).The result of previous study show the deoxynivalenol antagonized the antibiotic effect of enniatin in animal fecal microbiome (Novak et al., 2021).

Enzyme inhibitors

Enniatin B exposure inhibits acyl-CoA acyltransferase (ACAT), suppresses triglyceride synthesis and free fatty acid pools, and reduces cholesterol ester synthesis in $HepG_2$ cells. It also inhibits $_{3,5}$-cyclo-nucleotide phosphodiesterase, binds calmodulin, alters calmodulin-dependent signal transduction, and inhibits multidrug resistance proteins (Trenin et al., 2000). Enniatins also inhibit the catalytic activity of topoisomerases (Dornetshuber et al., 2009).

Oxidative stress

Enniatins induce reactive oxygen species, lipid peroxidation, and decrease glutathione levels in human intestinal epithelial cells, but possess antioxidant capacities in human promyelocytic leukemia and cervix carcinoma cells (Dornetshuber et al., 2009; Prosperini et al., 2013).

Cytotoxicity and apoptosis

Enniatins induce apoptosis by increasing cytoplasmic calcium concentration and intercalating with DNA, allowing the action of endonuclease (Chen et al., 2006). Enniatins can inhibit multidrug resistance proteins (Hiraga et al., 2005) and disrupt extracellular-regulated protein kinase (Wätjen et al., 2009), while reducing lysosomal function and mitochondrial metabolic activity in cells (Ivanova et al., 2012).

Immunotoxicity

Previous research studied the effects of beauvericin and enniatin B on the differentiation of human monocytes into macrophages or immature dendritic cells. They found that beauvericin did not affect the differentiation process, while enniatin B decreased the expression of maturation markers, and increased IL-10 secretion in dendritic cells (Ficheux et al., 2013). Additionally, exposure to beauvericin or enniatin B during the differentiation process into macrophages resulted in a decrease in endocytosis ability and an increase in CD71 expression in monocytes. Exposure to enniatin B in murine monocyte-macrophage cells led to increased levels of activated caspase-1 and the release of interleukin-1 beta (IL-1β), particularly in cells primed with lipopolysaccharide (LPS), suggesting the involvement of caspase-1 in IL-1β release and potential activation of the inflammasome through lysosomal damage and cathepsin B leakage (Gammelsrud et al., 2012).

In vivo, increased percentage of CD4+ T helper lymphocytes and decreased percentage of CD8+ cells were found in rats after oral exposure to enniatin A (Juan et al., 2014). The functional implications of these changes are unclear. Fusafungine, a mixture of enniatins, has been found to have anti-inflammatory effects in respiratory tract infections, improving symptoms related to both local and systemic inflammatory reactions in various respiratory tract diseases (German, 2001).

Hematotoxicity and myelotoxicity

***In vitro* studies**

Enniatin B was tested on human hematopoietic progenitors *in vitro* and showed cytotoxic effects on platelet progenitors at 1.8 µM, potentially leading to a decrease in platelet production and adverse effects on coagulation (Ficheux et al., 2012). Enniatin B also disrupted the differentiation of human red blood cell progenitors (Ficheux et al., 2012). In terms of myelotoxicity, beauvericin and enniatins were ranked lower than HT-2 toxin, T-2 toxin, diacetoxyscirpenol, deoxynivalenol, and moniliformin (Ficheux et al., 2012).

Genotoxicity

Enniatin B was tested for genotoxic potential in various *in vitro* assays, including *Salmonella* Typhimurium assays and the hypoxanthine-guanine phosphoribosyltransferase (HPRT) assay with V79 cells, and no mutagenicity or increase in mutant frequency was observed (Behm et al., 2009). Variation is observed in the studies reviewed in Table 1 for *in vitro* genotoxicity tests of enniatin B from EFSA Panel on Contaminants in the Food Chain. Additional tests using the micronucleus assay and the Comet assay also showed no significant

genotoxic potential of enniatin B, but did exhibit pronounced cytotoxicity in V79 cells (Behm et al., 2009). Enniatins, a mixture of A, A1, B, and B1 homologues, have been shown to intercalate into DNA and inhibit the activity of topoisomerase I and II, with cytotoxic effects independent of DNA repair pathways and unaffected by ATM protein kinase (Dornetshuber et al., 2009).

Table 1. *In vitro* genotoxicity tests of enniatin B from EFSA Panel on Contaminants in the Food Chain (CONTAM., 2014)

Test	Cell system	Dose (µM)	Metabolic activation	Exposure time (hours)	Result	Reference
Reverse gene mutation assay (Ames test)	*S.* Typhimurium TA98, TA100, TA102, and TA104	1–100	With and without S9	n.a.	Negative	(Behm, Degen, & Föllmann, 2009)
Gene mutation assay (HPRT locus)	Hamster lung V79 cells	0.1–20	With and without S9	4	Negative	(Behm, Degen, & Föllmann, 2009)
Micronucleus test	Hamster lung V79 cells	0.03–10	Without S9	18	Negative	(Behm, Degen, & Föllmann, 2009)
Comet assay	Hamster lung V79 cells	0.1–100	Without S9	3–18	Negative	(Behm, Degen, & Föllmann, 2009)
Comet assay	RAW 267–4 murine macrophages	2.5, 5	Without S9	24	Negative	(Gammelsrud et al., 2012)

n.a.: not applicable

2.3 Effects of mycotoxins on microorganisms

The effects of mycotoxins on the gut microbiota can lead to population changes at different levels, which can be the result of the antimicrobial properties of mycotoxins, or the toxic effect on gut cells and leakage of antimicrobial compounds, making it challenging to characterize the effects due to variations in mycotoxin concentration and gut microbiome composition (Sheth et al., 2019; Tang et al., 2020).

Antimicrobial Properties of Mycotoxins

Mycotoxins such as aflatoxin (AFB1) (Arai et al., 1967), beauvericin, and enniatins (Bertero et al., 2020) have been found to inhibit bacterial growth, but their use as antibiotics is discouraged due to their toxicity. *In vitro* studies with rumen fluid have shown that mycotoxins indirectly affect the microbiota by reducing digestibility, gas production, and ammonia-N concentrations in a culture system with ruminal microbial population collected from steers (Mojtahedi et al., 2013). Studies using ovine rumen fluid found that AFB1 partially inhibited microbial activity, leading to reduced digestion of alfalfa hay and decreased gas

production and ammonia-N concentrations (Westlake et al., 1989). Certain mycotoxins found in silage fed to ruminant species, such as patulin, glyotoxin, mycophenolic acid, and roquefortine C, were suspected to have health impacts at high concentrations, but some mycotoxins produced by *Monascus spp*. may reduce methane production without affecting fermentation and feed digestion, potentially reducing the environmental impact of ruminant production (Tapia et al., 2005). Deoxynivalenol (DON) showed no antimicrobial activity against *Ruminococcus albus* and *Methanobrevibacter ruminantium*, but their growth was inhibited by fusaric acid (May et al., 2000). However, DON reduced gas production and concentrations of ammonia-N and volatile fatty acids (Jeong et al., 2010), while increasing the fermentation of organic matter, crude protein, and neutral detergent fiber in a contaminated diet (Seeling et al., 2006). The presence of DON at a concentration of 5 mg/kg in the diet did not affect organic matter fermentation or microbial protein synthesis, but it did alter the composition of the *Clostridium* population, potentially reducing cellulolytic activity in the rumen microbiota. Additionally, the extent of organic matter fermentation and crude protein degradation varied depending on the rate of concentration in the diet, which could have a greater impact on the rumen microbiota than mycotoxins (Boguhn et al., 2010). Microbial efficiency in rumen did not change by 100 mg/kg of FB1 (Gurung et al., 1999). Studies using an *in vitro* model of colon fermentation in humans found that OTA has a weak effect on fermentation and minor changes in the *Bifidobacterium* population, suggesting that antimicrobial properties of mycotoxins on gut microbiota are observed at high concentrations (Ouethrani et al., 2013).

Changes in the Gut Microbiota Secondary to Mycotoxin Exposure

Changes in the gut microbiota due to mycotoxin exposure vary across animal species and are influenced by factors such as dose, duration, and experimental design. The composition of gut microbiota varies between species due to genetics and environmental factors, with differences in bacterial composition observed in different genus (Liu et al., 2020). The effects of mycotoxins on the gut microbiome vary depending on the portion of the gut analyzed, and these effects are primarily due to toxicity rather than antimicrobial properties (Cieplińska et al., 2019). Variation is observed in the studies reviewed in Table 2 in terms of the time of observation, length of exposure to the toxin, and age of the animal. Changes in microbiota composition have been reported with age and the composition of the feed, which varies with the age of the animals in farm conditions. The effect of mycotoxins on microbiota varies with time, with some studies reporting transient and reversible effects. The dose of mycotoxin is a determining factor in the results, although monotone dose-response effects were not always observed. *Bacteroides* and *Lactobacillus* genus are commonly affected by mycotoxins, which can alter the microbiota composition and subsequently affect mycotoxin toxicity.

Mycotoxin exposure (table 2.), including Aflatoxin B1 (AFB1), Deoxynivalenol (DON), Ochratoxin A (OTA), and Zearalenone (ZEN), significantly alters gut microbiota across species. In rats, AFB1 and DON affect microbial diversity and specific genera. OTA consistently reduces diversity and increases Lactobacillus. In mice, these mycotoxins alter key phyla and reduce diversity. Rabbits show decreased diversity and shifts in bacterial groups with ZEN and DON.

In pigs, DON and combined mycotoxins impact Firmicutes and Bacteroidetes, altering microbiota balance. Poultry exhibit increased harmful bacteria and reduced beneficial bacteria like Lactobacillus under mycotoxin exposure, with ducks showing increased Bacteroidetes with OTA.

Table 2. Effect of mycotoxins on gut microbiota composition

Species	Exposure *	Method of Analyze	Sample Analyzed	Result	Reference
Rat	AFB1: 5, 25, 75 µg/kg BW 28 days	16S rRNA gene sequencing	excreta	Phylogenic diversity declined. There is no discernible pattern of growth or decline at the phylum level.	(Wang et al., 2016)
Rat	AFB1: 25 µg/kg BW 28 days	16S rRNA gene sequencing	excreta	*Bacteroidetes* (13.5%), *Firmicutes* (82%) the most plentiful (3.3%) *Proteobacteria* 1.7 percent of actinobacteria and 1% of *Saccharibacteria* Microbiota richness is unaffected. *Prevotella_9* has less *Alloprevotella* spp., which is more prevalent.	(Liew, Mohd-Redzwan, & Than, 2019)
Rat	DON: 100 µg/kg BW 28 days	RT-PCR	excreta rats inoculated with human fecal flora	The microbiota changed over time; *Bacteroides* and *Prevotella* genera are more prevalent from days 10 to 20. Expression of *Escherichia coli* was down on day 27.	(Saint-Cyr et al., 2013)
Rat	DON: 60, 120 µg/kg BW 40 days	16S rRNA gene sequencing	cecal digesta	The most prevalent taxa were *Firmicutes* and *Bacteroidetes*, with the relative abundance of the *Coprococcus* genus increasing.	(Miró-Abella et al., 2018)
Rat	DON: 2, 10 mg/kg feed 28 days	16S rRNA gene sequencing	excreta	There hasn't been a major change in the microbiota's diversity or composition.	(Payros et al., 2017)
Rat	OTA: 70, 210 µg/kg BW 28 days	16S rRNA gene sequencing	excreta	Decreased diversity of the microbiota inside subjects; increased relative *Lactobacillus*	(Guo et al., 2014)

Table 2. continued. Effect of mycotoxins on gut microbiota composition

				abundance; reduced relative abundance of *Ruminococcus, Bacteroides, Dorea, Oribacterium*, and *Syntrophococcus*	
Mice	AFB1*: 100, 160, 400 µg/kg BW 60 days	16S rRNA gene sequencing	intestinal contents (from jejunum	The predominant bacteria are *Lactobacillus* and *Bacteroides*.	(Yang et al., 2017)
Mice	DON: 1, 5 mg/kg BW 14 days	16S rRNA gene sequencing	excreta	The microbiota changes over time. The four most common bacterial phyla are *Bacteroidetes, Firmicutes, Proteobacteria*, and *Verrucomicrobia*. Reduced relative abundance of the *Alistipes* genus and *Bacteroidaceae* family on day 14.	(Lin et al., 2020)
Mice	DON: 1, 5 mg/kg BW 14 days	shotgun sequencing	cecal digesta	The most prevalent genera were *Parabacteroides, Bacteroides, Mucispirillum, Mastadenovirus*, and *Lactobacillus*. relative abundance of *Firmicutes* increased at low dosages. *Bacteroidetes* become more prevalent overall when exposed to large amounts.	(J.-J. Wang et al., 2019)
Mice	DON: 10 µg/kg BW 280 days	16S rRNA gene sequencing	excreta	The majority of *Deferribacteres, Proteobacteria, Verrucomicrobia*, *Tenericutes*, and Cyanobacteria bacteria belong to the *Firmicutes* phylum. *Actinobacteria* and *Bacteroidetes* are less common. Different taxonomic abundances at the family and genus levels are rather noticeable.	(Vignal et al., 2018)

Table 2. continued. Effect of mycotoxins on gut microbiota composition

Mice	ZEN: 10 mg/kg BW 14 days	16S rRNA gene sequencing	colon digesta	The predominate phyla in the colon were *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria*; decreased microbiota diversity *Bacteroidetes* and *Firmicutes* are less common.	(Wang et al., 2018)
Rabbit	ZEN: 400, 800, 1600 µg/kg BW 28 days	16S rRNA gene sequencing	cecal digesta	Actinobacteria abundance decreased, whereas *Cyanobacteria, Synergistetes*, and *Proteobacteria* abundance rose. *Adlercreutzia, Blautia, Desulfitobacter, Lactobacillus*, and *Oxalobacter* become increasingly rare.	(Li et al., 2018)
Rabbit	DON: 1.5 mg/kg BW 24 day	16S rRNA gene sequencing	ileal, cecal and colon digesta	The ileum and caecum microbiota were less diverse, with *Firmicutes, Bacteroidetes*, and *Proteobacteria* dominating. *Firmicutes* and *Bacteroidetes* grew, with *Ruminococcaceae* being the biggest. *Proteobacteria, Actinobacteria*, and *Cyanobacteria* dropped	(Wang et al., 2020)
Pig	DON: 2.5 mg/kg feed 28 days	Bacterial culture Capillary electrophoresis	excreta	The evolution of the entire aerobic bacterial flora throughout time The maximum increase in total aerobic mesophilic bacteria occurs on day 7.	(Waché et al., 2008)
Pig	DON: 1,3 mg/kg feed 28 days	16S rRNA gene sequencing	small intestinal lumen digesta	*Firmicutes, Proteobacteria, Cyanobacteria*, and *Actinobacteria* were dominant phyla, with reduced *Firmicutes* and increased *Actinobacteria* in duodenum and ileum.	(Liu et al., 2020)

Table 2. continued. Effect of mycotoxins on gut microbiota composition

				Lactobacillus, Cupriavidus, Acinetobacter, Burholderia, Staphylococcus, Ochrobactrum, Corynebacterium, and *Streptococcus* were predominant genera.	
Pig	DON: 0.61, 1.28, 2.89 mg/kg feed 28 days	16S rRNA gene sequencing	cecal digesta	Decreased abundances of unclassified *f_Lachnospiraceae, Phascolarctobacterium, Ruminococcaceae_UCG-014*, increased *Prevotella_9*, and no rank *f_Prevotellaceae*, as well as a decrease in mesophilic aerobic bacteria and *C. perfringens, E. coli*, and *Enterobacteriaceae* family.	(S. Wang et al., 2019)
Pig	ZEN: 40 µg/kg BW DON: 12 µg/kg BW ZEN + DON: 40 + 12 µg/kg BW42 days	EcoPlate tests	ascending colon digesta	The study shows decreased abundances of unclassified *f_Lachnospiraceae, Phascolarctobacterium, Ruminococcaceae_UCG-014*, increased *Prevotella_9*, and no rank *f_Prevotellaceae*, and decreased *mesophilic aerobic bacteria* and *C. perfringens, E. coli*, and *Enterobacteriaceae* family	(Piotrowska et al., 2014)
Pig	ZEN: 0.8 mg/kg feed DON: 8 mg/kg feed 7 days	16S rRNA gene sequencing	colon digesta	*Firmicutes* and *Bacteroidetes* dominate the phyla *Lactobacillus*, *Megasphaera*, and *Fecalibacterium*, with the unclassified *Clostridiaceae* family being the most abundant. *Lactobacillus* is more abundant in DON and ZEN groups.	(Reddy et al., 2018)

Table 2. continued. Effect of mycotoxins on gut microbiota composition

Pig	ZEN: 5, 10, 15 µg/kg BW 7, 21, 42 days	Bacterial culture	duodenal cap, third duodenum part, jejunum, caecum, descending colon digesta	ZEN affects colony counts of microbiota rather than diversity, increasing yeast and mold counts in all intestinal segments, particularly in the colon.	(Cieplińska et al., 2019)
Pig	DON + ZEN: 3.02 + 0.76 mg/kg feed 7 days Repeated exposure	16S rRNA gene sequencing	excreta	The microbiota balance was altered by a single or repeated DON/ZEN challenge, resulting in reduced abundances of *Ruminococcaceae, Streptococcaceae*, and *Veillonellaceae*, as well as increased *Erysipelotrichaceae.*	(Le Sciellour et al., 2020)
Pig	FB1+FB2: 8.6 + 3.2 mg/kg feed 63 days	Capillary single-stranded conformation polymorphism analysis	excreta	A DON/ZEN challenge altered the microbiota balance, reducing the abundance of *Ruminococcaceae, Streptococcaceae*, and *Veillonellaceae*, and increasing *Erysipelotrichaceae.*	(Burel et al., 2013)
Pig	FB1: 12 mg/kg feed 0, 8, 15, 22, 29 days	16S rRNA gene sequencing	excreta	Exposure to a specific environment leads to a decrease in diversity index and changes in microbiota structure and composition, with increased Lactobacillus and reduced *Lachnospiraceae, Veillonellaceae* families.	(Mateos et al., 2018)
Broiler	AFB1: 40 µg/kg feed 21 days	Bacterial culture	ileal digesta	No effect on *Lactobacilli, Bifidobacteria, C. perfringens, E. coli.*	(Liu et al., 2018)
Broiler	Aflatoxins 0.5, 2 mg/kg feed 7 and 28 days	Bacterial culture	ileal digesta	Increased E. coli, *Salmonella, Klebsiella,* and total Gram-bacteria at day 28 of exposure. Changes persisted for 14 days after exposure stopped.	(Jahanian et al., 2019)

Table 2 continued. Effect of mycotoxins on gut microbiota composition

Broiler	AFB1: 1, 1.5, 2 mg/kg feed 21 days	Bacterial culture	cecal digesta	Increased total aerobic bacteria, total Gram- bacteria, variable effect on total lactic acid bacteria; Effects are not always dose-dependent.	(Galarza-Seeber et al., 2016)
Broiler	DON: 2.5, 5 and 10 mg/kg feed 35 days	16S rRNA gene sequencing	cecal digesta	*Firmicutes*' relative abundance increased, while *Proteobacteria*'s abundance decreased due to decreased *Oscillospira, Clostridiaceae, Clostridium*, and *Ruminococcaceae* genera, and *Clostridiales* genus.	(Lucke et al., 2018)
Broiler	FB1 + FB2: 10.4 + 8.2 mg/kg feed 15 days	16S rRNA gene sequencing	ileal digesta	After 15 weeks, *Candidatus Savagella* and *Lactobacillus* spp. abundance decreased, while total *Clostridium perfringens* increased. *Lactobacillus* and *Bifidobacterium* spp. decreased in intestinal content and excreta samples	(Antonissen et al., 2015)
Turkey	OTA: 199 to 462 µg/kg feed 21, 42, 63, 105 day	Bacterial culture	jejunum and cecal digesta, excreta	Over 15 weeks, *Candidatus Savagella* and *Lactobacillus* spp. abundance decreased, while total *Clostridium perfringens* increased, and *Lactobacillus* and *Bifidobacterium* spp. decreased in intestinal content and excreta samples.	(Śliżewska et al., 2020)
Duck	OTA: 235 µg/kg BW 14 days	16S rRNA gene sequencing	excreta	Increased *Bacteroidetes* (phylum level), *Bacteroide*s (genus level)	(W. Wang et al., 2019)

2.4 Human gut microbiota structure and composition

Gut microbiota structure and composition was the key controller of immune and metabolic functions (Thursby et al., 2017). Gut microbiota consist of bacteria, yeast, and viruses more than 160 species (Laterza et al., 2016). The most phyla that we found in human gut microbiota was *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria,* and *Verrucomicrobia* (Arumugam et al., 2011). Gut microbiota can now be studied better with advanced sequencing methods like targeting the 16S ribosomal RNA (rRNA) gene. (Poretsky et al., 2014). Shorter gene subregions are now focused on for more accurate results (Mizrahi-Man et al., 2013), but errors can occur with shorter read lengths (Poretsky et al., 2014). Whole-genome shotgun metagenomics provides more reliable microbiota composition estimates (Hugon et al., 2015). Human-associated microbial repertoire includes 2172 species, with Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes being dominant (Hugon et al., 2015). Gut microbiota is less diverse but functionally redundant, influenced by factors like diet and genetics, crucial for developing disease therapies (Li et al., 2014).

2.4.1 Microbiota composition or alteration and the host health

The relevance of the intestinal microbiome in the digestion of complex food particles, defense against the expansion of pathogenic organisms, development of the intestinal epithelium, and control of the local immune system and metabolic processes has long been acknowledged. The involvement of intestinal microbiota in the pathogenesis of various disorders has recently been highlighted (Costa et al., 2018). Changes in the intestinal microbiota of horses are probably related to several disorders, although it can be challenging to distinguish between cause and effect. Studies examining the microbiota before, during, and after sickness can aid in determining causality, but they are exceedingly challenging to carry out on sporadic illnesses. Therefore, it is crucial to keep in mind that, while it is valuable to identify variations between ill patients and healthy controls, these frequently may only be regarded as differences and not causative events. Intestinal microbiota in horses may be linked to various diseases, but determining cause versus effect is challenging. The diversity of gut microbiota identifies the normal physiological of health gut microbiota. However, the health of gut microbiota could change due to the age, lifestyle, and food. Dysbiosis is the change of gut microbiota composition and can cause of variation of disease and disorder in human health (Rinninella et al., 2019).

Dysbiosis

Dysbiosis is a condition characterized by an imbalance in the gut microbiota, leading to immune system activation and changes in intestinal epithelial cells(Yoon et al., 2018). Dysbiosis is often caused by the proliferation of pathobionts, such as Enterobacteriaceae, due to factors like medication, poor diet, and stress (Levy et al., 2017). Loss of resident microorganisms and decreased diversity in the gut microbiota are also associated with dysbiosis, which can contribute to various diseases including inflammatory bowel syndrome (Canakis et al., 2020), obesity (Maruvada et al., 2017), and Parkinson's disease (Dutta et al., 2019).

Liver disease

The gut and liver have a close relationship known as the "gut-liver axis," influenced by gut bacteria and their byproducts like ethanol and ammonia (Giannelli et al., 2014). Changes in gut bacteria can lead to liver damage and complications in cirrhosis patients, affecting microbial diversity and increasing certain bacterial species (Lakshmi et al., 2010). The gut microbiota can impact liver function, progression of liver damage, and complications in cirrhosis patients through various mechanisms involving bacterial endotoxins (Chen et al., 2011). Dysbiosis in Acute-on-chronic liver failure (ACLF) patients' gut microbiota was linked to mortality, with certain bacterial families and MELD score predicting outcomes (Chen et al., 2015). Changes in gut microbiota composition contribute to liver diseases like hepatocellular carcinoma (HCC) and autoimmune liver diseases such *as Veillonella, Streptococcus*, and *Clostridium* increase in liver cirrhosis Chinese patients (Qin et al., 2014). NAFLD (multifactorial disorder comprising a group of diseases) development involves genetic, epigenetic, and environmental factors, with gut microbiota alterations playing a key role (Tilg et al., 2017). Liver diseases are associated with increased Enterobacteriaceae and decreased Bifidobacterium, leading to endotoxemia and immune dysfunction (Boursier et al., 2016). Probiotics development is suggested for preventing and treating liver disease progression (Wesolowski et al., 2017).

Obesity

Gut microbiota and diet play a significant role in obesity and metabolic disorders (Le Chatelier et al., 2013). Studies show that the microbiota from lean or obese individuals can affect adiposity in mice, influenced by diet (Caesar et al., 2015). Gut microbiota impacts metabolic diseases (Sonnenburg et al., 2016) , circadian rhythms (Leone et al., 2015) , and various physiological processes like energy metabolism (Delzenne et al, 2008)and immune homeostasis (De Vos et al., 2013).

Type 2 diabetes

The gut microbiome composition is linked to Type 2 Diabetes (T2D) development (Qin et al., 2012). Altered gut microbiome with lower diversity may lead to diabetes through inflammation(Burcelin, 2016). Strategies like specific fibers or therapeutic microbes show promise in reducing insulin resistance and metabolic diseases (Mardinoglu et al., 2016).

Gastric carcinogenesis

Study investigated gut microbiome changes in a rat model of gastric cancer using various substances. Gut bacteria richness increased, diversity decreased during cancer progression, with notable changes in precancerous lesions. Rat model's gut microbiome changes mimic those in humans, aiding in testing new treatments for gastric cancer (Yu et al., 2020).

2.4.2 Microbiota structure analysis

The investigation of bacteria has shifted from focusing on pathogenic organisms to evaluating novel characterization methods for complex microbial populations containing

members of different Kingdoms. Culture-based methods for studying bacterial communities are limited in their ability to recover all species present, with estimates suggesting that only 5-20% of species can be cultured. However, recent studies have explored more complex and expensive approaches to grow additional species in select research laboratories (Eckburg et al., 2005). The realization of the limitations of culture-based studies led to the development of culture-independent approaches, such as molecular fingerprinting and sequence-based methods. Molecular fingerprinting generates DNA banding patterns to compare samples, while sequence-based methods allow for more specific identification of microbes by characterizing part of their genome. Sanger sequencing was the first method used to evaluate mixed microbial populations, but it was limited to sequencing one DNA strand at a time. Next-generation sequencing (NGS) technologies have reduced cost and time limitations by sequencing millions of DNA strands in parallel, allowing for studies with millions or billions of sequences. Limitations still exist, such as small sequence sizes affecting accuracy and the potential for bias introduced by different PCR primers, highlighting the need to understand the general concepts of microbiota assessment (Walters et al., 2016).

2.4.2.1 Methods for microbiota structure analysis

Molecular methods

Quantitative PCR (qPCR)

Quantitative PCR (qPCR) is a technique utilized for detecting specific groups of organisms by using specific primers for each organism or group of bacteria. One advantage of qPCR is that it can provide quantification of the DNA fragments being amplified, allowing for the absolute abundance of the populations investigated to be determined. However, the method requires different sets of primers to target each bacterial taxon, which can increase the time and expense required to obtain limited amounts of data (AlShawaqfeh et al., 2017).

Targeted amplicon sequencing

Next-generation sequencing is revolutionize of biology by allowing cost advantageous and fast genome sequencing of non-model organisms (Mardis, 2008), even though there is a require to maximize the technology for systematic and population genetic studies aim on specific genes from large individuals (Lennon et al., 2010). Next-gen technology has been used for targeted sequencing, but it absence a trustworthy method that is easily ascendable, time-no-labor-intensive, relatively economical, and can be applied to a broad diversity of organisms and genes (Rubin et al., 2010). To solve this, a targeted amplicon sequencing (TAS) approach has been suggested. TAS is a two-step PCR process that amplify a targeted gene region using traditional PCR, followed by an additional PCR that attaches a known 10 base pair tag, barcode, or multiplexing identifiers (MIDs) to classify amplicons from different samples. This results in tagged amplicons ready to be precisely sequenced using a next-gen platform without comprehensive amplicon preparation steps combination of MID-locus specific primer. TAS concede researchers to have complete control of PCR protocols and is fully scalable while still generating an MID tagged amplicon library (Bybee et al., 2011).

2.4.2.2 Limitation of next-generation sequencing (NGS)

The limitations of next-generation sequencing (NGS) for microbiota assessment include lack of absolute quantification and potential bias introduced at various steps, such as DNA extraction, primer selection, PCR amplification, and bioinformatics parameters, which can favor or underestimate certain bacterial taxa (Walters et al., 2016). Prey DNA detection in fecal or stomach samples is influenced by DNA degradation, PCR inhibitors, and preferential amplification of certain DNA fragments, highlighting the importance of optimizing PCR protocols for efficient primer usage and error reduction in NGS(Shehzad et al., 2012). The major limitation of NGS is the short size of DNA fragments it can sequence, which hampers classification at lower taxonomic levels; longer fragment sequencing technologies are available but still limited, and low abundance bacteria may be underrepresented after PCR amplification, with fewer PCR cycles reducing detection bias. Next-Generation Sequencing (NGS) only provides a relative abundance of bacterial populations, not actual counts, and warns that comparing changes in relative abundances can lead to erroneous conclusions. However, combining NGS with quantitative PCR (qPCR) can overcome this issue by providing total bacterial counts. Molecular methods for detecting bacterial DNA have limitations as the presence of DNA does not necessarily indicate the microorganism is a consistent part of the microbiota or that it is viable. Metagenomics provides information about the genetic capacity of a community, but gene expression is better measured through mRNA sequencing and protein analysis, although these methods are limited by cost, material instability, and database quality (Ari et al., 2016).

2.4.2.3 Microbiota analysis visualization

Alpha diversity and biomarker analysis

Habitat-level diversity is the most used metric for characterizing communities. It comprises two main components: species richness and equitability indices. Heterogeneity indices incorporate both of these components (Thukral, 2017).

Species richness measures the number of individuals per unit area or sample (e.g., quadrat) and the species present in that area. Also known as variety indices, species richness indices are higher in communities with more species. Equitability indices, on the other hand, reflect the relative abundance of different species within a community, indicating how evenly the individuals are distributed among the species. A community where species have an equal number of individuals will exhibit a higher evenness index. Conversely, a community dominated by one or a few species in terms of individual numbers will have a lower evenness index (Thukral, 2017).

Richness

Richness refers to the number of taxa, typically identified as operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) (Callahan et al., 2017)

Phylogenetic Diversity (PD)

Phylogenetic Diversity (PD) is a measure of richness that incorporates phylogenetic information. Despite the term "diversity," it does not consider the abundance of taxa. PD is calculated as the sum of the branch lengths on the phylogenetic tree that connect the members of the set, given the phylogenetic tree encompassing s taxa (Faith, 2006)

Chao index

Chao index estimates species richness based on observed taxa, giving more weight to rare species like singletons and doubletons (Chao, 1984). It assumes a Poisson distribution for taxa and is useful for datasets with low-abundance species (Kim et al., 2017), but singletons and doubletons are often removed from 16S rRNA datasets (Allen et al., 2016).

Shannon's Index

The Shannon index, also known as the Shannon-Wiener or Shannon-Weaver index, is a metric that quantifies community diversity by considering both species richness (the number of species) and evenness (how individuals are distributed among those species). In microbiome research, this index is frequently used to evaluate the diversity of microbial communities (Shannon, 2001). Shannon's Index is a way to measure the diversity of species in a sample by considering both the number of different species and how evenly they are distributed (Magurran, 2013).

Simpson's Index

Simpson's index (D) is a measure of taxa diversity that integrates both richness and evenness(Lemos et al., 2011) Here, s represents the number of OTUs/ASVs, and pi is the proportion of the community represented by the OTU/ASV. This index emphasizes taxa evenness more than taxa richness (Kim et al., 2017), indicating taxa dominance and providing the probability that two randomly chosen individuals belong to the same taxon. The index ranges from 0 to 1, with higher values indicating lower diversity.

Relative abundance plots

For plots of relative abundance, there are numerous ways to express the similarity of microbial community membership and structure. The most fundamental of them is a depiction of the relative abundance of the community's primary bacterial populations. The relative abundance of each taxon can also be visualized using heat maps, which compare a color scale between each sample and group. However, visualizing individual taxa becomes challenging in complicated environments such as feces, which contain hundreds of thousands of species. (Costa et al., 2019).

Principal coordinate analysis

Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) are the most often utilized methods for representing disparities between communities. The PCoA is derived from the creation of a distance matrix of each sample based on a database

listing the number of times a species appears in a community. However, following PCoA or NMDS analysis, this is a multi-dimensional figure that may be visualized in two or three dimensions (2D or 3D). This will also return the percentage of variance accounted for by each axis. For example, if the first axis explains 40% of data variance and the second 35%, a 2D plot will show 75% of that data set variation. If this number is low, it is preferable to add additional dimensions (3D) to better perceive the differences between the samples (Costa et al., 2019).

2.4 Humanized animal model for microbiota study

The physical fitness of animals' behavior, including that of humans, is impacted by the presence and make-up of the host's microbial populations. As a model organism for examining the processes of various diseases, the mouse has received most of the attention in microbiome research to date activities taking place in microbial communities. Additionally, mouse microbiome models have been the most popular alternative for preclinical research into the connections between host physiological, metabolic, immunological, and neurological characteristics and the microbiome. These have also been employed in the creation of approaches to treat functional deviations in populations that cause illness. However, the mouse is not a great model for examining many facets of the microbiome as well as host stimuli and environmental reactions. As a result, scientists have also begun studying the microbiomes of other species, such as zebrafish, pigs, and Drosophila. This chapter provides an overview of the microbiome research done using various models and an explanation of its benefits.

Humanized animal models, such as germ-free mice colonized with human microbiota, are widely recognized for their effectiveness in studying the human microbiota. These mice are bred under sterile conditions and subsequently introduced to human-derived microbial communities. This approach facilitates the exploration of human-specific interactions and functions of the microbiota within a live organism (Turnbaugh et al., 2009). These models are instrumental for investigating the impact of human microbiota on health and disease in a controlled environment, and for studying its interactions with the host's immune system, metabolism, and other physiological processes (Hugenholtz et al., 2018).

2.4.1 Rodents

The role of rodent models in exploring host-microbiota interactions, including immune responses and microbial metabolism. Researchers use these models to assess the impacts of diet, antibiotics, and various interventions on gut microbiota composition and function. The necessity of considering species-specific differences and employing meticulous experimental designs to ensure that the findings are relevant and translatable to human health (Casteleyn et al., 2010). Nonetheless, this ratio fluctuates between these two species in various gut regions. In mice and humans, the ratio of the small intestine to the colon is 2.5 and 7, respectively (Treuting et al., 2017). The cecum in mice is larger than in humans and serves as a site for fermenting plant material and synthesizing vitamins, while the human cecum is small and has no clear function (Treuting et al., 2017). There are considerable variations between the human and mouse digestive tracts at the microscopic level (Treuting et al., 2017). The colon of mice has thin *muscularis mucosae*, while the human colon has a thicker mucosal wall

and transverse folds along the colonic mucosa, which are only found in the cecum and proximal colon of mice, potentially creating different microbial communities. Mucin-producing goblet cells are plentiful on the surface of mouse intestinal crypts in the proximal colon, but their numbers diminish near the base of the crypt in the distal colon and rectum. Goblet cells are widely distributed in humans from the cecum to the rectum. The presence and role of Paneth cells differ between mice and humans, with Paneth cells being present in the cecum and proximal colon in humans but only in the cecum in mice. The location and amount of defensins produced by Paneth cells differ between humans and mice, which may impact local immune responses and the composition of intestinal microbiota (Cunliffe et al., 2001; Ghosh et al., 2002). Mice serve as a valuable model system for the investigation of host-microbiota interactions relevant to human biology (Spor et al., 2011) since humans and mice have about 99% of the same genes and these genes share significant phylum through family-level similarities with the human gut microbiome.

A gnotobiotic rat model was study with seven or eight bacterial species defining major members of the human gut microbiota. The choice of bacteria was based on their incidence in humans, evaporation products formed, genomic sequence availability, and ability to form a stable community in the rodent gut (Becker et al., 2011). Another study, the stability of bacterial communities in rats associated with human fecal bacteria for 12 months using PCR-denaturing gradient gel electrophoresis. The main human fecal microbiota can be found in the human microbiota-associated rat model for at least one year, suggesting the model's long-term stability (Alpert et al., 2008). The simplified human microbiota, was used in dietary research to study the effects of different diets on cell count, fermentation products, and microbiota-associated characteristics in the rat's caecal contents, comparing them to conventional and germfree rats (Becker et al., 2011).

The main outcome demonstrates that mice, despite anatomical and physiological differences from humans, remain valuable for modeling host-microbiota interactions relevant to human biology. Meanwhile, gnotobiotic rat models successfully mimic human gut microbiota, offering insights into microbial community stability and dietary impacts, supporting their use in biomedical and dietary research.

Germ-Free Rodent Models

In vivo models capable of capturing the complexity of the human microbiome in combination with a physiologically appropriate host are required. For several reasons, they are among the most widely used animal models in the main goal of biomedical research to improve human health. Mice may be generated with reasonable ease. Despite considerable duplication, there are significant distinctions between the main types of microbes that colonize lab mice rather than people, which may create mice with a gut microbiome that is unique to mice, but less translationally relevant for gut microbiota. Therefore, gut microbiota of germ-free rodents is a commonly utilized tool to improve human translation. In research using germ-free animals, Table 3 shows examples of germ-free animal models that enable colonization success for human microbiota study. Human gut bacteria linked to human health demonstrate varying degrees of colonization success.

Table 3: Human microbiota-associated rodent models for microbiota analysis

Organism	Colonization efficiency of organism		Recipient	Detection method	Reference
Bifidobacteriaceae	Despite significant abundance in inoculum (72.8%) completely lost 2 days postcolonization	L	Mouse	16S rRNA sequencing	(Nejrup, Licht, & Hellgren, 2017)
***Bifidobacterium* spp.**	Lost almost completely	L	Mouse	16S rRNA sequencing	(Zhang et al., 2017)
	Less prevalent than in the inoculum	L	Mouse	qPCR	(Zeng et al., 2013)
	Did not compare with the inoculum but showed more abundance than pig with pig microbiota	E	Pig	qPCR	(Che et al., 2009)
	Did not compare with the inoculum but showed more abundance according to high-bran diet	E	Mouse	Culturing	(HIRAYAMA et al., 1994)
***Fecalibacterium* spp.**	Lost almost completely	L	Mouse	16S rRNA sequencing	(Zhang et al., 2017)
	Well developed in recipient rats	E	Rat	DGGE + sequencing	(Licht, Madsen, & Wilcks, 2007)
	Did not compare with the inoculum but established in mice	E	Mouse	16S rRNA sequencing	(Zhang et al., 2017)
***Lactobacillus* spp.**	Increased abundance in recipient mice	M	Mouse	qPCR	(Zeng et al., 2013)
	Did not show 7 days postcolonization	L	Mouse	Culturing	(Wong, Hentges, & Dougherty, 1996)
	Did not show 14 days postcolonization	L	Mouse	qPCR	(Imaoka et al., 2004)
***Pseudobutyrivibrio* spp.**	Lost almost completely	L	Mouse	16S rRNA sequencing	(Zhang et al., 2017)

M: recipient animals had more abundance compared to the donor inoculum

L: recipient animals had less abundance compared to the donor inoculum

E: recipient animals had the same abundance compared to the donor inoculum

2.4.2 Pigs

Pigs are an appropriate animal model because of their functional and compositional parallels to human gastrointestinal systems (GI). The humans and pigs are similar in terms of minimal nutrient requirements. Pigs and humans have comparable nutrient-sensing systems. As a result, pigs' ability to taste acids (sour), carbohydrates (sweet), glutamic acid (umami), and fatty acids influences how much they eat. Pigs are useful for research on dietary taste testing and appetite management because of the similarities in their nutrient-tasting systems. Pigs and humans both employ the colon as the primary location for the fermentation of food fibers, as opposed to the cecum. Due to this common physiology, digesta transit periods and nutrient absorption mechanisms are comparable (Roura et al., 2016). The similarity of human functional pathway in porcine GI microbiota is 96% (Xiao et al., 2016). To assess the impact of gut microbial composition on diet-induced obesity and nonalcoholic steatohepatitis, researchers employed genetically modified pigs with variable obesity susceptibility (Pedersen et al., 2013). These gnotobiotic pig models with completely identified bacterial communities may be utilized to study the interactions between the host and the microbiota as well as the results of probiotic and dietary treatments. Although expensive and logistically challenging, these models can accurately replicate the human intestinal environment Mice are advantageous for genetic manipulation, cost-effectiveness, and ease of handling, making them suitable for controlled experiments. However, pigs offer advantages in mimicking human gastrointestinal anatomy, physiology, and diet, which are critical for studying gut microbiota interactions relevant to humans(Pluske et al., 2018). Helicobacter pylori is one such bacterium, and the process gastritis and stomach ulcers as a result of this (Roura et al., 2016). Pigs can be utilized to characterize microbiome alterations following neurological events because of comparable microbiota-gut-brain connections (Michael et al., 2021).

Because of the larger size of pigs and the simplicity of gavage feeding, a model of this illness has been successfully created in newborn piglets, which is favored over mice models (Burrin et al., 2020). Short bowel syndrome, which is frequently an infantile consequence of necrotizing enterocolitis, is brought on by a surgical resection of the intestine and results in poor nutritional absorption as well as imbalance. Because repeated measures, such as stool output and plasma proteins, may be taken for testing of long-term effects and probes of control systems, the pig model has been utilized to research *in vivo* processes and is superior to the mouse in this regard (Burrin et al., 2020).

Obesity, dyslipidemia, hypertension, and insulin resistance are all aspects of the metabolic syndrome. Ossabaw and Landrace pigs have created effective models for this condition (Renner et al., 2020). Pig intestinal endotoxin transit was affected by a diet high in polyunsaturated fatty acids (Mani et al., 2013). This endotoxin has been connected to obesity, inflammation, and metabolic disorders including diabetes. Additional dietary research has revealed that a high-calorie diet promotes the growth of colonic proliferative and stem cell

zones and that the pig colon, like that of humans, has two different resident stem cells (ASCL-2 and BMI-1) (Charepalli et al., 2017). The pig model could be utilized to research colon cancer and other diseases since a high-calorie diet has been proven to boost stem cell populations in the colon (Charepalli et al., 2017).

Future research might examine the possible therapeutic impacts of the gut microbiome on diet or, conversely, the detrimental consequences of an incorrect diet on dysbiosis, such as the deleterious effects of the microbiome on women with recurrent vaginal infections. There is a chance to learn more about how GI tract injury physically changes the microbial composition. The GI model for pigs might be used to study the impact of climate change on the microbiota and gut health.

2.4.3 Other Models

Zebrafish (*Danio rerio*)

Over the past few decades, zebrafish have been increasingly used in research. Zebrafish are useful because they have little space requirements, are widely available, are inexpensive, and reproduce quickly. Additionally, zebrafish are preferred because of the similarities of genetic, anatomical, and physiological similarity between zebrafish and their mammalian hosts (Wong et al., 2013). The digestive system and the adaptive immune system exhibit homology. Zebrafish have a pancreas, gall bladder, liver, and intestines that are comparable to those of mammals. Additionally, the goblet cells, enteroendocrine cells, and absorptive enterocytes that make up the intestinal epithelial cells are comparable to those seen in mammals. The transparent nature of zebrafish allows for real-time visualization of gut microbes (Rawls et al., 2007), making it an attractive experimental system for studying host genes and signaling pathways regulated by the microbiota (Patton et al., 2001). Mutagenesis, retrovirus-based insertional mutagenesis, zinc finger nucleases, morpholino-based gene knockdown, and the involvement of RNA interference in function loss are a few techniques used for genetic screening (De Rienzo et al., 2012). As a result, the zebrafish possesses several characteristics that make it a desirable experimental system. Using straightforward techniques for the growth of green fluorescent and gnotobiotic zebrafish, (Pham et al., 2008) conducted investigations on the early postembryonic development of zebrafish. Green fluorescent larvae use antibiotics to maintain the sterilization of embryos (Davis et al., 2016). The transparent body is the method that labeled in larvae or the microflora of the larvae (Singer et al., 2010). These methods may be used to identify the function of microbiota in a number of illnesses and disorders, including inflammatory bowel disease (IBD) (Yang et al., 2014). Probiotic bacteria may impact anxiety- and stress-related behavior, immunity and pathogen resistance (Qin et al., 2017), metabolism and reproduction (Qin et al., 2014). There are restrictions on using zebrafish as a study model for microbiota-related research because of the variations in exposure and environmental variables.

Fruit Fly (*Drosophila*)

Numerous studies have been conducted on the gut microflora of *Drosophila* to understand host-microbe interaction, with the microbial community being less complex compared to mammals (Chandler et al., 2011). Diet has a significant impact on the makeup of

the gut microflora in Drosophila, with prominent bacteria from the groups Acetobacteraceae, *Lactobacillales*, and Enterobacteriaceae (Corby-Harris et al., 2007). Drosophila is used as a model for the study of symbiosis and host-symbiont relationships (Shin et al., 2011).

Dogs (*Canis familiaris*)

Due to their similarity to humans in terms of gastrointestinal tract anatomy (Misic et al., 2015), mouth flora (Harvey, 1998), and lung microbiology (Ericsson et al., 2016), dogs have been chosen as study models. They are perfect for examining the microbiota in diverse internal organ systems and for examining the health benefits of probiotics and gut flora. Dogs can be used as research models, although there are restrictions due to their expense and living needs.

Rabbits (*Oryctolagus cuniculus*)

Because rabbits are more expensive than rats or mice, they are not frequently utilized as animal models. To explore the impact of pathogens like *Vibrio cholerae* on the small intestine, in particular, rabbits have been utilized in studies on infectious disorders brought on by GI tract infections (Melling et al., 1976). Moreover, there are drawbacks to using rabbits as model animals in host-microbiota interaction research, chief among them being their higher cost than rodents.

CHAPTER 3

RATIONALE, HYPOTHESIS AND OBJECTIVES

3.1 Rationale

Enniatin mycotoxins are emerging contaminants that have been detected in various food commodities. Moreover, the heat from the cooking baking, and frying could not eliminate these mycotoxins. Enniatin in food and feed have not been regulated due to lack of the scientific support. Antifungal, antibacterial, and anthelmintic capacity in enniatin could affect to the human gut microbiota structure and diversity. The human gut microbiota plays a crucial role in the metabolism of dietary compounds, including mycotoxins. Enniatin mycotoxins may undergo biotransformation by gut microbes, influencing their toxicity and bioavailability. Investigating these interactions in a murine model that harbors human intestinal microbiota enables a more realistic assessment of the potential metabolic transformations and the resulting impact on human gut microbiota structure and diversity. *In vivo* analysis using the murine model of human microbiota allows for a deeper exploration of the enniatin effects to the composition and diversity of human microbiota. The use of a murine model with human intestinal microbiota enhances the translatability of findings to humans. While *in vitro* studies provide valuable insights, the *in vivo* approach allows for a more comprehensive understanding of the interactions in a living organism. Investigating the effects of enniatin mycotoxins in a murine model with humanized microbiota ensures that the results are more reflective of the potential impact on human health. Mycotoxin exposure has been linked to alterations in gut microbial composition, leading to dysbiosis. Studying the effects of enniatin mycotoxins in a murine model with human intestinal microbiota enables the assessment of potential shifts in microbial populations and their implications for gut health. This is particularly relevant considering the established connection between gut microbiota and overall host well-being. A comprehensive understanding of the *in vivo* effects of enniatin mycotoxins is vital for informing regulatory measures and developing effective mitigation strategies. Data generated from this study can contribute to the establishment of safety guidelines and regulations, ensuring the protection of human health against the potential adverse effects associated with exposure to enniatin mycotoxins.

3.2 Hypothesis

We hypothesized that exposure to enniatins in a humanized Sprague Dawley model of intestinal microbiota would lead to dose-dependent alterations in microbiota.

Objectives

This study aimed to analyze the impact of dose-dependent exposure to enniatins on the human intestinal microbiota.

CHAPTER 4

ARTICLE

Manuscript preparation for submission to the journal Archives of Toxicology

Title:

Investigation of oral enniatin exposure on murine humanized gut microbiota.

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Keywords:
Mycotoxin, enniatin, gut microbiota, toxicity, rat, murine model

ABSTRACT

Enniatin, a toxic substance found in various food sources, is an emerging mycotoxin known for its harmful effects on humans when ingested through contaminated food due to its antimicrobial and antibiotic properties, which may impact human intestinal microbiota. The intestinal microbiota is crucial for maintaining human health. Still, the specific effects of enniatin on the microbiota are not fully understood, and studies on the interactions between microbiota and emerging toxins are limited. This study aimed to assess the impact of low-dose and high-dose exposure to enniatin mycotoxins on the human intestinal microbiota using a humanized murine model. An experiment involving 24 male Sprague Dawley rats, aged 7 weeks, was conducted. The rats underwent microbiota depletion using a cocktail of antibiotics and antifungals, followed by the transplantation of human intestinal microbiota from two donors. The rats were divided into three groups and treated with either 2 µg/kg, 200 µg/kg of enniatin or assigned to a control group. The animals were euthanized using isoflurane, followed by cardiac venipuncture. Fecal samples and colonic contents were then collected for analysis. Microbial communities in the samples were analyzed using 16S rRNA gene sequencing. The results indicated that the antibiotic treatment successfully depleted the rat intestinal microbiota, and fecal microbiota transfer established a human-like microbiota in the rats, with some donor-specific bacteria present in the recipients. Treatment with Enniatin did not influence alpha diversity (Chao, Shannon, and Simpson indexes) nor beta-diversity (Jaccard and Bray-Curtis indices), suggesting a limited impact on community composition (PERMANOVA, $P < 0.0001$). The LEfSe analysis identified significant differences in certain bacterial genera among treatment groups. In rats that received fecal transplants from donor 1, Muribaculaceae*, Proteobacteria,* and *Turicimonas* decreased in the low-dose group, while *Romboutsia* and *Ligilactobacillus* increased in the high and low-dose groups, respectively. In the colon, *Clostridium* and *Fecalibacillus* were more abundant in the low-dose group, and *Hungatella* was more abundant in the control group. In rats transplanted with feces from donor 2, *Escherichia/Shigella* was increased in the low-dose group, Clostridiaceae was decreased in the high-dose group, and Bifidobacteriaceae was more abundant in the control group. In conclusion, enniatin exposure was associated with changes in specific bacterial groups without impacting overall microbial composition.

Keywords:
Mycotoxin, enniatin, gut microbiota, toxicity, rat, murine model

Introduction

Enniatins (ENNs) are emergent mycotoxins produced by the secondary metabolism of various *Fusarium* fungi, predominantly contaminating grains and their derivatives (Fraeyman et al., 2017). ENNs have been identified in alternative food items, including fish, dried fruits, nuts, spices, cocoa, and coffee products (Prosperini et al., 2017). The ENNs frequently detected as natural contaminants in grains are ENNA, ENNA1, ENNB, and ENNB1 (Feifel et al., 2007). The mean chronic exposure for humans to the combined ENNA, ENNA1, ENNB, and ENNB1 is estimated at 0.42–1.82 μg/kg body weight (BW) per day, with the 95th percentile dietary exposure in adults estimated to be 0.91–3.28 μg/kg BW per day (Chain, 2014).

The cytotoxicity of ENNs relies on their ionophore characteristics, and studies have demonstrated that low micromolar concentrations of ENNs can adversely affect various cell lines (Gruber-Dorninger et al., 2017). Despite the European Commission requesting a scientific opinion on the risks of ENNs in food and feed, no limits have been set so far. Human and animal health concerns arise from the potent cytotoxicity of ENNs observed in vitro. However, a limited number of in vivo toxicity studies have been conducted, and none have succeeded in reproducing the robust cytotoxic effects of ENNs (Rodríguez-Carrasco et al., 2016). While the European Food Safety Authority determined in 2014 that short-term exposure to ENNs posed no risk to human health, uncertainties persist regarding the impact of prolonged exposure. Comprehensive toxicity data are essential for a thorough risk assessment (Chain, 2014).

Enniatins belong to the cyclohexadepsipeptides class and have demonstrated effectiveness as antifungal and antibacterial activity (Malachova et al., 2011; Meca et al., 2010). Dysbiosis is an altered composition of intestinal microbiota, which has been linked to numerous pathological conditions, including autism, asthma, cardiovascular incidents, colon cancer, Crohn's disease, diabetes, eczema, food allergies, irritable bowel syndrome, obesity, hepatic encephalopathy, and psychiatric illnesses (Fung et al., 2017 ; Schnabl et al., 2014).To obtain more precise toxicity data, it is essential to conduct general toxicity studies in accordance with appropriate test guidelines, considering the dose interval and inadequate dose-response relationship. This study aimed to investigate the effect of enniatin on the intestinal microbiota using a humanized murine model, hypothesizing that enniatin would induce dose-dependent changes in the intestinal microbiota of humanized Sprague Dawley rats.

Materials and methods

Approval

The Institutional Animal Care and Use Committee form Division Fermes et Animaleries (FANI), the Faculty of Veterinary Medicine, University of Montreal authorized all animal care and experimental techniques. Approve number Rech-2208.

Animals

Twenty-four seven-week-old male Sprague Dawley strain rats supplied by Charles River Laboratory, were fed with Charles River Rodent Diet 5075, and trained to accept oral administration with diluted syrup (1 ml of syrup per 100 ml of sterile water). Feces and urine were collected on day 0 and every week, using individually housed metabolic cages. Animal weights were recorded every week. At the end of enniatin treatment all the rats were subjected to put down by an overdose of isoflurane asphyxiation. Decapitator and heart venipuncture were used to collect the serum, colon contents and cecal contents, which were frozen in liquid nitrogen and kept in a refrigerator at -80 Celsius.

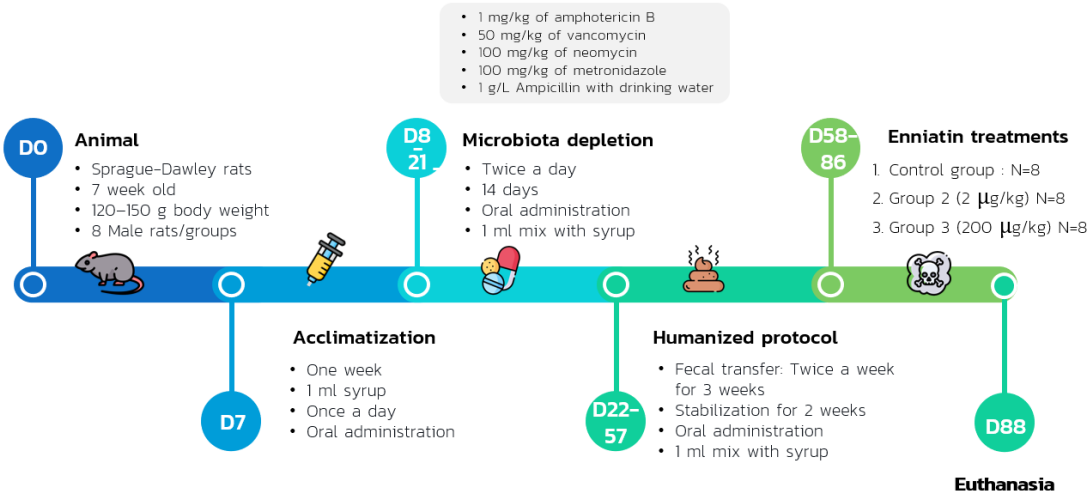


Figure 8. Graphical abstract of the summary of materials and methods.

Microbiota depletion

Rats were subjected to oral administration every 12 hours with diluted syrup (1 ml of syrup per 100 ml of sterile water) combined with 1 mg/kg amphotericin-B for 3 days, followed by an antibiotic combination previously described (Reikvam et al., 2011) consisting of 50 mg/kg vancomycin, 100 mg/kg neomycin, and 100 mg/kg streptomycin, 1 mg/kg metronidazole and 1 mg/kg amphotericin-B were diluted with Dimethyl sulfoxide (DMSO) 1 ml for 14 days. Ampicillin was given ad libitum in drinking water (1 g/L).

Human donors

An ethical authorization was obtained from Comité d'éthique de la recherche en sciences et en santé of Université de Montréal under the number 2022-1673. The fecal inocula were collected from two healthy adults (one male and one female). These participants ate an unrestricted Western diet and were not on antibiotics or other medications. No additional medicines known to impact the fecal microbiota were taken for a minimum of three months before sampling. None of the subjects had any history of metabolic or gastrointestinal illnesses.

Human fecal collection, processing, and transfer into rats

Human volunteers were given disposable coffee filter-like fecal collection equipment. The human feces were collected and immediately put in an anaerobic environment (Anaerobic atmosphere generation bags; Thermo Scientific). Samples were transported to an anaerobic workstation (MRC Laboratory instruments) for microbiological processing in the laboratory. Fecal samples from donors were diluted with saline water 1:99 (weight/volume) and mixed with 5 ml for all rats in each group. syrup before being administered orally to the rats in a volume of 1 ml per rat .

Humanization protocol

Rats receiving fecal material transfer were given oral administration mixed with syrup 12 hours after the final antibiotic gavage with fecal material reconstituted in diluted syrup from one of two human donors (Donor 1 or 2). Rats in the weekly fecal transfer treatments were given oral administration with fecal material from their respective donors twice a week for 3 weeks and stabilization of 2 weeks.

Treatment with enniatin

Twenty-four**,** seven-week-old male Sprague Dawley strain rats were randomly divided into vehicle controls (*N* = 8), 2 µg/kg (*N* = 8) and 200 µg/kg body weight of enniatin-treatment groups (*N* = 8). After two weeks of microbiota stabilization, 2 µg/kg and 200 µg/kg body weight of enniatin were administered orally with diluted syrup (1 ml of syrup per 100 ml of sterile water) to the two groups of rats daily for four weeks.

Fecal samples were obtained on day 0 (before the start of the treatment), every week (during the treatment), and on day 29 (one day after the conclusion of the treatment). Samples were kept frozen at -80 C for microbiota analysis.

DNA extraction

A QIAamp DNA stool micro kit (Qiagen) was used to extract DNA from the rat's fecal and colon content, and from 6 fecal samples of donors by following the manufacturer's protocol. The DNA quantification was estimated by spectrophotometry with a NanoDrop™ 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

Microbiota analysis

The DNA samples from rats were used to study the microbiota. The V4 hypervariable region of the 16S rRNA gene was PCR-amplified using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806F (5'-GGACTACHVGGGTWTCTAAT-3') (Klindworth et al., 2013) (Klindworth et al., 2013). Paired-end sequencing of the amplicons was conducted using an Illumina MiSeq (Illumina, San Diego, CA, USA) platform for 250 cycles from each end at the McGill Génome Québec Innovation Centre (Montréal, QC, Canada) Bioinformatic analysis was conducted using Mothur software (Kozich et al., 2013) Sequencing reads were aligned with the SILVA reference database, clustered at 97% similarity, and classified with the Ribosomal Database Project (RDP).

Richness and diversity were defined using the Chao richness estimator, Simpson's diversity index, and Shannon index. Membership analysis considers only presence or absence of bacterial taxa, while structure analysis includes relative abundance. A 2-dimensional Principal Coordinate Analysis (PCoA) plot was assembled to anticipate sample similarities. Bar charts showing the relative abundance of the most abundant bacteria (>1%) were created to show the main phyla and genera in each rat.

Statistical analysis

Statistical analysis was performed using Sigma Plot V.14.5. A one-way ANOVA followed by Tukey's post-hoc test was applied to compare fecal DNA content and qPCR 16S rRNA gene data. The alpha-diversity indices reflecting the effects of enniatin on animal average weights were also analyzed using one-way ANOVA with Tukey's post-hoc test. The data are presented as mean and standard deviation (SD).

A generalized linear model was used to assess the impact of FMT and enniatins on the microbiota alpha diversity, with results displayed through box plots. Beta diversity, which evaluates differences in taxonomic composition among samples, was assessed using the Jaccard and Bray-Curtis indices to examine community membership and structure, respectively. The Permutational multivariate analysis of variance (PERMANOVA) was employed to determine the significance of clustering, using the adnois2 command in R! The time of sampling, level of enniatin exposure, sample nature (feces or cecum content), and donor source (donor 1 or donor 2) were used as variables.

The Linear Discriminant Analysis Effect Size (LEfSe) method (Segata et al., 2011), was used to identify biologically relevant features by comparing their abundance across all treatment groups at different sampling days. LEfSe incorporates a factorial Kruskal-Wallis sum rank test followed by a pairwise Wilcoxon rank-sum test. A P value of less than 0.05 was considered statistically significant.

Results

Animal Weight

At the initial time point (week 1: Acclimatization, week 2-3: Microbiota depletion, week 4-6: Humanized protocol, week 7-8: Stabilization, week 9-12: Enniatin treatments), both the control and mycotoxin-treated groups exhibited similar baseline weights. Over the following weeks, the trends in average weights for the control, low-dose, and high-dose enniatin-treated groups were tracked. The analysis revealed no statistically significant differences in the average weights among rats receiving human feces from donor 1 (Figure 9.) and donor 2 (Figure 10.) across the control, low-dose, and high-dose enniatin-treated groups.

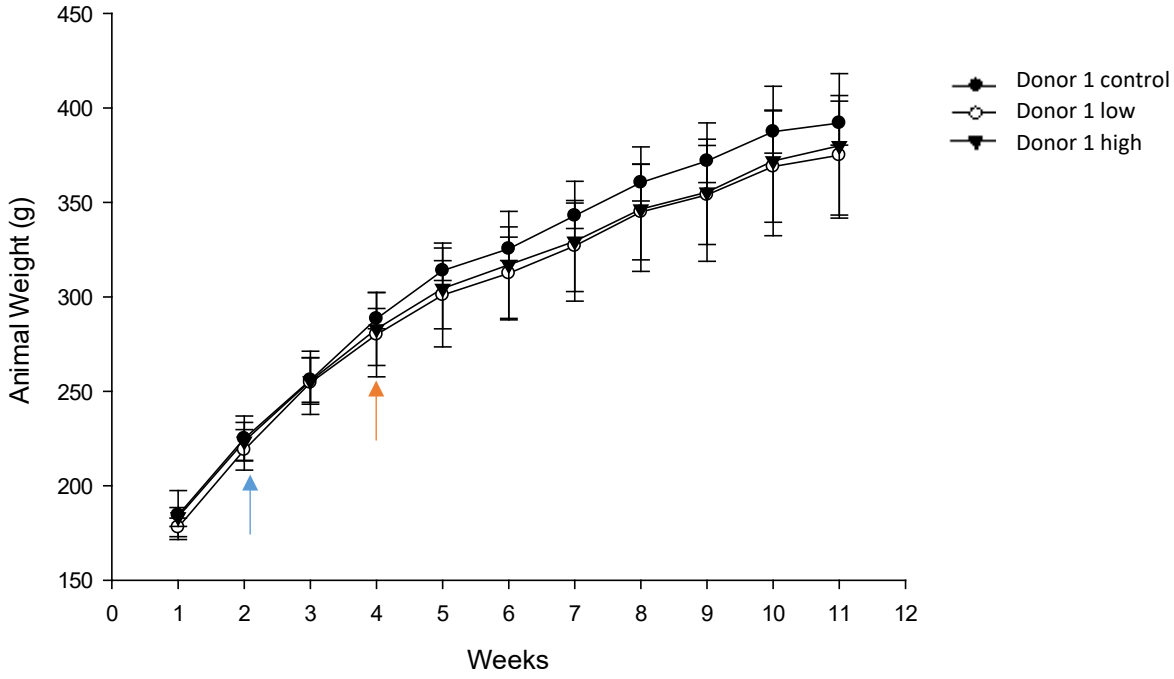


Figure 9. Body weight obtained from rats receiving human feces from donor 1, week 1: Acclimatization, week 2-3: Microbiota depletion (blue arrow), week 4-6: Humanized protocol (orange arrow), week 7-8: Stabilization, week 9-12: Enniatin treatments). Differences were assessed by the non-parametric Kruskal–Wallis test. * $p < 0.05$.

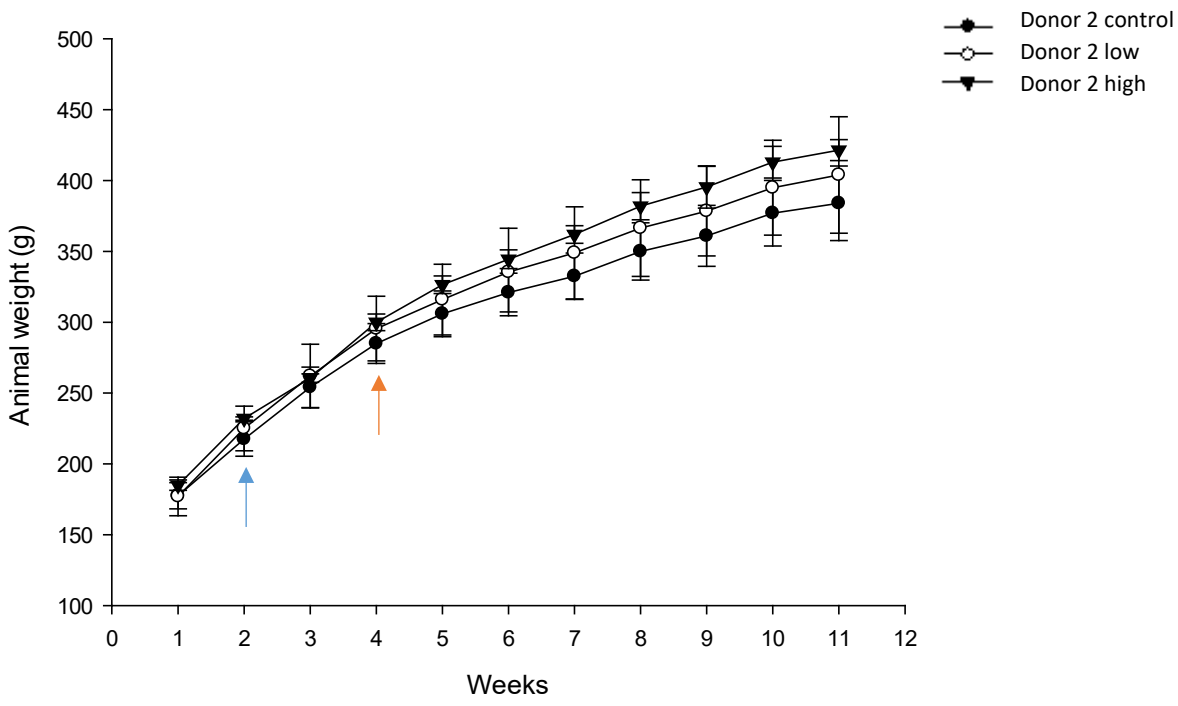


Figure 10. Body weight obtained from rats receiving human feces from donor 2, week 1: Acclimatization, week 2-3: Microbiota depletion (blue arrow), week 4-6: Humanized protocol (orange arrow), week 7-8: Stabilization, week 9-12: Enniatin treatments). Differences were assessed by the non-parametric Kruskal–Wallis test. * $p < 0.05$.

DNA concentration in feces

NanoDrop® instrument were used to measure fecal DNA concentration showed a significant decrease after microbiota depletion protocol compared to before and after fecal microbiota transplantation ($p < 0.05$, Figure 11). As a result of the quantitative PCR (qPCR) analysis of the 16S rRNA gene, the quantification cycle (C_q) of the fecal DNA samples after microbiota depletion increased to 37.47±1.13, compared with 32.94±1.19 before microbiota depletion and 33.18±1.79 after FMT from the human donors, which indicates a reduced DNA load in samples after antibiotic treatment, and almost normalization of DNA load following the FMT ($p < 0.05$, Figure 12).

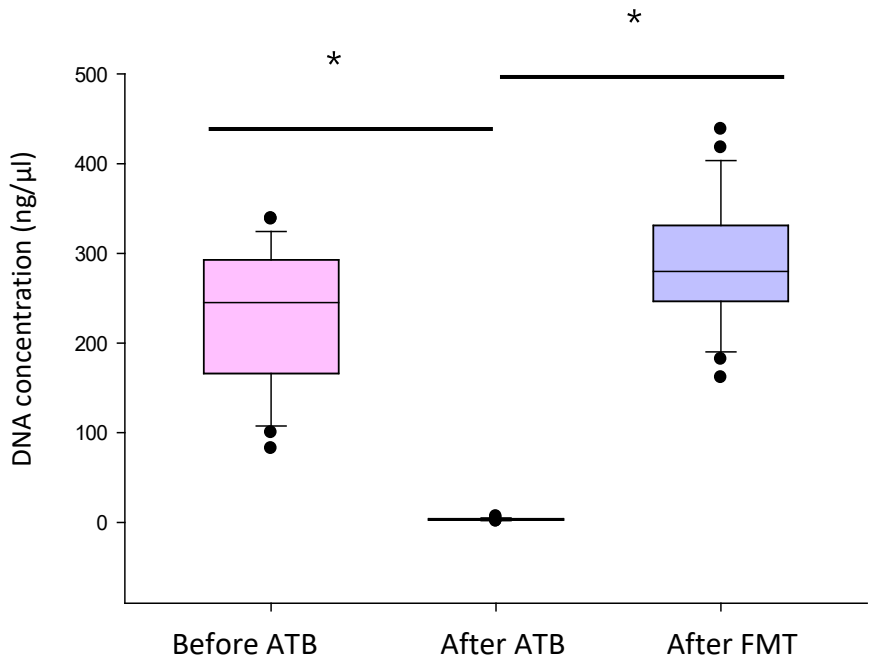


Figure 11. Effect of antibiotic treatment and fecal microbiota transplantation on fecal DNA concentration. Box plots showing bacterial DNA concentration in feces of rats before antibiotics (Before ATB), after antibiotics (After ATB) and after fecal microbiota transfer (After FMT). Differences were assessed by the non-parametric Kruskal–Wallis test. * p <0.05.

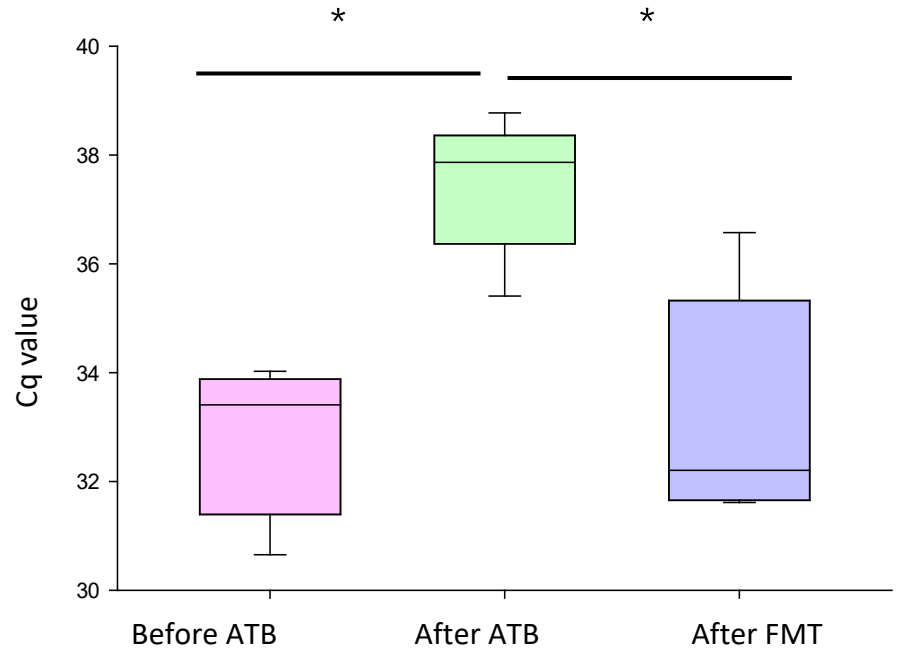


Figure 12. Distribution of Cq values of 16S rRNA genes before antibiotics (Before ATB), after antibiotics (After ATB)and after fecal microbiota transfer (After FMT) in 6 rats. Differences were assessed by the non-parametric Kruskal–Wallis test. * p <0.05.

Establishment of human gut microbiota in rats

Alpha diversity before Microbiota depletion, after Microbiota depletion, and after fecal microbiota transplantation.

Alpha diversity characterizes the species richness and diversity within each sample. Figure 13 illustrates the computation of the number of observed genera, Chao, Shannon, and Simpson indices in samples collected prior to microbiota depletion (Before-ATB), after microbiota depletion (After-ATB), and following fecal microbiota transplantation (After-FMT). The Chao index was employed to assess fecal microbial richness, with no statistically significant distinctions observed among the three samplings (Figure 13B). Conversely, the observed genera, the evenness (Shannon index) and overall diversity (Simpson index) of the fecal microbiota exhibited notable variations in rats subjected to antibiotic exposure in comparison to before-ATB and after-FMT conditions ($p < 0.05$, Figure 13 A, C, D).

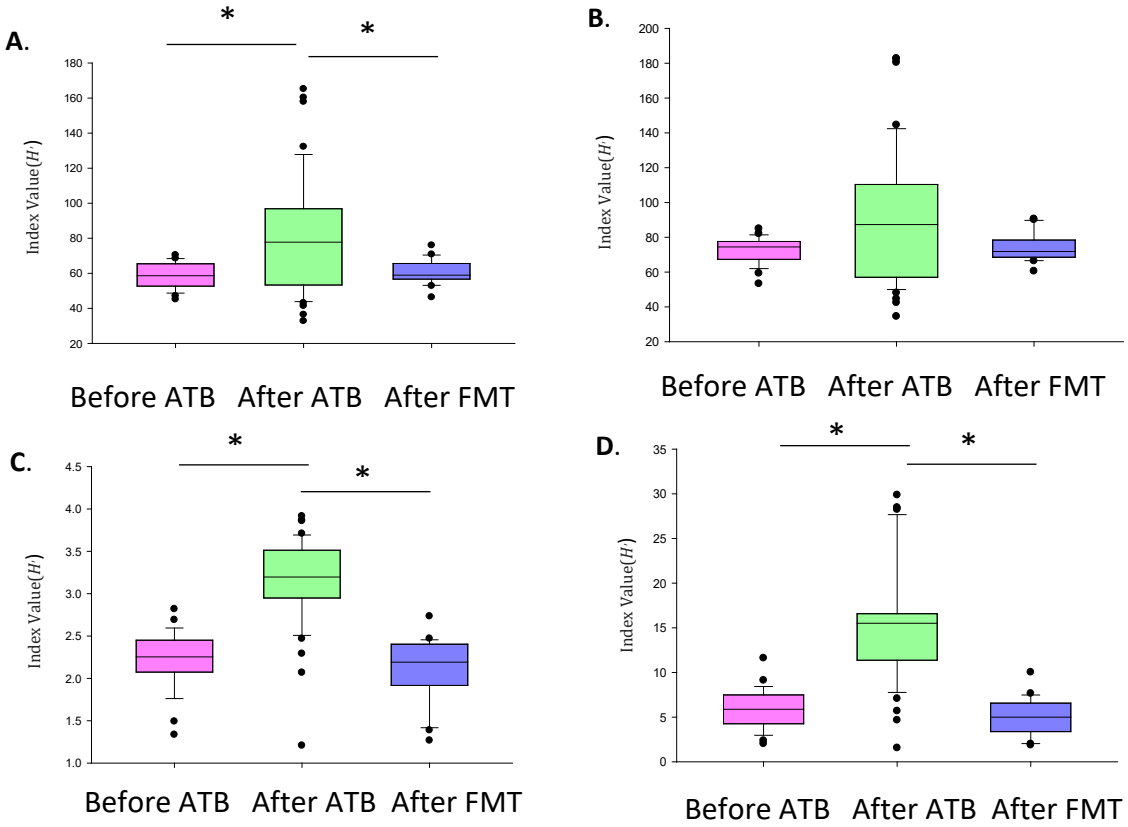


Figure 13. Effect of fecal microbiota transplantation in microbiota community and diversity. Box plots showing alpha diversity indexes (Observed OUT, Chao, Shannon, and Simpson) in fecal microbiota before antibiotics (Before ATB), after antibiotics (After ATB) and after fecal microbiota transfer (After FMT). Observed OUT (A), Chao index (B), Shannon index (C), and Simpson index (D), reflect the evenness and global diversity of OTUs, respectively. Values are expressed as average ± standard deviation (n = 24). Differences were assessed by the non-parametric Kruskal–Wallis test, * $p<0.05$.

Beta diversity analysis from overall samples collected before microbiota depletion, after Microbiota depletion and after fecal microbiota transplantation.

Beta diversity elucidates the variances in microbial composition across distinct samples. In the context of beta-diversity, principal coordinate analysis (PCoA) plots were constructed based on the Jaccard and Bray–Curtis dissimilarity indices to compare community membership and structure, respectively. The PCoA plots depicted discernible dissimilarities in both microbiota membership (Figure 14) and structure (Figure 15) among samples collected at each stage: before microbiota depletion (before-ATB), after microbiota depletion (after-ATB), and after fecal microbiota transplantation (after-FMT). The PERMANOVA test revealed a significant impact on community structure (Bray-Curtis) of the sampling time ($p<0.001$), the origin of the donor ($p<0.001$) and the type of sample ($p=0.003$), but not from exposure to toxins ($p=0.926$). However, there was no interaction between variables. The pairwise comparison revealed a significant difference between samples collected before and after antibiotics, and after antibiotics and after FMT (both $p<0.001$). There was a significant difference between human donors ($p=0.036$) and between rats receiving the microbiota from different donors ($p=0.004$).

For community membership, there was a statistical difference between the different sampling times ($p<0.001$), the origin of the donor ($p<0.001$) and the type of sample ($p=0.003$), but not from exposure to toxins ($p=0.942$). Similarly, there was no interaction between the variables. The pairwise comparison revealed a significant difference between samples collected before and after antibiotics, and after antibiotics and after FMT (both $p<0.001$). There was a significant difference between human donors ($p=0.003$) and between rats receiving the microbiota from different donors ($p=0.002$).

Figure 14. Principal coordinate analysis (PCoA) of fecal microbiota (membership) before ATB, after ATB and after FMT based on Jaccard distances. A distinct clustering of samples by each group was observed.

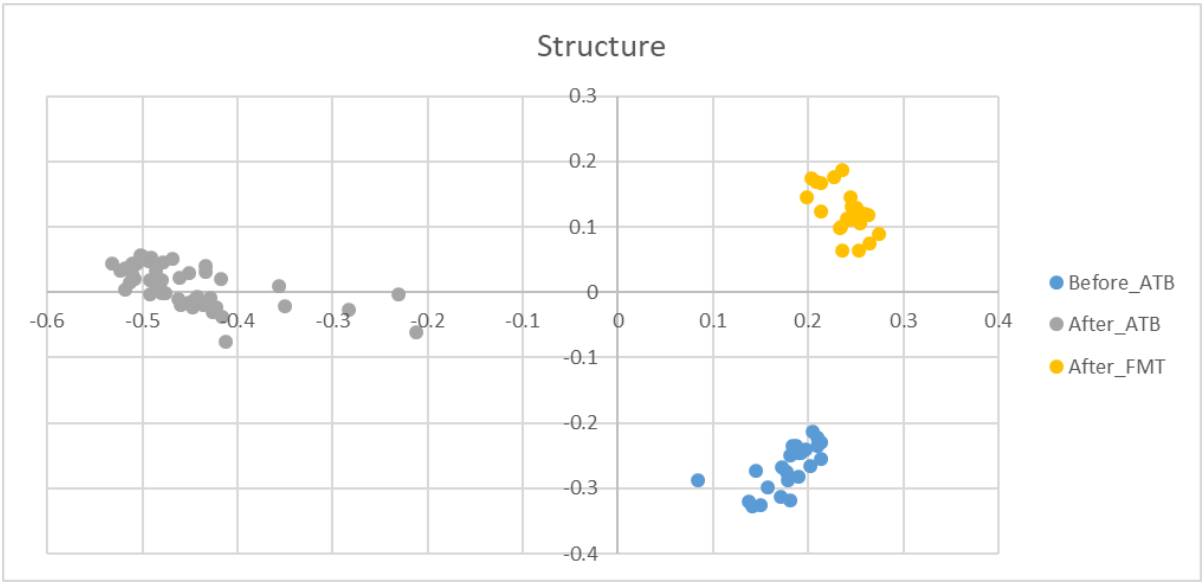


Figure 15. Principal coordinate analysis (PCoA) of fecal microbiota (structure) before ATB, after ATB and after FMT based on Bray–Curtis distances. A distinct clustering of samples by each group was observed. Groups are distinguished by colors.

Figure 16. shows the relative abundances of the most common genera found before microbiota depletion (before ATB), after microbiota depletion (after ATB), and after fecal microbiota transplantation (after FMT). At the genus level, *Lactobacillus* was the most

abundant taxon in the feces of rats from all groups, with averages of 34.01% before ATB groups, 12.82% after ATB group and 33.46% after the FMT.

Delving into the finer granularity at the genus level, the intestinal microbiota in the before-ATB group was characterized by a higher prevalence of *Lactobacillus, Turicibacter, Romboutsia,* Lachnospiraceae, and *Limosilactobacillus*. In contrast, the after-ATB group exhibited a distinct composition dominated by *Lactobacillus, Escherichia Shigella,* Lachnospiraceae*, Romboutsia, and Leuconostoc*. Similarly, the after-FMT group showcased a unique profile, with *Lactobacillus,* Lachnospiraceae, Ruminococcaceae*, Bacteroides,* and Muribaculaceae.

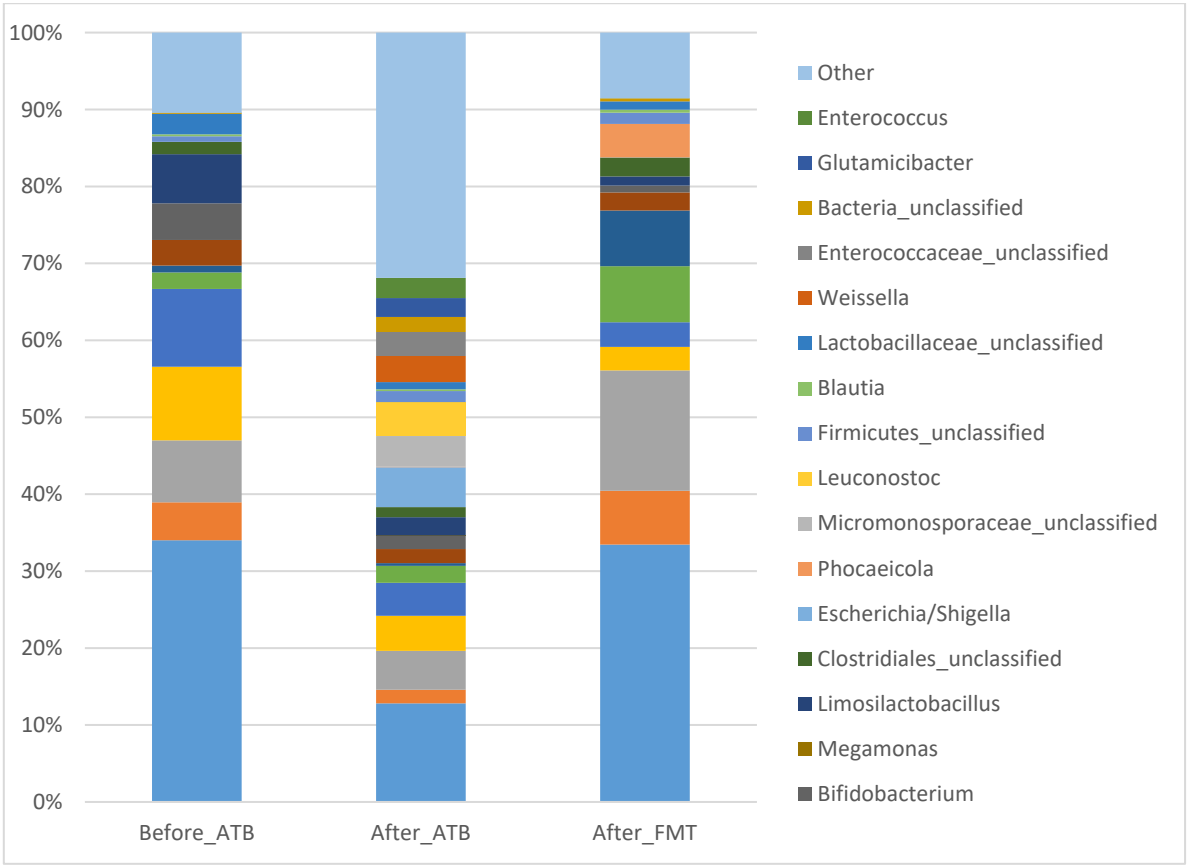


Figure 16. Relative abundances of the main taxa found in feces of rats before antibiotics (Before ATB), after antibiotics (After ATB) and after fecal microbiota transfer (After FMT); the percentage on the vertical axis indicates the relative abundances.

Beta diversity analyzed by donor: Donor 1.

Further analysis dividing samples according to the donors was carried out. The PCoA plots depicted discernible dissimilarities in both microbiota membership (Figure 17) and structure (Figure 18) among samples collected at each sampling.

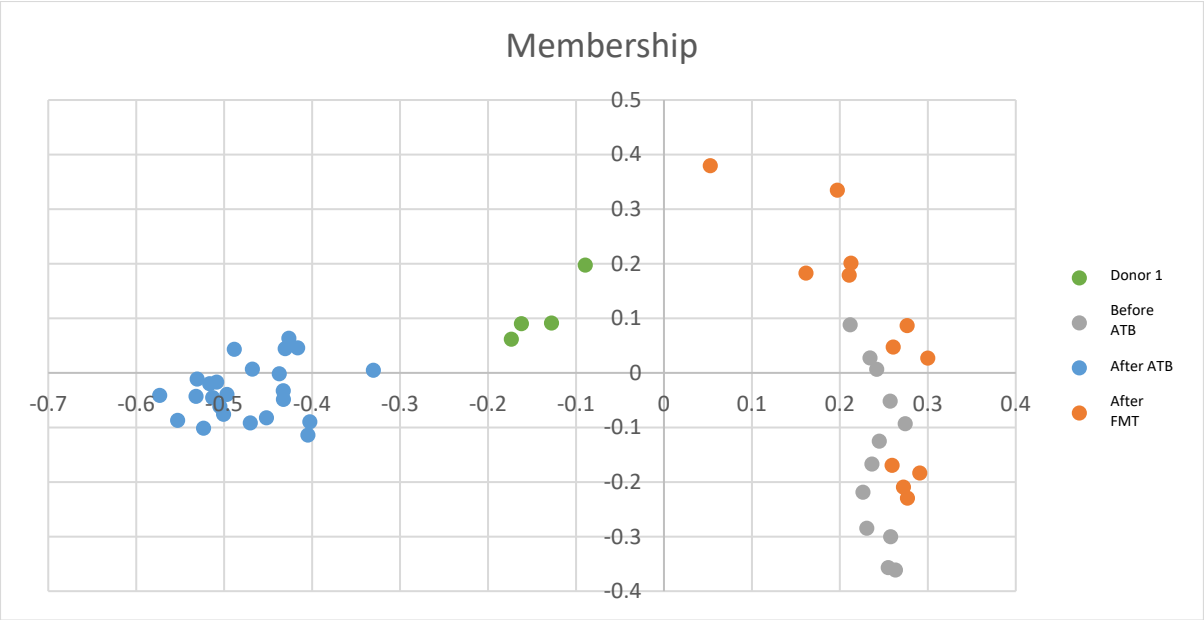


Figure 17. Principal coordinate analysis (PCoA) of fecal microbiota (membership) from Donor1, before ATB, after ATB and after FMT based on Jaccard distances.

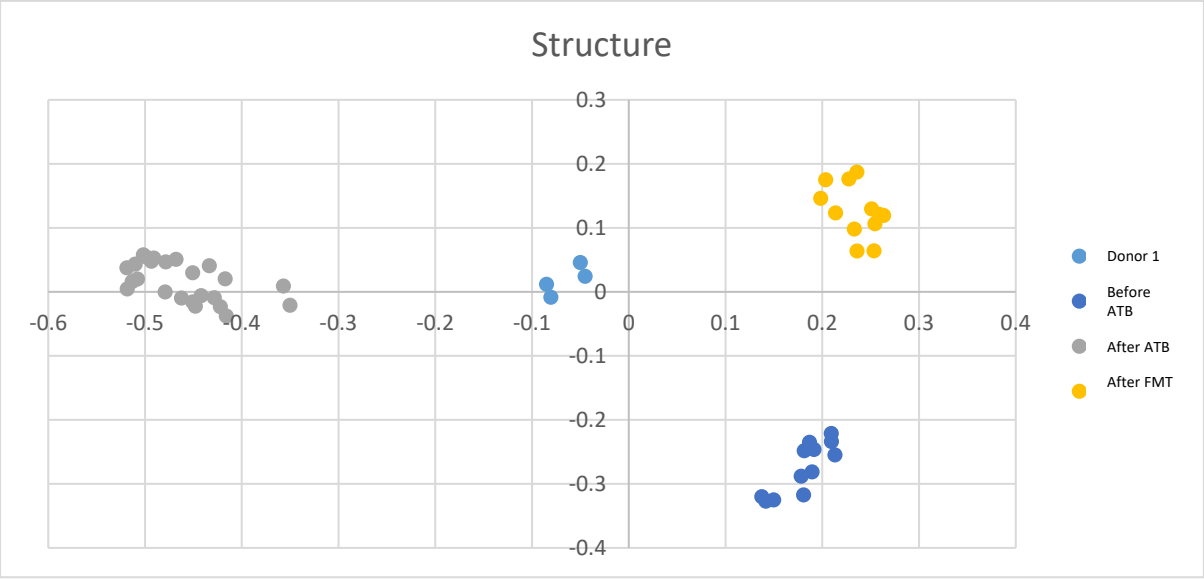


Figure 18. Principal coordinate analysis (PCoA) of fecal microbiota (structure) from Donor1, before ATB, after ATB and after FMT based on Bray–Curtis distances.

The *Blautia* was the most abundant taxon in the feces of Donor 1 (Figure 19).

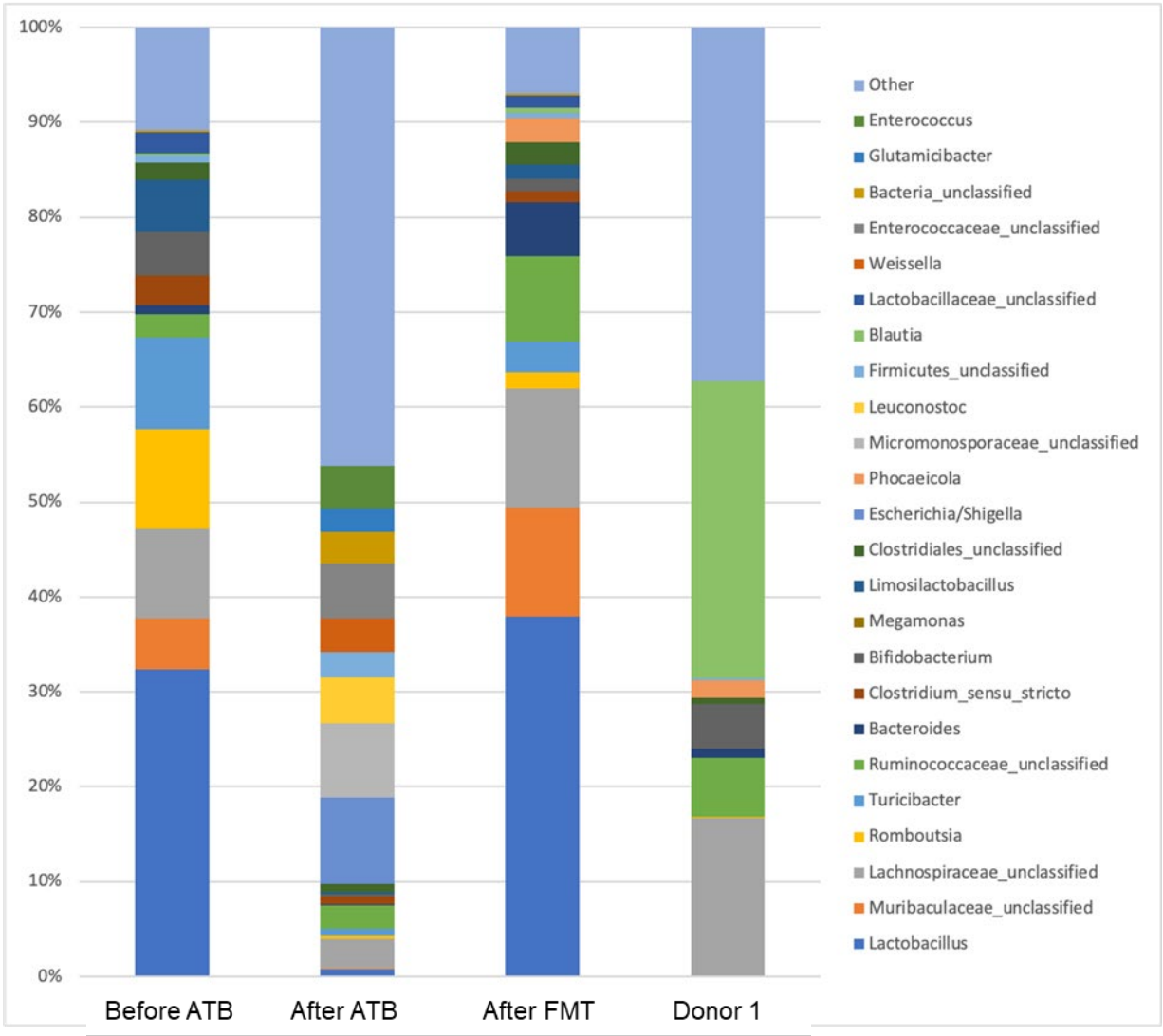
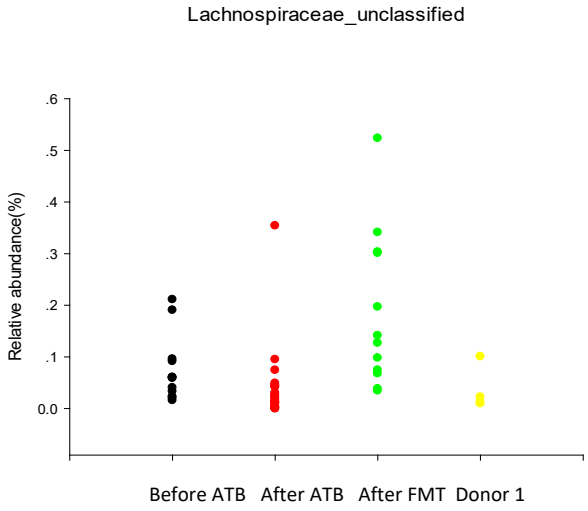
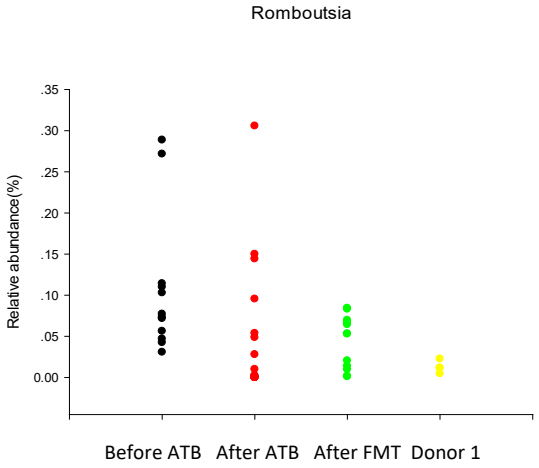
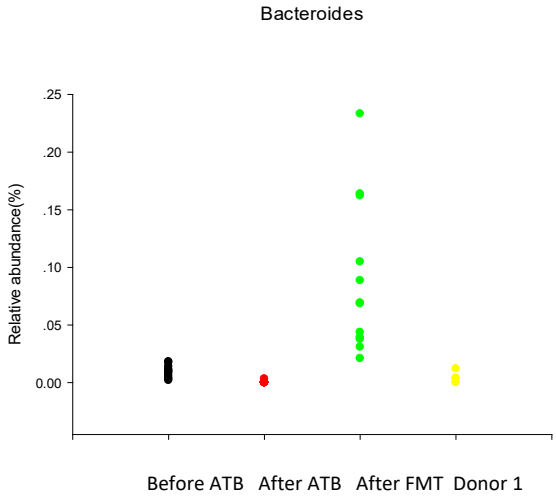


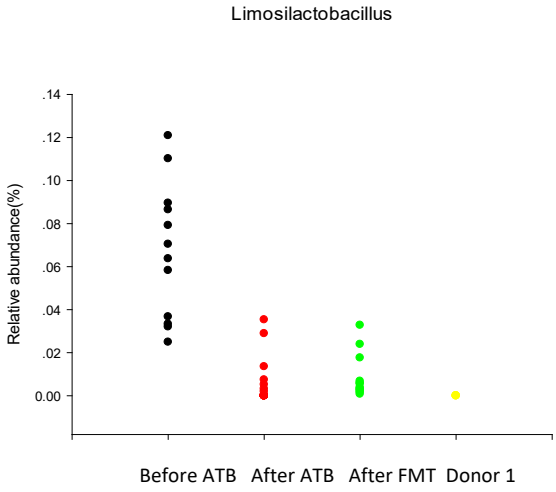
Figure 19. Relative abundances of the main taxa found in feces of rats before antibiotics (Before ATB), after antibiotics (After ATB), after fecal microbiota transfer (After FMT) and human feces donor 1; and the percentage on the vertical axis indicates the relative abundances.

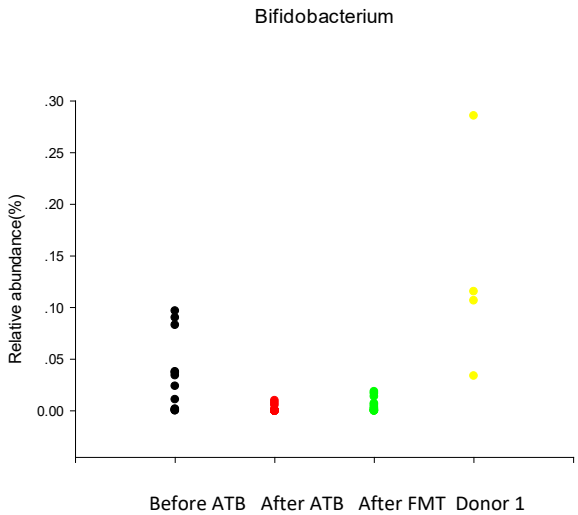
Some bacteria were found to be significantly different among groups of treatment, such as, as shown the changes in the fecal microbiota induced by before and after antibiotic depletion, fecal microbiota transplantation from Human donor feces number 1 (Donor 1) were observed. The LEfSe analysis was used to determine which genera were statistically different between groups (Figure 20): *Bacteria unclassified,* Bacteroidaceae*, Bacteroidales, Bacteroides, Barnesiella, Bifidobacterium, Desulfovibrionales, Eggerthella,* Enterococcaceae*, Hungatella,* Lachnospiraceae, Lactobacillaceae*, Limosilactobacillus, Mycobacteriales,* Peptostreptococcaceae*, Romboutsia* and *Streptococcus*

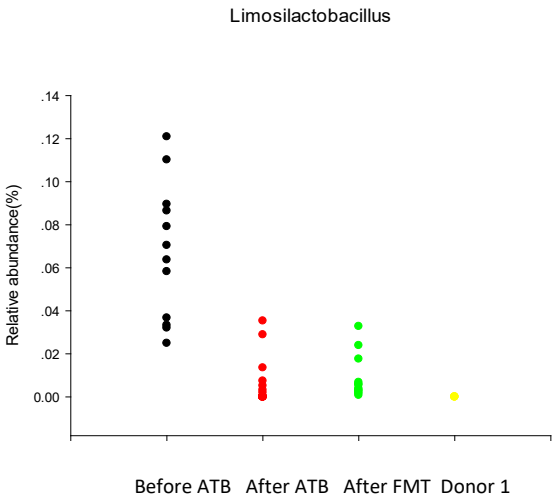

Lachnospiraceae_unclassified
Relative abundance(%)
.6
.5
.4
.3
.2
.1
0.0
Before ATB
After ATB
After FMT
Donor 1

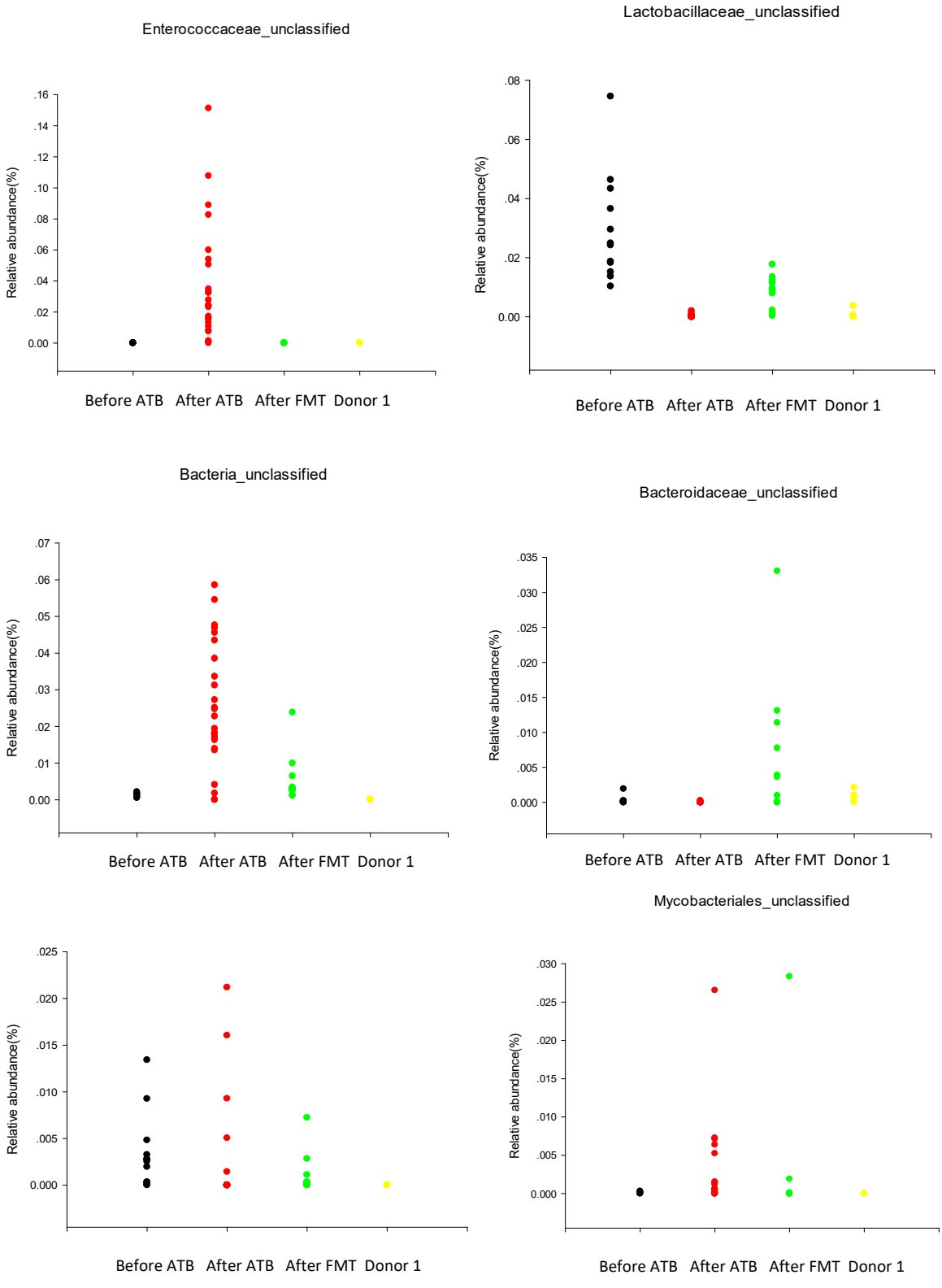

Romboutsia
Relative abundance(%)
.35
.30
.25
.20
.15
.10
.05
0.00
Before ATB
After ATB
After FMT
Donor 1

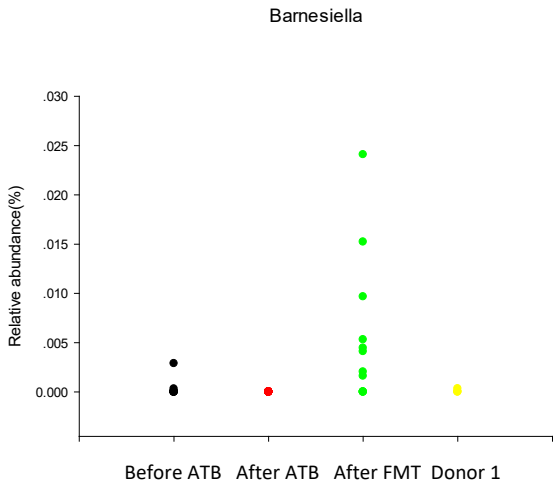

Bacteroides
Relative abundance(%)
.25
.20
.15
.10
.05
0.00
Before ATB
After ATB
After FMT
Donor 1

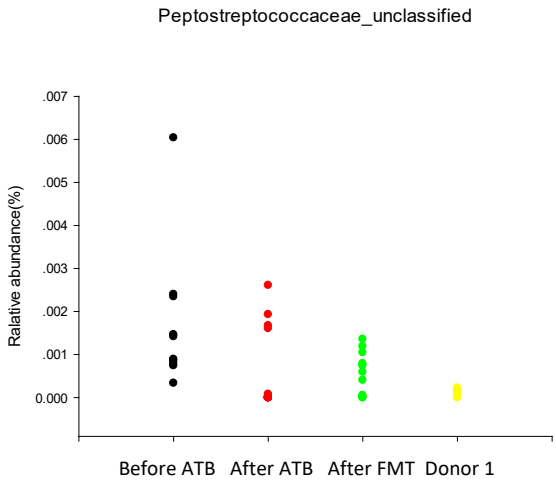

Limosilactobacillus
Relative abundance(%)
.14
.12
.10
.08
.06
.04
.02
0.00
Before ATB
After ATB
After FMT
Donor 1

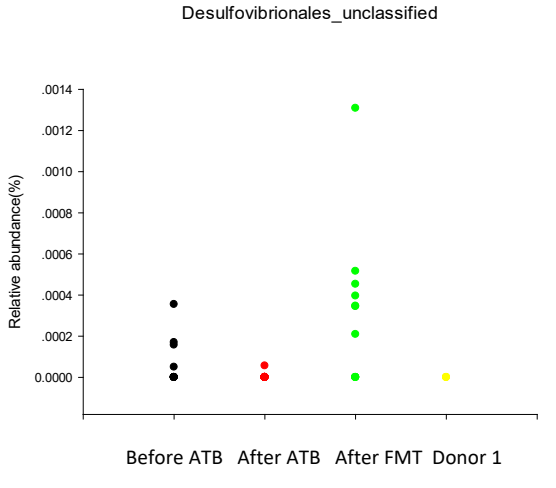

Bifidobacterium
Relative abundance(%)
.30
.25
.20
.15
.10
.05
0.00
Before ATB
After ATB
After FMT
Donor 1

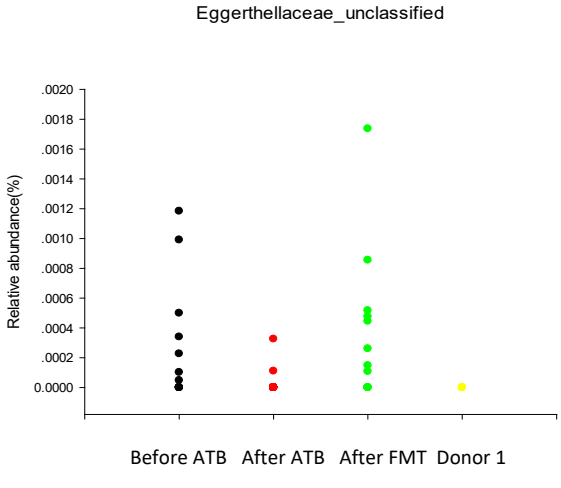

Limosilactobacillus
Relative abundance(%)
.14
.12
.10
.08
.06
.04
.02
0.00
Before ATB
After ATB
After FMT
Donor 1

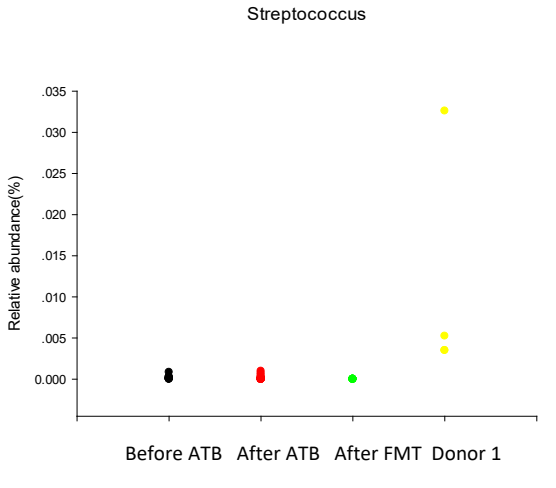

Enterococcaceae_unclassified
Relative abundance(%)
.16
.14
.12
.10
.08
.06
.04
.02
0.00
Before ATB
After ATB
After FMT
Donor 1
Lactobacillaceae_unclassified
Relative abundance(%)
.08
.06
.04
.02
0.00
Before ATB
After ATB
After FMT
Donor 1
Bacteria_unclassified
Relative abundance(%)
.07
.06
.05
.04
.03
.02
.01
0.00
Before ATB
After ATB
After FMT
Donor 1
Bacteroidaceae_unclassified
Relative abundance(%)
.035
.030
.025
.020
.015
.010
.005
0.000
Before ATB
After ATB
After FMT
Donor 1
Relative abundance(%)
.025
.020
.015
.010
.005
0.000
Before ATB
After ATB
After FMT
Donor 1
Mycobacteriales_unclassified
Relative abundance(%)
.030
.025
.020
.015
.010
.005
0.000
Before ATB
After ATB
After FMT
Donor 1

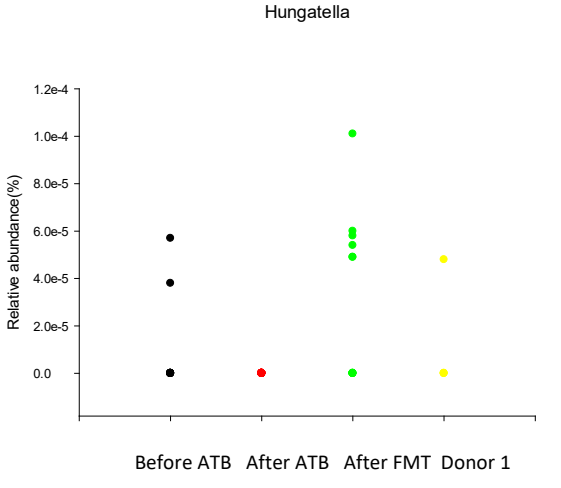

Barnesiella
Relative abundance(%)
.030
.025
.020
.015
.010
.005
0.000
Before ATB
After ATB
After FMT
Donor 1


Peptostreptococcaceae_unclassified
Ralative abundance(%)
.007
.006
.005
.004
.003
.002
.001
0.000
Before ATB
After ATB
After FMT
Donor 1


Desulfovibrionales_unclassified
Relative abundance(%)
.0014
.0012
.0010
.0008
.0006
.0004
.0002
0.0000
Before ATB
After ATB
After FMT
Donor 1


Eggerthellaceae_unclassified
Relative abundance(%)
.0020
.0018
.0016
.0014
.0012
.0010
.0008
.0006
.0004
.0002
0.0000
Before ATB
After ATB
After FMT
Donor 1


Streptococcus
Relative abundance(%)
.035
.030
.025
.020
.015
.010
.005
0.000
Before ATB
After ATB
After FMT
Donor 1


Hungatella
Relative abundance(%)
1.2e-4
1.0e-4
8.0e-5
6.0e-5
4.0e-5
2.0e-5
0.0
Before ATB
After ATB
After FMT
Donor 1

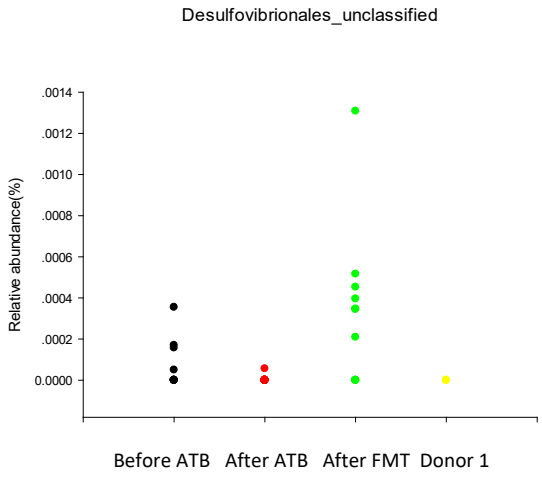


Figure 20: Linear discriminant analysis effect size (LEfSe) analysis. A, LEfSe analysis showing genera that were significantly differentially abundant between before and after antibiotic depletion, fecal microbiota transplantation from Human donor feces number 1 (Donor 1)

Beta diversity analyzed by donor: Donor2.

The PCoA plots depicted discernible dissimilarities in both microbiota membership (Figure 21) and structure (Figure 22) among samples collected at each sampling.

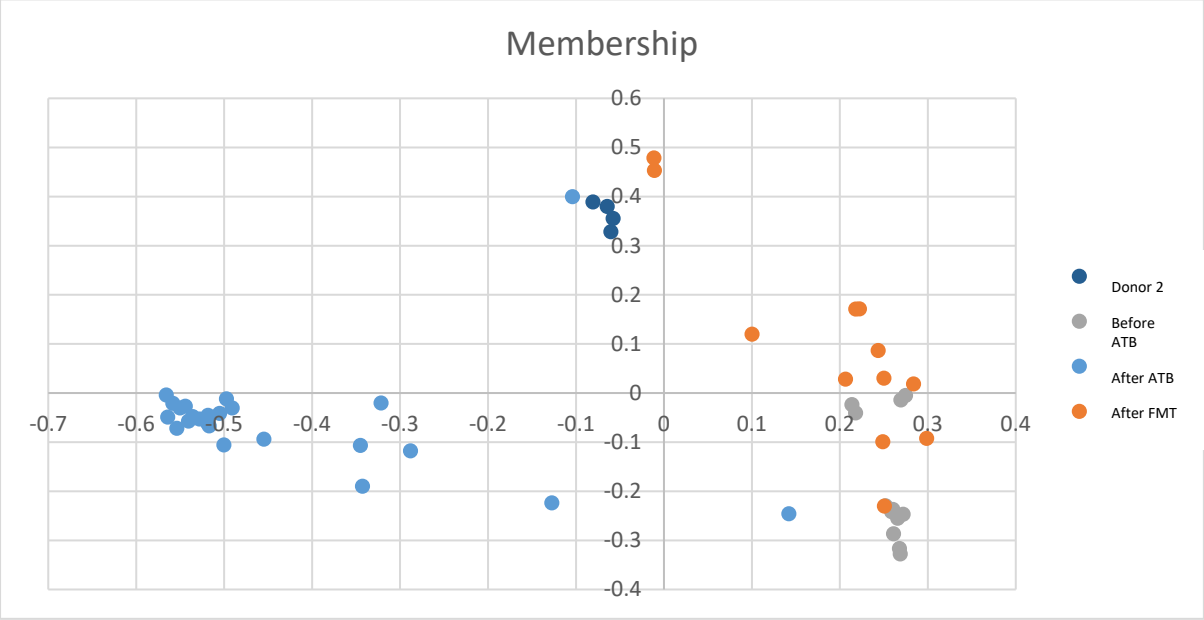


Figure 21. Principal coordinate analysis (PCoA) of fecal microbiota (membership) from Donor2, before ATB, after ATB, and after FMT based on Jaccard distances.

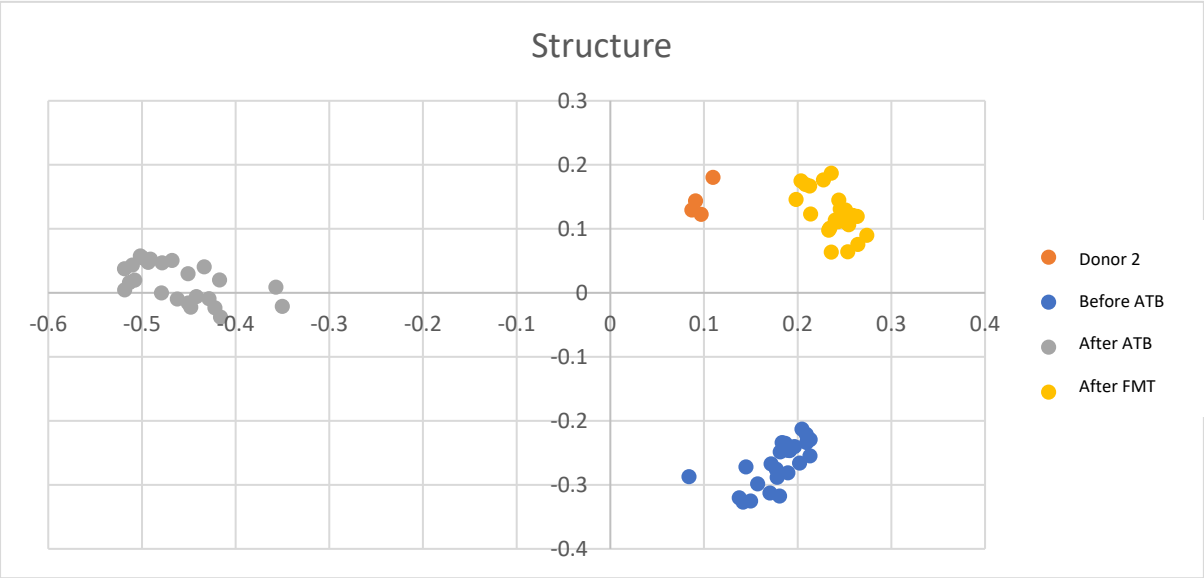


Figure 22. Principal coordinate analysis (PCoA) of fecal microbiota (structure) from Donor2, before ATB, after ATB, and after FMT based on Bray–Curtis distances.

The *Megamonas* were the most abundant genus in the feces of Donor 2 (Figure 23).

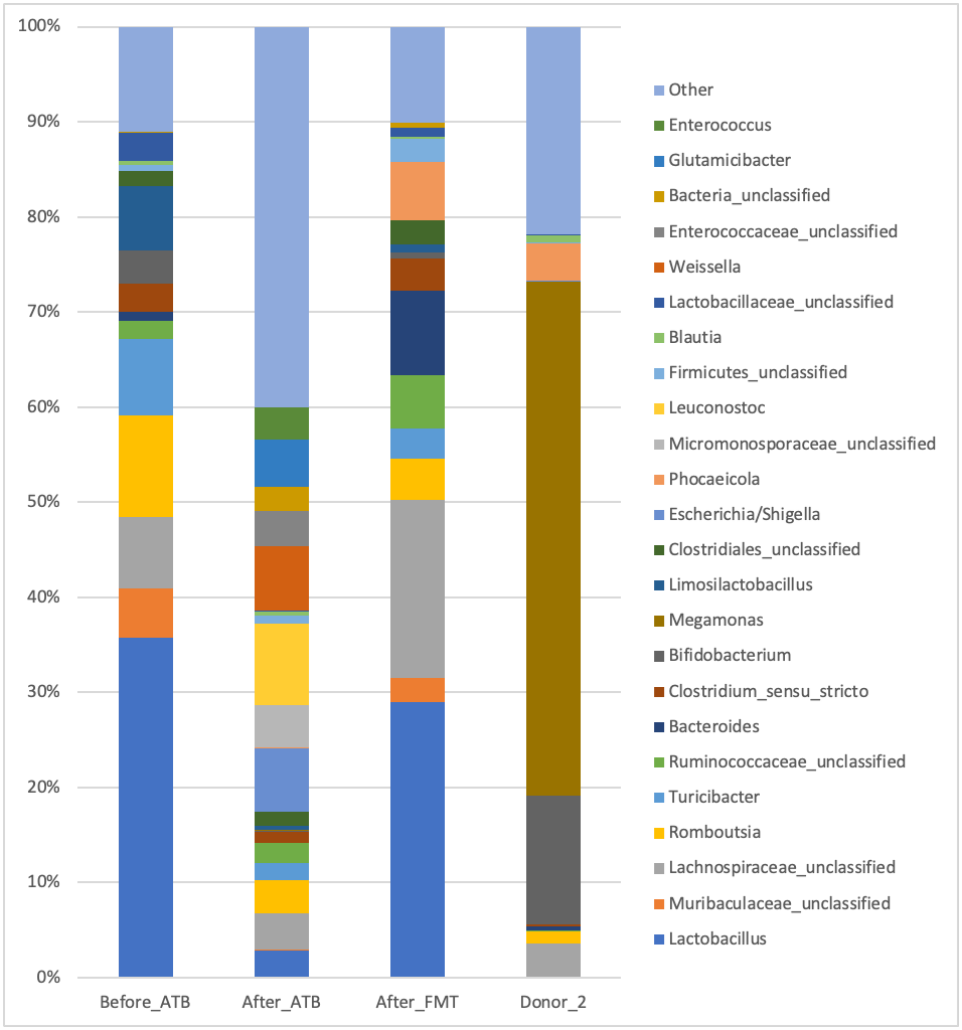
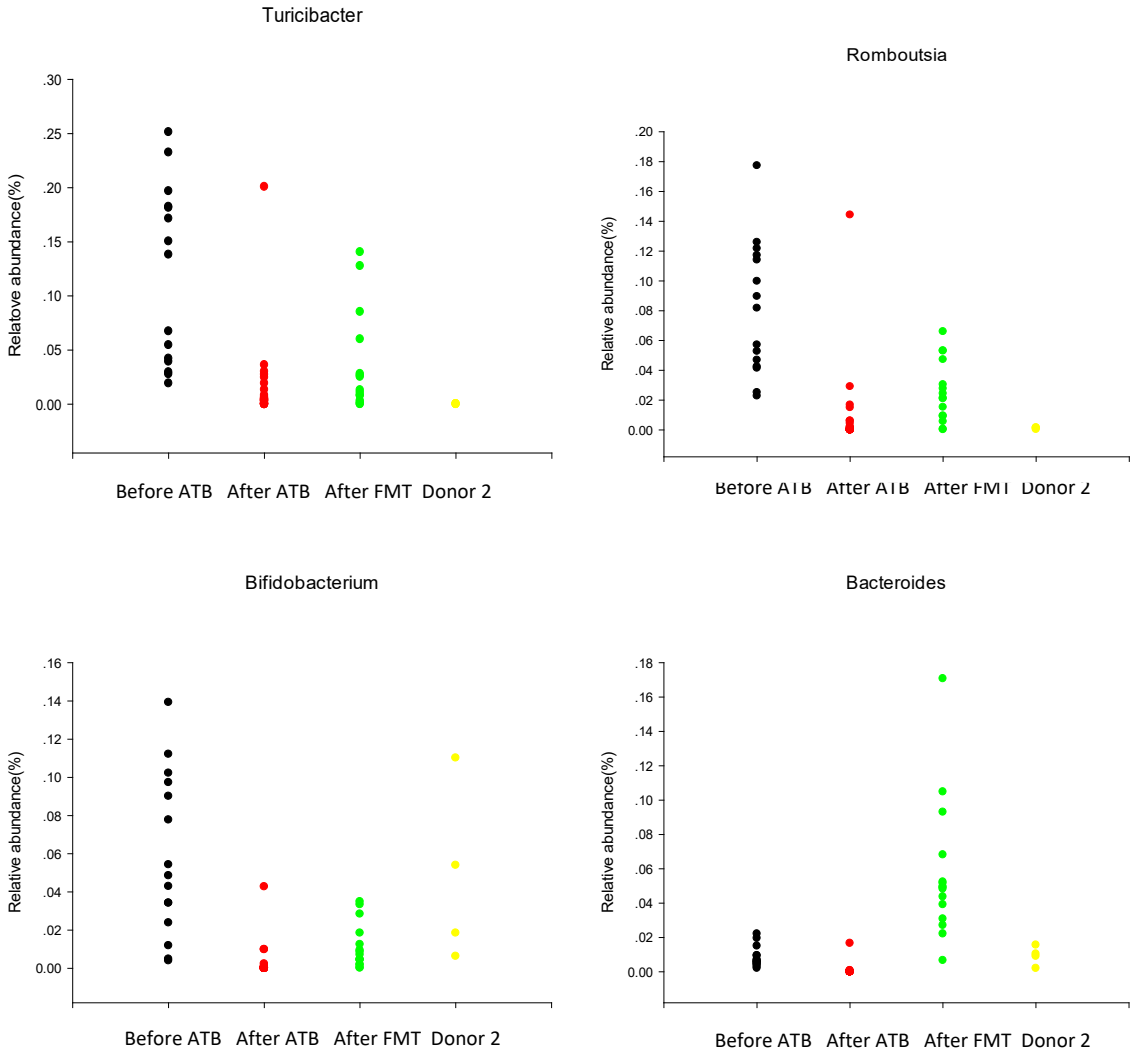


Figure 23. Relative abundances of the main taxa found in feces of rats before antibiotics (Before ATB), after antibiotics (After ATB), after fecal microbiota transfer (After FMT) and human feces donor 2; and the percentage on the vertical axis indicates the relative abundances.

The LEfSe analysis was used to determine which genera were statistically different between groups (Figure 24): *Romboutsia, Turicibacter, Bacteroides, Bifidobacterium, Limosilactobacillus, Phocaeicola, Firmicutes,* Lactobacillaceae and *Bacteria_unclassified*, as shown in the table attachment



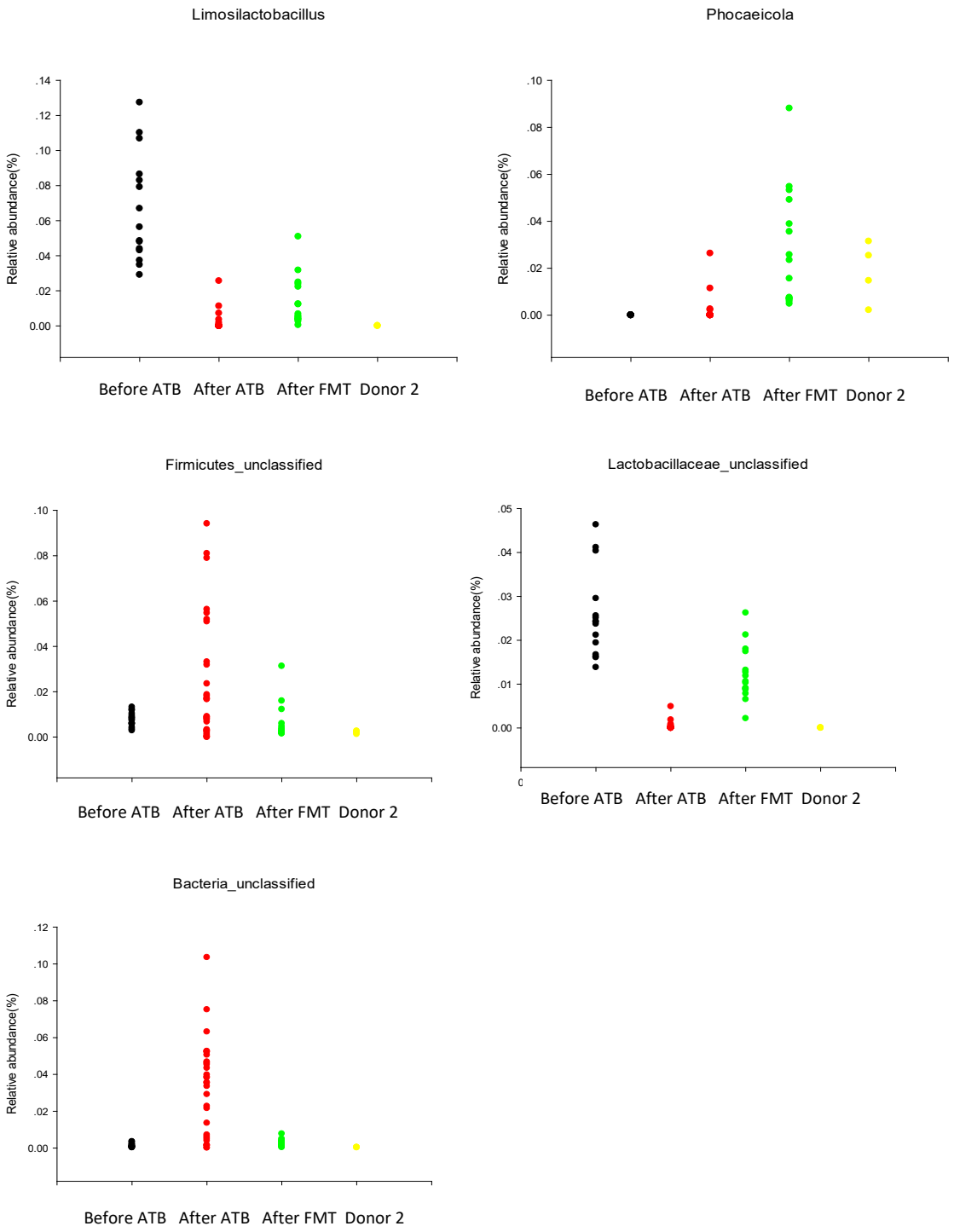


Figure 24: Linear discriminant analysis effect size (LEfSe) analysis. A, LEfSe analysis showing genera that were significantly differentially abundant between before and after antibiotic depletion, fecal microbiota transplantation from Human donor feces number 2 (Donor 2)

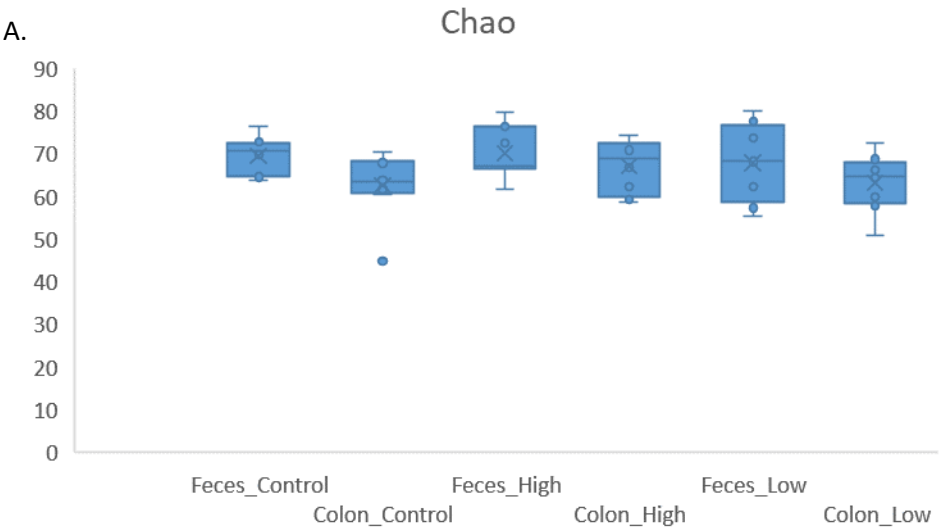
Changes of the gut microbiota in response to enniatin treatment

The impact of oral enniatin exposure on the human intestinal microbiota was investigated using a humanized rodent model with control and enniatin-treated rats. High-quality readings were successfully generated from 24 fecal samples, including content from the colon. These samples were divided into distinct groups, with 8 samples each for the control group, enniatin at 2 µg/kg group, and enniatin at 200 µg/kg group.

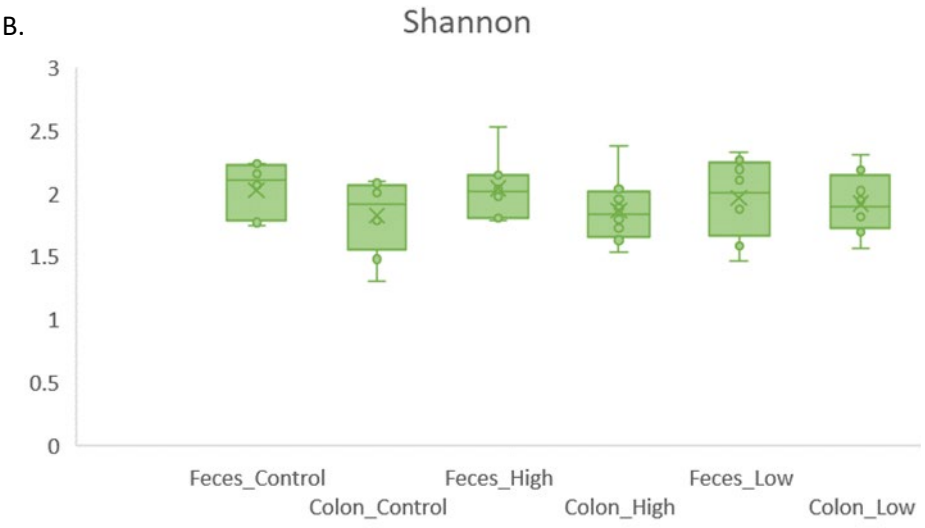
Alpha diversity

There was no statistical difference between groups for any of the alpha diversity indices utilized in this study (all $p > 0.05$, Figure 25).

A.



B.



C.

Figure 25. Box plots showing alpha diversity indexes (Chao, Shannon, and Simpson) in fecal microbiota of control and enniatin 2 and 200 µg/kg treated rats. Chao index (A) Shannon index (B), and Simpson index (C) reflect the evenness and global diversity of OTUs, respectively. Values are expressed as average ± standard deviation (n = 24). Differences were assessed by the non-parametric Kruskal–Wallis test.

Beta diversity

Within the beta-diversity framework, principal coordinate analysis (PCoA) plots revealed no clear distinctions within fecal microbial communities across control, low-dose, and high-dose enniatin treatment groups in both fecal and colon samples from 24 rats. The PCoA plots delineated evident dissimilarities in microbiota membership (Figure 26) and structure (Figure 27) among feces, as well as microbiota membership (Figure 28) and structure (Figure 29) among colon samples at each stage.

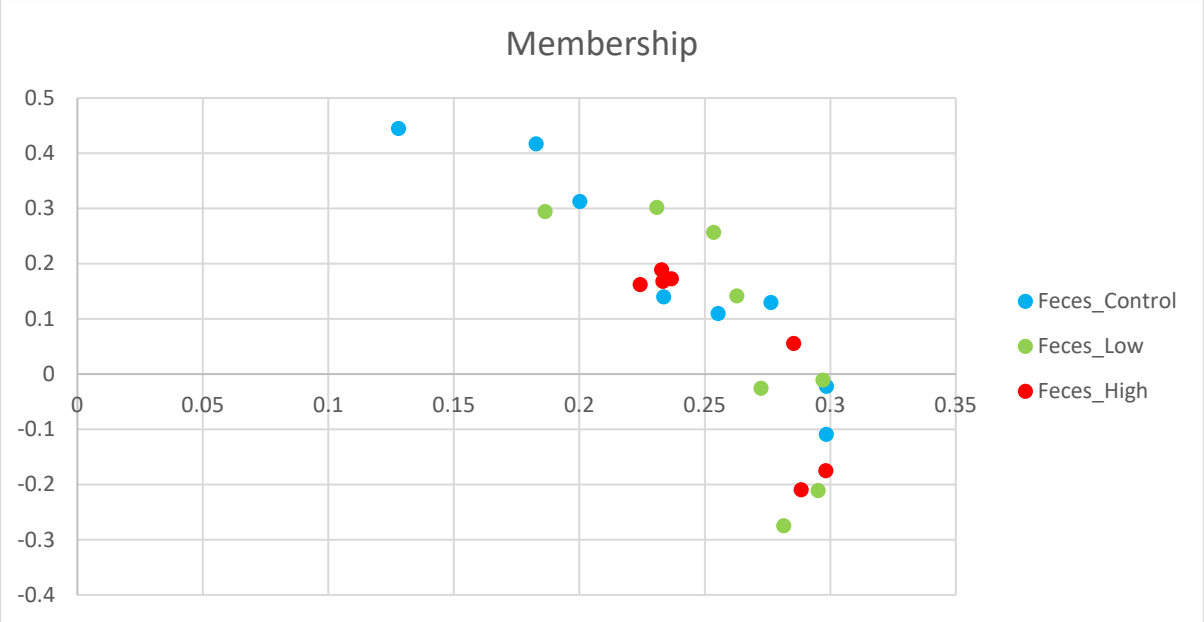


Figure 26. Principal coordinate analysis (PCoA) of fecal microbiota (membership) from control and 2 µg/mg (feces low) and 200 µg/mg (feces high) based on Jaccard distances.

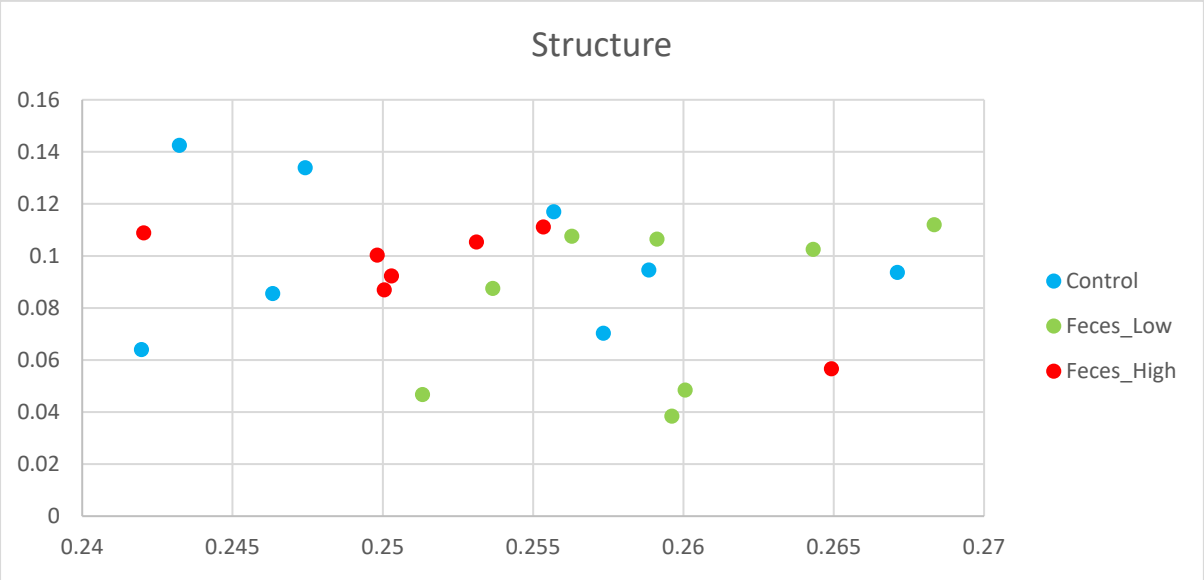


Figure 27. Principal coordinate analysis (PCoA) of fecal microbiota (structure) from control and 2 µg/mg and 200 µg/mg based on Bray–Curtis distances.

Figure 28. Principal coordinate analysis (PCoA) of colon microbiota (membership) from control and 2 μg/mg and 200 μg/mg based on Jaccard distances.

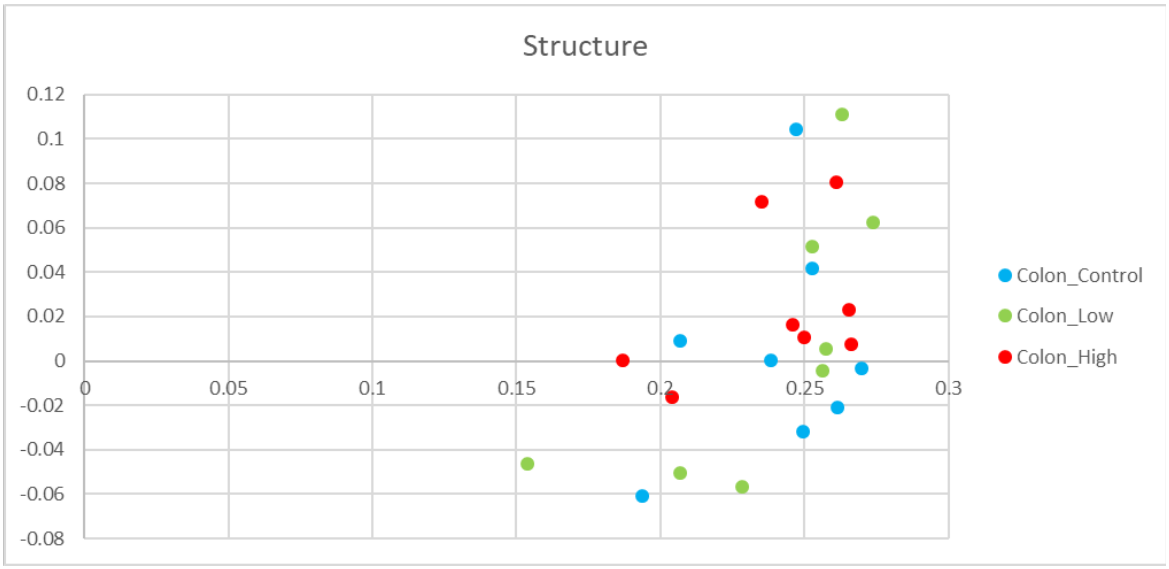


Figure 29. Principal coordinate analysis (PCoA) of fecal microbiota (structure) from control and 2 μg/mg and 200 μg/mg based on Bray–Curtis distances.

To explain changes in the fecal microbiota induced by enniatin exposure in more detail, we determined the relative abundance at the phylum and family levels. At the phylum level, a total of nine bacterial genera were identified in all fecal samples. Further, several bacterial genera were identified in all fecal samples, with *Lactobacillus* being the most abundant genus in the feces of rats from all groups, accounting for an average of 24.36% in controls, 36.77% in low exposure and 30.05% in high exposure (Figure 30).

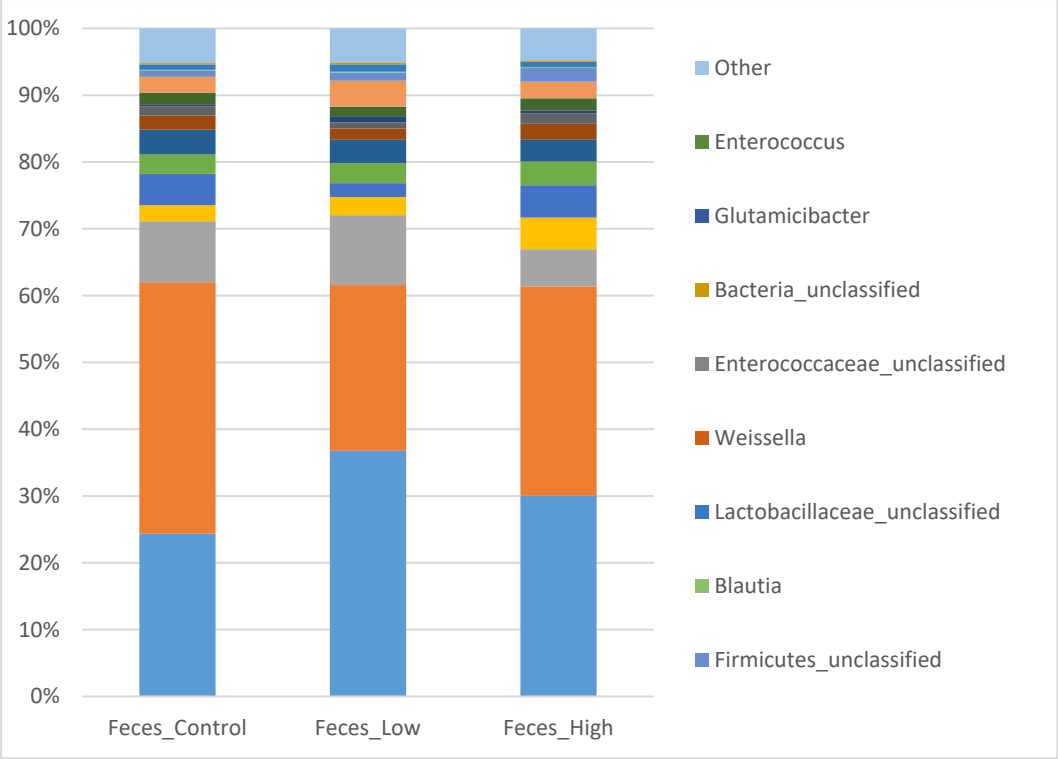


Figure 30. Relative abundances of the main taxa found in feces of rats control, low enniatin treatment and high enniatin treatment group; and the percentage on the vertical axis indicates the relative abundances.

Colonic samples collected at the moment of euthanasia were collected for further comparison of long-term exposure to enniatin. *Lactobacillus* was the most abundant genus in the colon of rats from all groups, accounting for averages of 35.43% in colon controls, 31.73% in the low exposure and 32.41% in the high exposure (Figure 31).

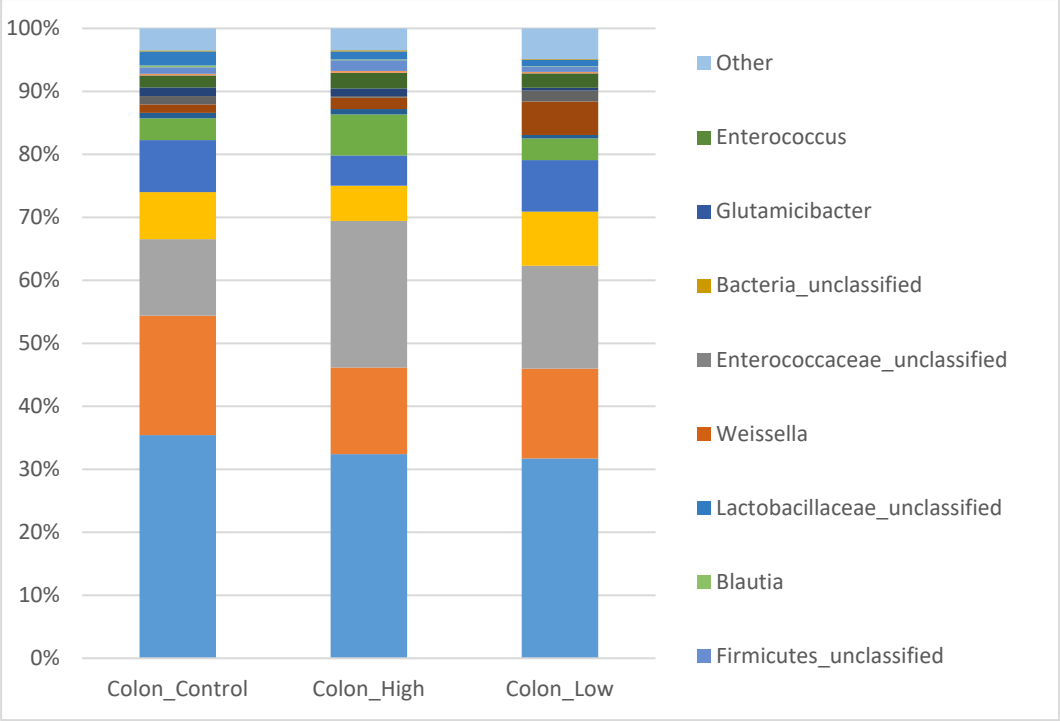


Figure 31. Relative abundances of the main taxa found in colon of rats control, low enniatin treatment and high enniatin treatment group; and the percentage on the vertical axis indicates the relative abundances.

Fecal and colonic samples were further divided according to the donor to account for the compositional differences observed in the microbiota of donors. The most abundant taxa in the feces and colon of rats receiving FMT from donors 1 and 2 are presented in Figure 32 and Figure 33, respectively.

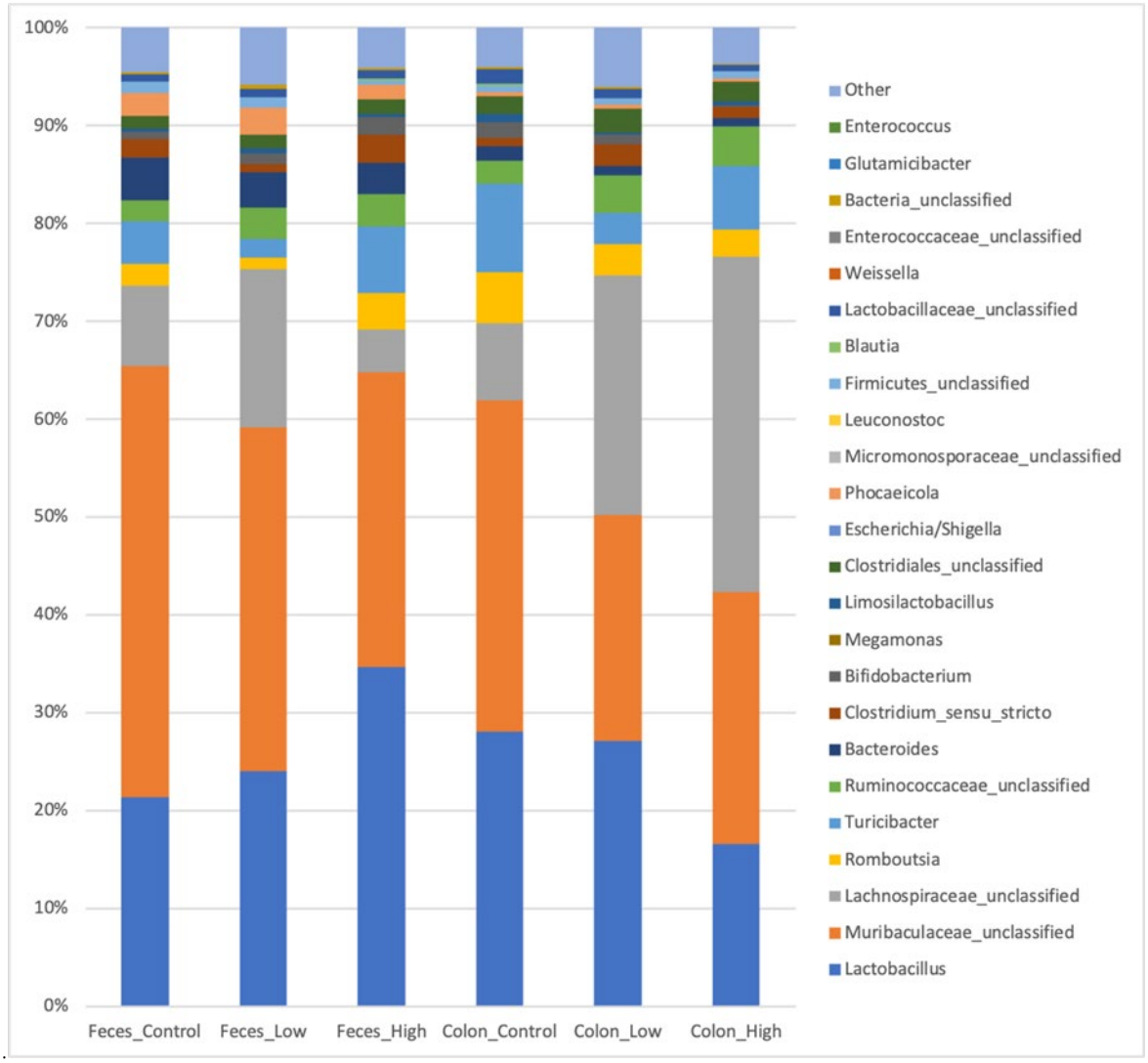


Figure 32. Relative abundances of the main taxa found in feces and colon of rats receiving human feces transfer from donor 1 and treat with control, low enniatin treatment and high enniatin treatment group; and the percentage on the vertical axis indicates the relative abundances.

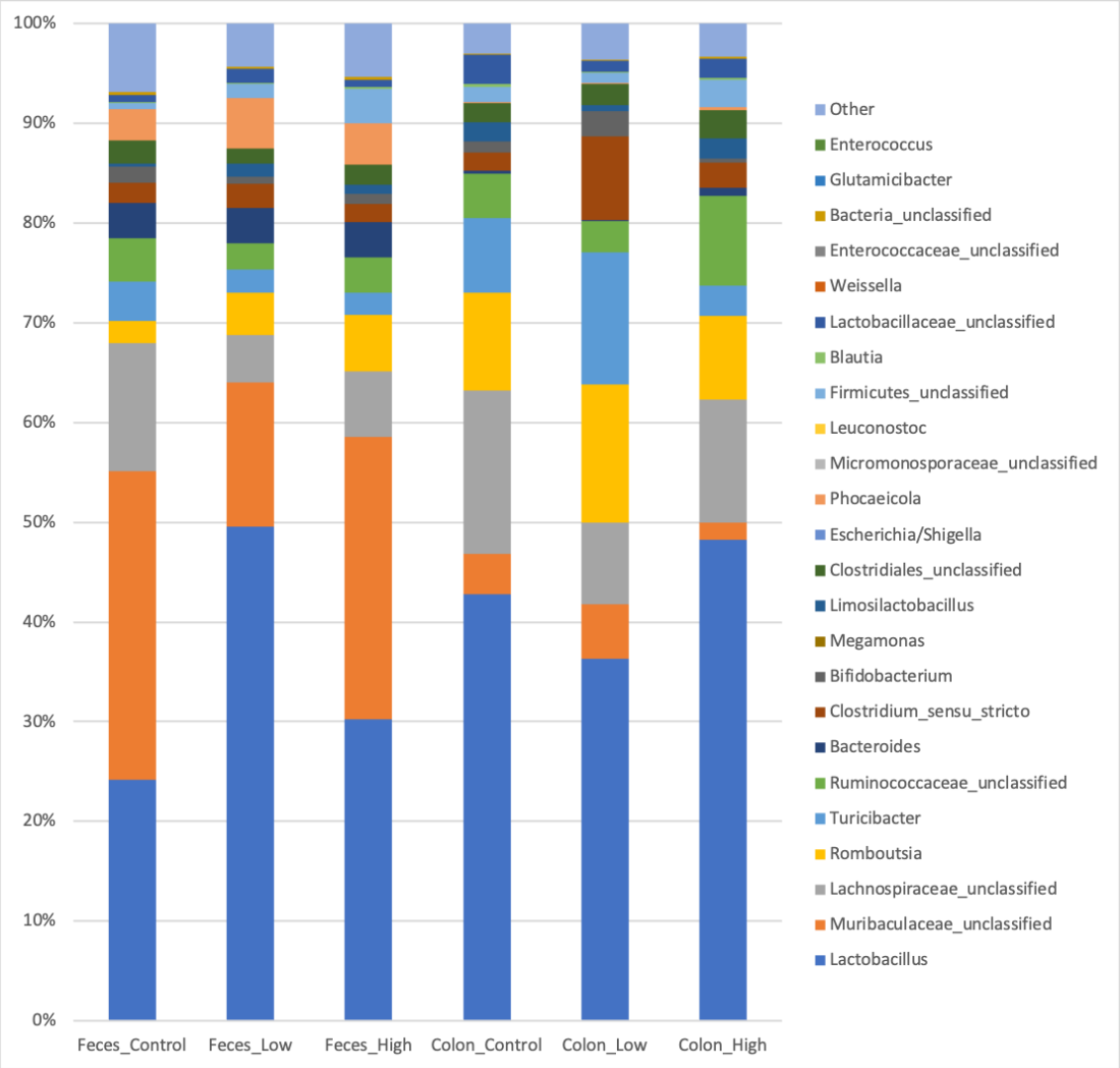
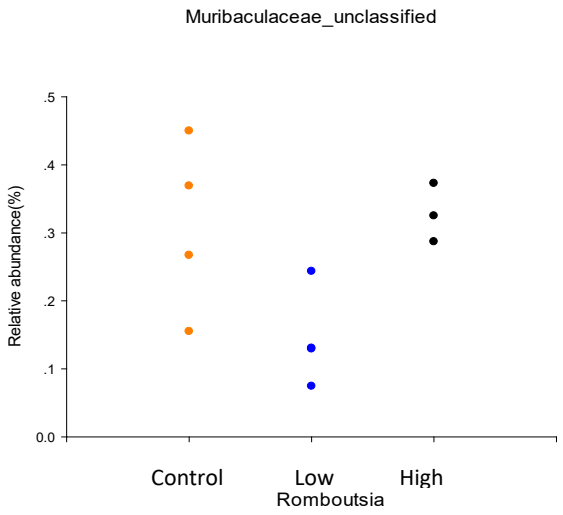
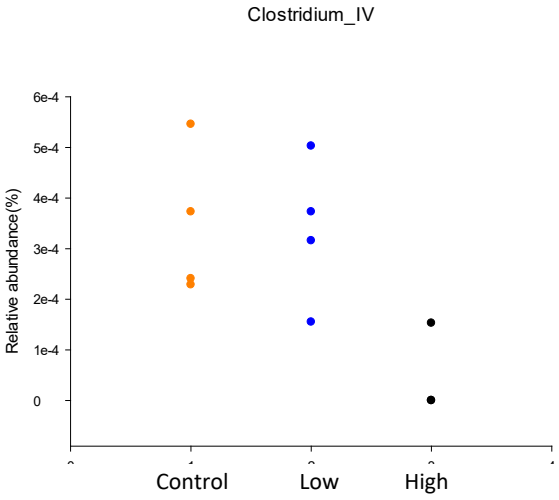
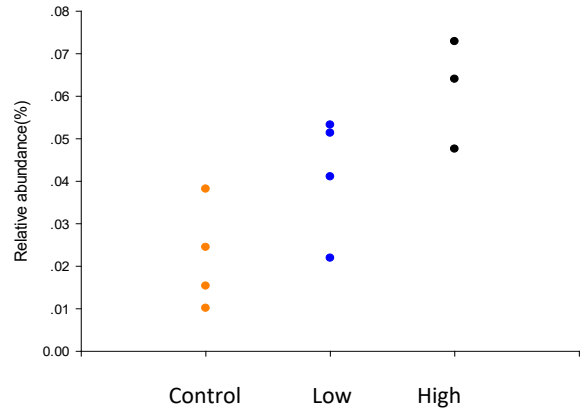


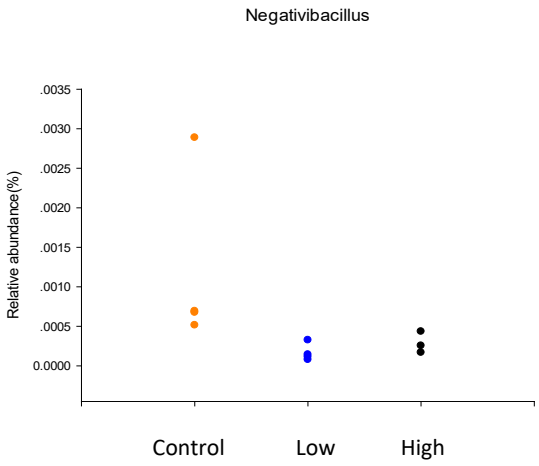
Figure 33 . Relative abundances of the main taxa found in feces and colon of rats receiving human feces transfer from donor 2 and treat with control, low enniatin treatment and high enniatin treatment group; and the percentage on the vertical axis indicates the relative abundances.

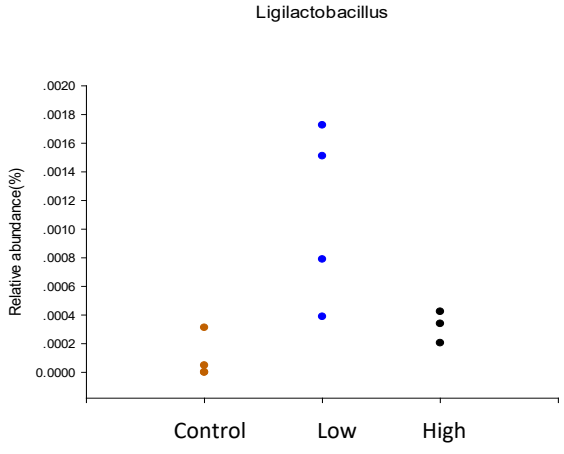
Some bacteria were found to be significantly different among the treatment groups evaluated by the LEfSe analysis: Unclassified Muribaculaceae*, Romboutsia, Ligilactobacillus, Negativibacillus, Unclassified Proteobacteria, Turicimonas, and Clostridium_IV* were more abundant in the feces and colon of rats receiving FMT from donor 1 (Figure 34 and 35, respectively). Moreover, *Clostridium sensu stricto*, *Faecalibacillus*, and *Hungatella* were more abundant in the colon of those rats.

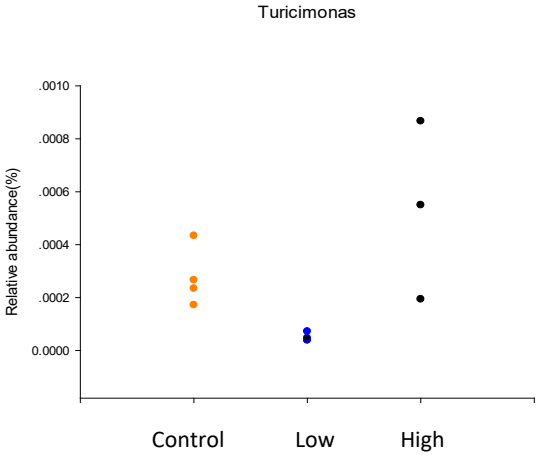

Muribaculaceae_unclassified
Relative abundance(%)
.5
.4
.3
.2
.1
0.0
Control
Low
High


Clostridium_IV
Relative abundance(%)
6e-4
5e-4
4e-4
3e-4
2e-4
1e-4
0
Control
Low
High


Romboutsia
Relative abundance(%)
.08
.07
.06
.05
.04
.03
.02
.01
0.00
Control
Low
High


Negativibacillus
Relative abundance(%)
.0035
.0030
.0025
.0020
.0015
.0010
.0005
0.0000
Control
Low
High


Ligilactobacillus
Relative abundance(%)
.0020
.0018
.0016
.0014
.0012
.0010
.0008
.0006
.0004
.0002
0.0000
Control
Low
High


Turicimonas
Relative abundance(%)
.0010
.0008
.0006
.0004
.0002
0.0000
Control
Low
High

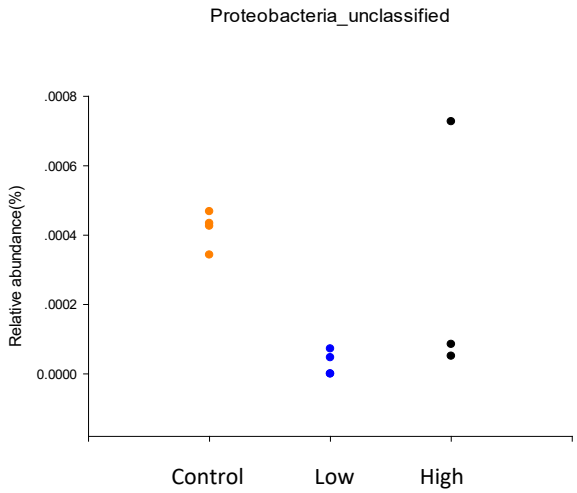


Figure 34: Linear discriminant analysis effect size (LEfSe) analysis showing the genera that were significantly differentially abundant between treatments groups from rat's feces receiving human feces transfer from donor 1.

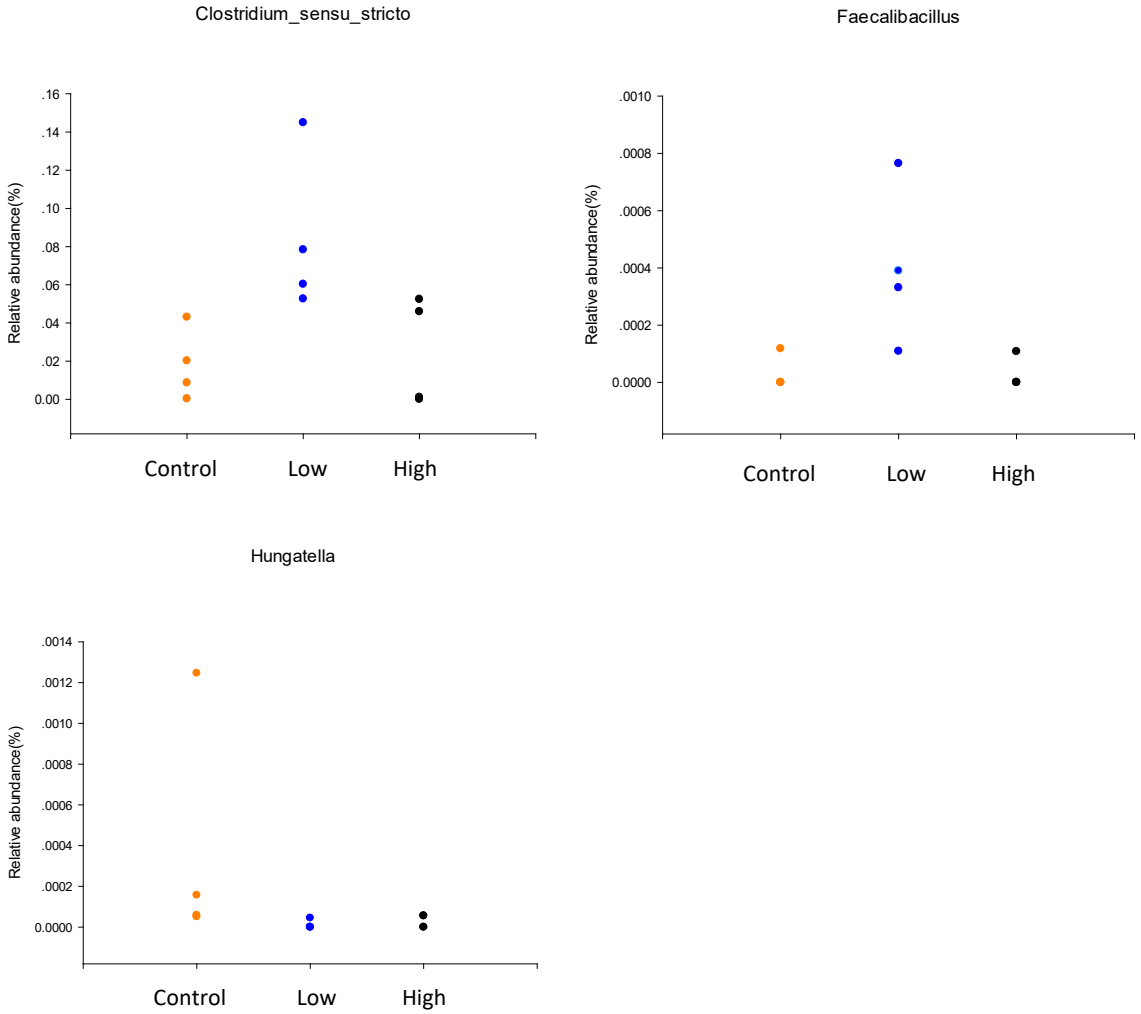


Figure 35: Linear discriminant analysis effect size (LEfSe) analysis shows genera that were significantly differentially abundant between treatment groups in rats' colons receiving human feces transfer from donor 1.

Escherichia Shigella, Unclassified Clostridiaceae and Unclassified Bifidobacteriaceae were more abundant in the feces of rats receiving FMT from donor 2 (Figure 36).

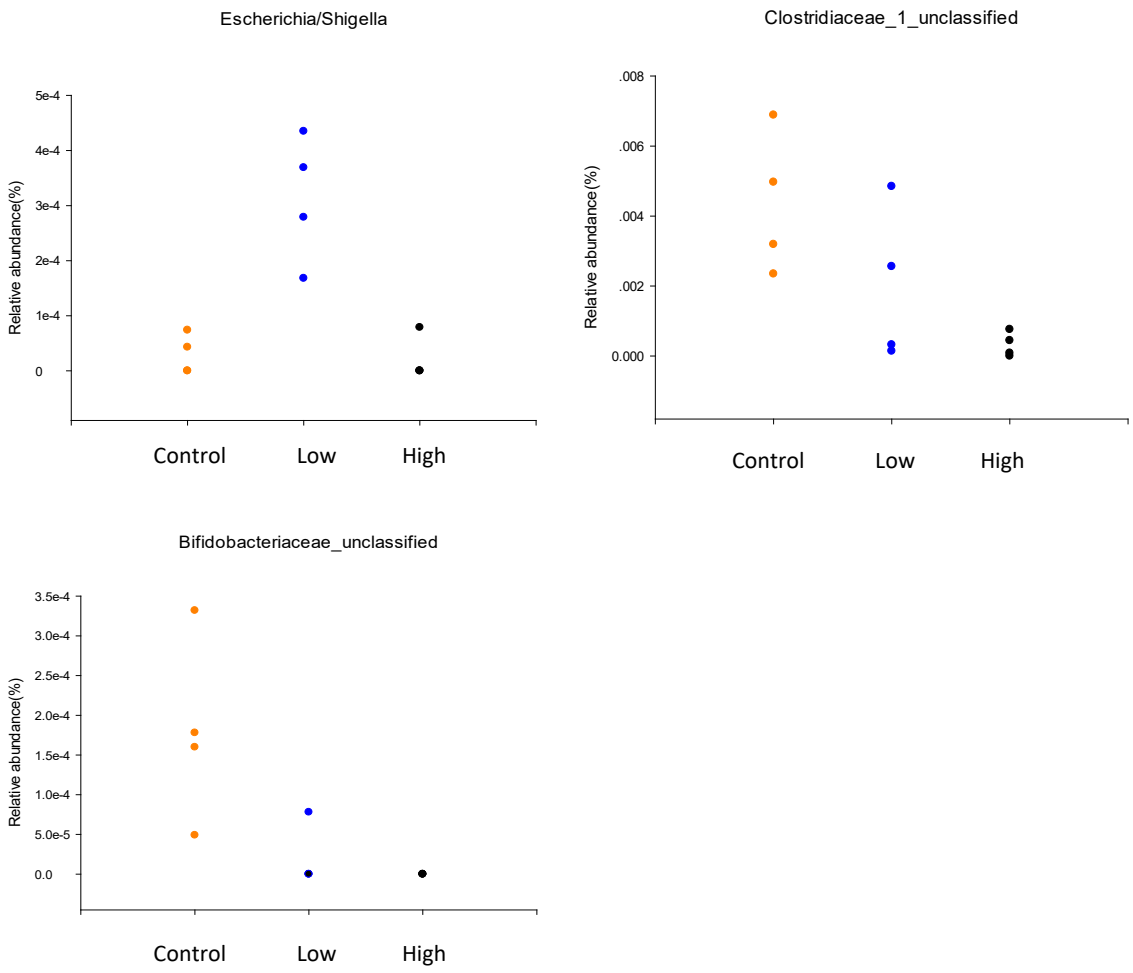


Figure 36: Linear discriminant analysis effect size (LEfSe) analysis showing genera that were significantly differentially abundant between treatment groups from rat's feces receiving human feces transfer from donor 2.

DISCUSSION

Summary

Our result showed notable variations were observed in the microbiota composition pre- and post-antibiotic treatment. Furthermore, we successfully established a humanized rat model utilizing human intestinal microbiota, even though the two human donors displayed markedly distinct microbiota communities and diversity. The PERMANOVA test showed significant effects of sampling time, donor origin, and sample type on both community structure and membership, while toxin exposure had no impact. Pairwise comparisons revealed differences before and after antibiotics, after FMT, and between donors and recipient rats, with no interactions between variables. In a repeated oral dose toxicity assessment of the enniatin complex in Sprague-Dawley rats, enniatin exposure influenced the architecture of the humanized microbiota. The relative abundances of particular bacterial genera demonstrated a dose-dependent effect of enniatin, although the syrup employed might have homogenized the microbiota, potentially obscuring some impacts of enniatin on microbiota structure. No clinical changes or alterations in body weight were detected during the experiment.

Microbiota depletion

The microbial composition exhibited a significant difference between the pre-antibiotic and post-antibiotic groups. As highlighted in previous research, the gut microbiota is intricately individualized and subject to influences such as environmental factors, dietary choices, medications, and genetic predispositions (Anwar et al., 2019). Antibiotics, a known perturbator of the gut microbiota, induce both transient and permanent changes, with short-term usage leading to alterations in resistome composition and an increased potential for horizontal gene transfer (Palleja et al., 2018; Isaac et al., 2016). Notably, the gut microbiota demonstrates a capacity for recovery following short-term exposure to broad-spectrum antibiotics (Palleja et al., 2018). A study showed significant and long-lasting disruptions in the microbiota after a 7-day clindamycin treatment. The impact included decreased diversity of certain bacteria, persistent antibiotic resistance genes, and lasting changes even 2 years after treatment. (Jernberg et al., 2007). To effectively deplete gut bacteria, a cocktail of multiple antibiotics providing broad-spectrum activity is required. Following a two-week course of broad-spectrum antibiotics, a reduction in bacterial load ranging from 72% to 86% was reported (Kiraly et al., 2016; Reikvam et al., 2011). One study evaluated the depletion of gut microbes by administering individual broad-spectrum antibiotics (Ampicillin, Doxycycline, or Ciprofloxacin) and found that each induced distinct taxonomic change. This supports the idea that an antibiotic cocktail is more effective than a single antibiotic. Another study measured 16S rRNA gene copies in fecal pellets after two weeks of treatment with either an antibiotic cocktail (1 g/L ampicillin, 1 g/L metronidazole, 1 g/L neomycin, 0.5 g/L vancomycin), a single antibiotic (1 g/L ciprofloxacin), or combinations of antibiotics (1 g/L neomycin and 1 g/L metronidazole; 0.5 g/L vancomycin and 1 g/L ampicillin). Only the cocktail and the vancomycin/ampicillin combination showed significant differences compared to the vehicle-treated animals (Baldridge et al., 2015).

Establishment of human gut microbiota in rats

Rats inoculated with human microbiota serve as a model to investigate microbial interactions. A research study tracked the stability of bacterial communities in rats colonized with human fecal bacteria over a 12-month period. Findings indicated a high degree of similarity between the rat and human microbiota, implying that human fecal microbiota can be sustained in rats for at least one year (Alpert et al., 2008). Inbred rodent models are utilized to investigate microbiome-related diseases, yet discrepancies arise in the colonization of human gut microbiota between germ-free (GF) rats, GF mice, and antibiotic-treated mice. GF rats demonstrated superior colonization of human-donor bacteria compared to GF mice, underscoring the significance of the recipient rodent's genetic background on gut microbiota composition (Wos-Oxley et al., 2012) The impact of dietary habits on gut microbiota composition is evident, as chronic consumption of a high-sugar diet induces metabolic dysfunctions and alters the composition of the bacterial kingdom. Specifically, there is an increase in Proteobacteria phylum, Enterobacteriales order, Enterobacteriaceae family, and Escherichia genus in response to a high-sugar diet (Shin, Whon, & Bae, 2015). This dietary shift also leads to a reduced abundance of the Pediococcus genus and an elevated abundance of *Helicobacteriaceae*, particularly in rats subjected to a high-fat diet (Moon et al., 2014; Higashikawa et al., 2016; Rong et al., 2016).

Changes of the gut microbiota in response to enniatin treatment

A study conducted on CD1(ICR) mice investigated the 28-day repeated oral dose toxicity of an enniatin complex. The mice received enniatin B, B1, and A1 in a 4:4:1 ratio at doses of 0, 0.8, 4, and 20 mg/kg/day. Life parameters remained stable, with only minor reductions in food intake observed in males at 4 and 20 mg/kg and in females at 20 mg/kg. There were no significant changes in body and organ weights, hematology, blood biochemistry, or histopathology. Under the experimental conditions, the no-observed-adverse-effect level (NOAEL) was determined to be 20 mg/kg/day for both sexes (Okano et al., 2021). Another study examined the effects of oral exposure to an enniatin complex in 2-month-old female Wistar rats over a 28-day period. On day 28, blood samples and tissues from the gastrointestinal tract, liver, and kidneys were collected to detect enniatin A. No adverse effects were noted at a dose of 20.91 mg/kg body weight/day (Maranghi et al., 2018).

In vivo toxicity studies on enniatins (ENNs) remain limited. A novel method was developed and validated for the simultaneous analysis of ENNs A, A1, B, and B1 in Wistar rat plasma, serum, and feces using liquid chromatography-tandem mass spectrometry. The method demonstrated satisfactory recoveries (70–106%), precision, and linearity, with detection and quantitation limits of 1 and 10 ng/ml, respectively. When applied to samples from rats administered ENNs, no adverse effects were observed, and ENNs were detected in feces, peaking at 6 hours post-administration (Escrivá et al., 2015). In another study, the pharmacokinetics and 28-day repeated-dose oral toxicity of enniatin B (ENNB) were evaluated in CD1 (ICR) mice. Male mice received a single oral or intravenous dose of ENNB, revealing a

bioavailability of 139.9%, an elimination half-life of 5.1 hours, 5.26% fecal excretion, and upregulation of liver enzymes. In the 28-day study, ENNB was administered via oral gavage at doses of 0, 7.5, 15, and 30 mg/kg body weight/day. Females showed decreased food consumption without clinical changes, while males at 30 mg/kg exhibited lower red blood cell counts, elevated blood urea nitrogen levels, and increased kidney weights. ENNB did not induce toxicity, and the NOAEL was identified as 30 mg/kg body weight/day (Ojiro et al., 2023). Uhlig et al., (2007) emphasized the dual nature of enniatins, highlighting their potent antifungal and antibiotic properties alongside significant toxicological effects. Their research underscores enniatins' potential as antimicrobial agents in agricultural and medical applications, but also cautions that their toxicity can lead to gastrointestinal damage, inflammation, and immune dysregulation. The study stresses the need to balance the beneficial antimicrobial properties of enniatins with their potential health risks to ensure their safe and effective use. Importantly, the study employed oral administration to simulate natural intake, recognizing that alternative administration methods, such as intraperitoneal injection, could lead to differences in the compound's structure and the animals' responses (Juan et al., 2014).

This study evaluated the effects of low and high doses of enniatin mycotoxins on gut microbiota using a humanized Sprague-Dawley rat model. The approach involved inducing microbiota depletion, successfully establishing a humanized model through fecal transplants, and assessing microbiota changes following enniatin exposure. While the study confirmed the dose-dependent modification of certain bacterial genera, the overall impact of enniatins on gut microbiota was limited, likely due to the homogenizing effect of the syrup used across all groups. These findings highlight the complexity of assessing mycotoxin-induced dysbiosis in a humanized model and underscore the importance of considering factors like dietary components when interpreting results.

DATA AVAILABILITY STATEMENT

All data pertinent to this study are contained in the article or available upon request. For all requests, please contact Dr.Imourana Alassane-Kpembi (Imourana.alassane-kpembi @umontreal.ca).

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CONFLICTS OF INTEREST

The authors declare that there were no conflicts of interest.

ETHICS STATEMENT

The experimental protocol was reviewed and approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine and the Comité d'éthique de la recherche en sciences et en santé (CERSES) at the University of Montreal. The rats were cared for in accordance with the Canadian Council on Animal Care guidelines.

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Additional information

The authors declare that there were no competing interests.

CHAPTER 5

GENERAL DISCUSSION

5.1 General Discussion

The challenge in replicating toxicity results between *in vivo* and *in vitro* models arises from multiple factors, including the intricacies of biological systems, variations in metabolic pathways, differences in pharmacokinetics and pharmacodynamics, the presence of immune responses, and the impact of the cellular microenvironment. Additionally, genetic, and epigenetic diversity and species-specific differences contribute to these discrepancies. *In vivo* models possess a complex and dynamic environment that is difficult to emulate *in vitro*, leading to variations in observed toxicity outcomes. This highlights the necessity for ongoing improvements in *in vitro* models to better simulate *in vivo* conditions and enhance the accuracy of toxicological predictions (Hartung, 2009).

5.1.1 Microbiota depletion

Variations in host genetics, diet, and digestive tract physiology present significant challenges in developing rat models for the human gut microbiome. In this research, we examine the effects of emerging mycotoxins, specifically enniatins, on altering the human intestinal microbiota structure and diversity, utilizing humanized non-germ-free rats created through antibiotic treatment and human fecal transplantation. The microbiota depletion method outlined in this study involves the administration of an antibiotic cocktail combined with an antifungal mixture in syrup form to all rats. As we were not allowed to use the gavage method for treatment administration, we mixed all treatment components with syrup for oral delivery. The syrup itself may have influenced the microbiota structure and diversity. However, the results indicate significant differences in microbiota structure and diversity before and after antibiotic administration. Individualized responses to antibiotics are influenced by the individualized microbiota at baseline (Falony et al., 2016) (Leónidas Cardoso, 2020). Antibiotic treatment can affect the absolute abundance of the gut microbiota, with some studies showing lower total microbial abundances during treatment, while others found no significant effect or even an increase in abundance (Dubinsky et al., 2020). The relative abundance of actinobacteria is often negatively affected by antibiotic treatment, particularly the genus Bifidobacterium (Pokusaeva et al., 2011). Different responses to antibiotics may be influenced by the gram-negative or gram-positive nature of the bacterial groups (Wacklin et al., 2014). Antibiotic treatment can induce cascading changes in the gut microbiota, leading to amplification and suppression of species not directly affected by antibiotics (Raymond et al., 2016). Studies involving larger sample sizes and more extensive sampling have found statistically significant, long-term disturbances in the composition of the gut microbiota (Pennycook et al., 2021).

5.1.2 Human feces transfer

Taxonomic analyses of gut microbiota rats colonized with bacteria from two human donors demonstrate that this method produces unique bacterial profiles in the recipient rats that mirror those of their respective donors. Similarly, the antibiotic method described herein also yielded an imperfect transfer of either human donor microbiome. There were many types of bacteria phylum from human donor 1 to recipient rats that were generally successful, such as *Bacteria unclassified,* Bacteroidaceae, *Bacteroidales, Bacteroides, Barnesiella,*

Bifidobacterium, Desulfovibrionales, Eggerthella, Enterococcaceae, *Hungatella,* Lachnospiraceae, Lactobacillaceae*, Limosilactobacillus, Mycobacteriales,* Peptostreptococcaceae, *Romboutsia* and *Streptococcus*. There were also many types of bacteria phylum from human donor 2 to recipient mice that were generally successful, such as *Romboutsia, Turicibacter, Bacteroides, Bifidobacterium, Limosilactobacillus, Phocaeicola, Firmicutes*, Lactobacillaceae and *Bacteria_unclassified*. FMT resulted in the transfer of specific bacterial species from the human donors to the mice, including *Bifidobacterium, Collinsella, Odoribacter, Bacteroides, Parabacteroides, and Roseburia. Fecalibacterium prausnitzii* was detected in mice receiving repeated rounds of FMT, while *Bifidobacterium* was detected in mice receiving FMT the first week. FMT once a week for four weeks is the best strategy as it allows engraftment of *Fecalibacterium* and a higher diversity of bacteria belonging to the *Bacteroidales* order (Wrzosek et al., 2018). Mice transplanted with feces diluted in skim milk + BHI had a similar microbiota composition to freeze-dried feces during FMT (Wrzosek et al., 2018). For the model in this study, the authors observed a decrease in the bacterial load after providing an antibiotic cocktail, similar to (Wrzosek et al., 2018) using polyethylene glycol for bowel cleansing. (Jalanka et al., 2016) and (Satokari et al., 2014) described a similar decrease in patients undergoing a bowel cleansing also using macrogol. Polyethylene glycol alone can lead to a similar decrease in the quantity of intestinal microbiota bacteria as a combination of antibiotics. Indeed, a decrease of up to four Logs of the 16S DNA using a combination of vancomycin, metronidazole, and neomycin or one ampicillin or vancomycin alone(Ubeda et al., 2010) or a combination of vancomycin and imipenem (Manichanh et al., 2010) were reported for antibiotic-treated rodents. A study that compared the efficacy of various gut preparation procedures commonly used before FMT in patients, such as antibiotic treatment, bowel cleansing, and no pre-treatment (He et al., 2017), led to the conclusion that the best intestinal microbiota transfer was obtained following antibiotic treatment. Human OTUs were detected in recipient mice four weeks later, even after only one FMT. FMT twice a week for several weeks can affect the engraftment and stability of the newly formed ecosystem. FMT once a week during the whole protocol may be the best compromise (Wrzosek et al., 2018).

5.1.3 Enniatins treatment

This study comprised a repeated oral dose study of enniatin complex (2 µg/kg and 200 µg/kg) in rats. However, no body weight was changed, and the microbiota structure and diversity were not significantly different among control, low-dose and high-dose treated rats. These results suggest that 28-day exposure to enniatin complex at doses of 2 µg/kg and 200 µg/kg may alter the humanized gut microbiota in rats. The syrup might have homogenized the microbiota masking the enniatin effect on the microbiota structure. Nonetheless, relative abundances of some bacteria genera indicate the dose dependent effect of enniatin exposures which is consistent with previous research on the toxic effects of eniatins (Ens) on Wistar rats (Manyes et al., 2014). Enniatin B caused stunted growth and anemia. High levels of Enniatin B (0.3, 5.2, 83 mg ENNB kg− 1 feed wet weight (ww) for ENNB diet) in commercial salmon feed could pose a risk to animal health in salmon. Enniatin A1 was effective against both M. tuberculosis H37Rv and M. bovis BCG. Enniatins, primarily a mixture of secondary metabolites of cyclohexadepsipeptides isolated from Fusarium species fungi, exhibit

antifungal, antibacterial, and anticancer effects (Malachova et al., 2011; Meca et al., 2010). However, their antibacterial mechanisms of action remain unknown.

As early as the 1960s, research reported that enniatins exhibited ionophoric activity (Shemyakin et al., 1969). Thus, the antibacterial activity of enniatin A1 may be related to its ionophore properties. Enniatin A1 exhibited significant bactericidal activity against M. tuberculosis H37Rv and M. bovis BCG, without affecting other tested strains (G. Wang et al., 2019). Enniatin A1 has been reported to function as an antibiotic with ion carrier characteristics (Kamyar et al., 2004). Currently, ionophore antibiotics are mainly used in the livestock and veterinary industry for anti-parasite treatment(Russell & Strobel, 1989). The cytotoxicity and poor selectivity of these antibiotics limit their development into antibacterial agents. However, advancements in molecular biology have led to the development of enniatins as antibacterial agents for treating upper respiratory tract infections (German-Fattal, 2001). Uhlig et al., (2007) highlighted the dual nature of enniatins, which exhibit potent antifungal and antibiotic properties alongside significant toxicological effects. Their research underscores the potential of enniatins as antimicrobial agents, useful in controlling fungal and bacterial pathogens in agricultural and medical settings. However, the study also emphasizes the importance of thoroughly evaluating the health risks associated with enniatin exposure, as their toxicity can lead to gastrointestinal damage, inflammation, and immune dysregulation. Balancing the beneficial antimicrobial properties of enniatins with their potential health hazards is essential for their safe and effective application. A recent review of the literature on beauvericin and enniatins indicates that the bioavailability of these mycotoxins is generally reduced by the action of the gut microbiota and probiotics, though the mechanisms behind this reduction remain imperfectly understood (Bertero et al., 2020).

5.2 Limitations and Perspectives

Microbiota research encounters challenges and opportunities in three key areas. First, the oral administration of mycotoxins with syrup presents a limitation, as the components of syrup, especially glucose, may influence the microbiota structure. Despite employing control groups and baseline microbiota information, a comprehensive perspective emphasizes the need for meticulous considerations of factors like absorption, metabolism, and oral kinetics for valid interpretation. Second, individual variation between donors in fecal microbiota transplantation introduces a limitation due to factors such as diet and genetics. A broader perspective involves recognizing the impact of functional differences, host-specific interactions, and health status on the transferred microbiota, urging the consideration of long-term stability and larger donor cohorts. Lastly, individual manipulation of human donors' microbiota in fecal transfer studies may introduce unintended consequences, impacting composition and diversity. Researchers must account for alterations in diet and lifestyle to ensure a representative state, highlighting the importance of standardized protocols to avoid interference with experimental objectives. Overall, addressing these limitations and adopting strategic perspectives are essential for advancing our understanding of microbiota dynamics and their implications for host health.

In conclusion, this study sheds light on analyzes the impact of low and high dose exposure to enniatins on the structure of human intestinal microbiota. The findings

emphasize the importance of considering individual variations in microbiota composition, as evidenced by exposure of enniatin show significantly alter some genera of microbial structure or diversity, it induced specific changes in bacterial genera abundance, warranting further exploration of its potential systemic toxic effects. This research contributes to our understanding of the complex interplay between emerging mycotoxin name as enniatins and gut microbiota, paving the way for more targeted and personalized approaches in medical treatments. Investigating the mechanisms underlying enniatin-induced gut microbiota alterations and the impact of systemic toxic effects of enniatins is worth further attention. Future research will focus on understanding the long-term impacts of enniatin exposure on host health, particularly its role in gastrointestinal disorders and immune dysregulation. By investigating the molecular pathways of enniatin-induced intestinal damage, inflammation, and microbial dysbiosis, we aim to elucidate its toxicological profile. These insights will be crucial for developing risk assessments and mitigation strategies, ultimately informing regulatory policies to protect public health from the potential hazards of prolonged enniatin exposure.

CHAPTER 7

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