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Influence of *Lactiplantibacillus plantarum* SNI3 on the gut microbiome: Unravelling its role in the gut-brain and gut-testis axis

PhD thesis

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Abbreviations

11β-HSD1 - 11β- Hydroxysteroid dehydrogenase type 1

Abx – Antibiotic treatment

ACTH – adrenocorticotropic hormone

ARC – Arcuate nucleus

AVPV – Anteroventral periventricular

BBB – Blood-brain barrier

BTB – Blood-testis-barrier

CASA – Computer-aided sperm analysis

CBUT – *Clostridium* tyobutyricum

CFU – Colony forming unit

CLRs – C-type lectin receptors

CNS - central nervous system

CORT – corticosterone

CRH – Corticotropin-releasing hormone

CVS – Chronic variable stress

DAPI – 4', 6 diamidino-2-phenylindole

DHT – Dihydrotestosterone

DMG – Dimethylglycine

ENS – enteric nervous system

EPM – elevated plus maze

F/B ratio – *Frimicutes* and *Bacteroidetes* ratio

FFARS – fatty acid receptors

FMT – Fecal microbiota transplantation

FOS – Fructo-oligosaccharides

FSH – Follicle-stimulating hormone

Fsh β – Follicle-stimulating hormone beta

GABA – Gamma-aminobutyric acid

GAPDH – Glyceraldehyde 3-phospate dehydrogenase

GCL – Glutamyl cysteine ligase

GF – Germ-free mice

GGT – γ-glutamyl transferase

GnRH – Gonadotropin-releasing hormone

GnRHR – Gonadotropin-releasing hormone receptor

GOS – Galacto-oligosaccharides

GPR109A – G-protein coupled receptor 109A

GR – Glucocorticoid receptor

GSSG – Oxidized glutathione

H&E – Haematoxylin and eosin

HFD – High-fat diet

HMDB – Human metabolome database

HPA axis – Hypothalamus-pituitary-adrenal axis

HPG axis – Hypothalamus-pituitary-gonadal axis

HSD – High-salt diet

IBD – Inflammatory bowel disease

IgA – Immunoglobulin A

Inha – Inhibin alpha

Inhba – Inhibin beta A

Inhbb – inhibin beta B

IPA – Indolepropionic acid

L. plantarum SNI3 – *Lactiplantibacillus plantarum* SNI3

LbSNI3 – *Lactiplantibacillus plantarum* SNI3

LH – Luteinizing hormone

Lhβ – Luteinizing hormone beta

MAPKS – mitogen-activated protein kinases

MD – Minimal Disease

MRS – deMan, Rogosa and Sharpe broth

MS – Maternal separation

MSI – Mass spectrometry imaging

ND – Normal fed

NF-kB – Nuclear factor-kB

NGS – Next-generation sequencing

NON – non-obese-diabetic mice

OF – Open field

Oxt – Oxytocin

PD – Parkinson's disease

PVN – Paraventricular nucleus of the hypothalamus

qRT-PCR – Quantitative real-time PCR

ROS – Reactive oxygen species

SCFAs – Short-chain fatty acids

SNS – Sympathetic nervous system

SPF – Specific pathogen free

STAT3 – Signal transducer and activator of transcription 3

STZ – Streptozotocin

T2D – Type 2 Diabetes

TESTO – Testosterone

Th1 – T helper cells 1

Th17- T helper cells 17

TLRs – Toll-like receptors

Treg – Regulator T cell

ZO-2 – Zonula occludens 2

γ-GluGlu – γ glutamyl glutamate

1. Introduction

1.1 Microbiome in general

The microbiome refers to the collection of genomes from all microorganisms in the environment (1). Microbiota, on the other hand, usually refers to microorganisms that are found within a specific environment. Microbiota can refer to all the microorganisms found in an environment, including bacteria, viruses, and fungi (2). These microbial communities inhabit various parts of the body such as the oro-naso-pharyngeal cavity, skin, and vagina, while the largest and most diverse communities are present in the gastrointestinal tract, from the stomach to the colon (3). The normal gut microbiota primarily comprises anaerobic bacteria, which outnumber aerobic and facultative anaerobic bacteria by 100 to 1000-fold (4). The composition of the gut microbiome is dominated mainly by *Firmicutes* and *Bacteroides*, along with smaller proportions of *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Cyanobacteria* (5).

1.2. Male reproductive physiology

The progressive decline in sperm counts and overall semen quality is a critical concern for male fertility (6). Over the past several decades, studies have highlighted a significant reduction in sperm count, estimated at approximately 50-60%, among men in North America, Europe, Australia and New Zealand since the 1970s (7, 8). This alarming trend is largely attributed to environmental factors, including lifestyle and diet changes or exposure to endocrine-disrupting chemicals (9, 10). These findings have profound implications as men account for nearly half of all infertility cases, underscoring the importance of male reproductive health in overall fertility outcomes. Male reproductive physiology is governed by tightly regulated hormonal, cellular and metabolic mechanisms. Each component plays a key role in maintaining fertility by coordinating the interactions between the hypothalamus, pituitary gland, and testes.

1.2.1. Regulation of hypothalamus-pituitary-gonadal (HGP) axis

The hypothalamus-pituitary-gonadal (HPG) axis, a complex hormonal network that regulates testosterone production and spermatogenesis, plays a central role in regulating male reproductive physiology (11). Activation of the HPG axis begins with the pulsatile

release of gonadotropin-releasing hormone (GnRH) from neurons in the hypothalamus primarily located in the preoptic area (12). The pulsatile release of GnRH is regulated by kisspeptin-expressing neurons in the arcuate nucleus (ARC) and anteroventral periventricular (AVPV) regions (13). These neurons co-express neurokinin B and dynorphin in ARC, ensuring precise regulation of GnRH release (14). GnRH is transported through the hypophyseal portal circulation to the anterior pituitary, where it binds to GnRH receptors (GnRHR) on gonadotroph cells. This interaction triggers the synthesis and release of follicle-stimulating hormone (FSH) and luteinization hormone (LH) (15). FSH signaling plays a crucial role in maintaining the spermatogonia pool, promoting differentiation via paracrine actions, facilitating entry to meiosis and supporting germ cell survival (16). While LH acts on Leydig cells in the testis, stimulating testosterone production. Testosterone is the primary androgen responsible for maintaining spermatogenesis and reproductive health. Testosterone and its more potent derivative dihydrotestosterone (DHT) provide negative feedback to the hypothalamus and pituitary, suppressing Gnrh, LH and FSH secretion (17) . Estrogen synthesized from testosterone via aromatase, similarly, inhibits the *Gnrh* and gonadotropin release (18). Additionally, FSH directly targets Sertoli cells, which are essential for supporting testosterone production and spermatogenesis. These cells also produce inhibin B, which downregulates FSH secretion from the anterior pituitary. In contrast, activins promote FSH synthesis, counteracting the effects of inhibin to maintain hormonal balance (19).

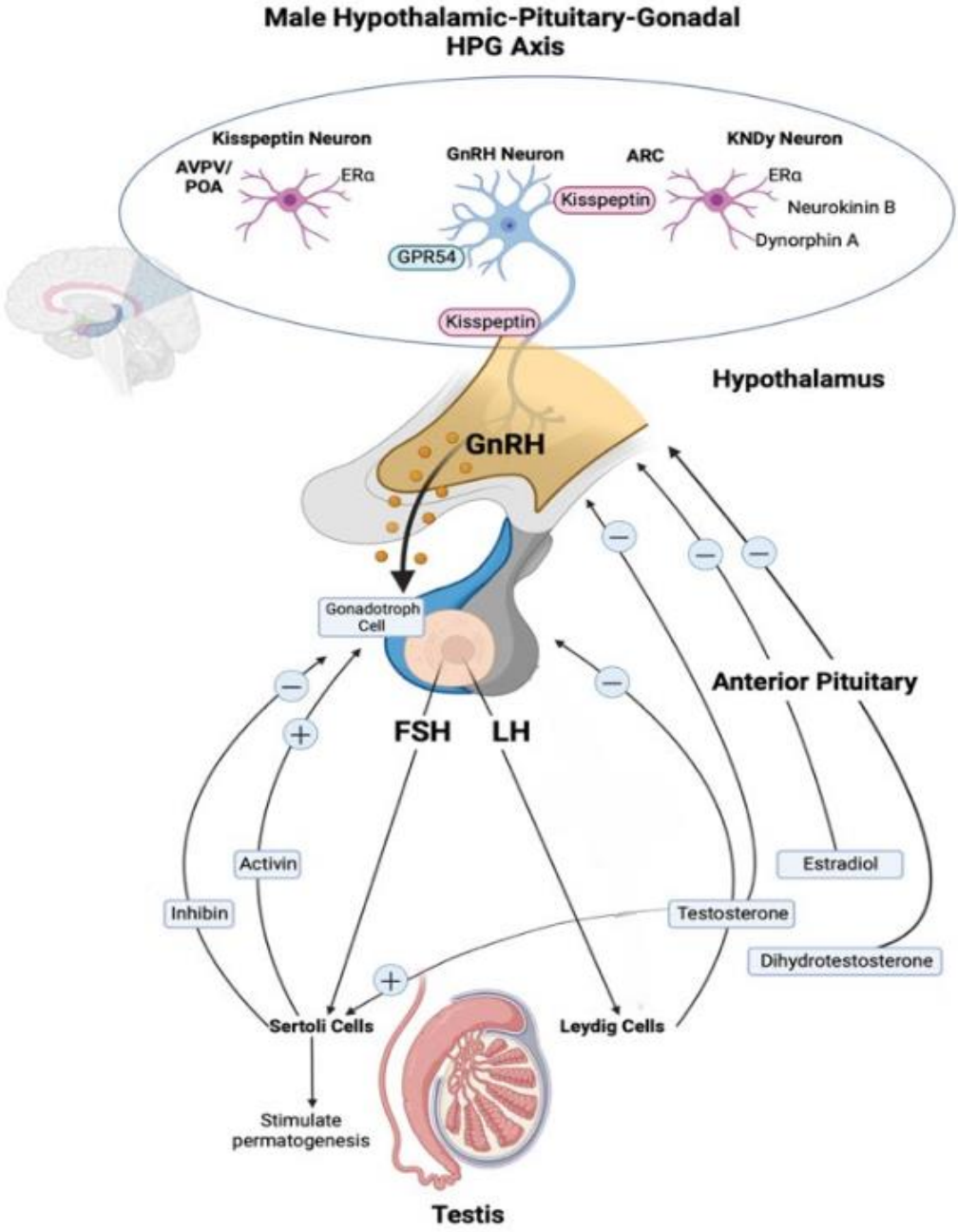


Figure 1. ***The regulation of the hypothalamus-pituitary-gonadal axis (HPG) in males (20).***

1.2.2. The spermatogenesis

Spermatogenesis is regulated by hormonal signals from the HPG axis and local interactions between germ cells, Sertoli cells and Leydig cells. Sertoli cells provide mainly structural and metabolic support to developing germ cells, while Leydig cells produce testosterone, which is responsible for the progression of spermatogenesis (21).

Spermatogenesis can be divided into three phases in the seminiferous tubule of the testis, which takes approximately 35 days in male mice (22). The initial phase begins with mitotic divisions of spermatogonial stem cells to produce primary spermatocytes, providing a continuous pool of germ cells for sperm production. In the meiotic phase, primary spermatocytes undergo meiosis, resulting in haploid spermatids. The final stage of spermatogenesis involves the transformation of round spermatids into mature spermatozoa (23). Mature spermatozoa are then transported to the epididymis, where they undergo further functional and structural modifications necessary for motility and fertilization competence (24).

1.3. Hypothalamus-pituitary-adrenal (HPA) axis

In response to acute stress, the sympathetic nervous system (SNS) is rapidly activated through inputs from the cortex and limbic system. These signals stimulate the adrenal medulla, leading to the prompt release of catecholamines (adrenaline and noradrenaline). This “fight or flight” reaction prepares the body by raising blood glucose levels through glycolysis and gluconeogenesis, while also increasing heart rate, respiratory rate and blood pressure (25).

The HPA axis is triggered in parallel, beginning with the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (PVN). Hypophysiotropic CRH neurons in the PVN project to the external layer of the median eminence, where CRH and co-stored peptides are released into the hypophyseal portal circulation (26). These hormones stimulate the secretion of adrenocorticotropic hormone (ACTH) from the adrenocorticotropic cells of the anterior pituitary gland.

ACTH then travels via systemic circulation to act on the adrenal cortex, where it stimulates the synthesis of glucocorticoids, cortisol in humans and corticosterone in rodents (27). Elevated glucocorticoids serve a key adaptive mechanism, mobilizing energy stores while modulating inflammation and immune response (28). Furthermore, glucocorticoids exert negative feedback via glucocorticoid receptors (GRs) located in the hypothalamus, pituitary and hippocampus suppressing further CRH and ACTH secretion to restore homeostasis (29). However, during chronic stress, the increased stress intensity and duration induce hyperactivation of the HPA axis, leading to persistently elevated plasma corticosterone levels and impairing feedback regulation (30). The elevated

corticosterone levels contribute to various pathophysiological outcomes, including metabolic dysfunction, immune dysregulation and psychological disorders (31).

1.4. Chronic stress

Stress responses are vital for adaptation to changing external or internal environments. However, excessive or prolonged stressors can overwhelm the body's defense mechanisms. Depending on their duration, stressors are classified as acute or chronic (spanning weeks to months) (32). Chronic stress often results in maladaptive processes, including passive, avoidant behaviours and a range of psychological and physiological changes. Selye's General Adaptation Syndrome identifies the alarm phase as the first stage of stress, involving activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis (33). Over time, the body's metabolic reserves become depleted. This leads to exhaustion and the emergence of pathophysiological conditions, such as metabolic syndrome and depression (34).

1.5. The impact of the gut microbiome and host physiology

Recent studies have highlighted the complex interplay between the gut microbiome and the host. The gut microbiome contributes to a range of physiological processes, including the maturation of the immune system, maintenance of intestinal homeostasis, protection against pathogens and regulation of energy homeostasis (35-37). Studies indicate that the composition of the microbiota remains relatively stable in healthy individuals (38). However, it is highly responsive to various factors, including environmental changes, diet, ageing and diseases. External influences such as stress and the use of drugs, especially antibiotics, can also disrupt microbial stability (39). This indicates that the gut microbiome is not merely a passive inhabitant but an active participant in the physiological processes of the host in health and disease (40). For instance, studies in mice have demonstrated that a shift from a high-fat diet to a low-fat/high-fibre diet results in notable changes in gut microbiome composition within just 24 hours (41).

The gut microbiota produces metabolites, including hormones, short-chain fatty acids (SCFAs), secondary bile acids, vitamins, choline, and amino acid derivatives, which extensively interact with the host's metabolism, immune response and even behavior (42).

Among SCFAs, acetic acid, propionic acid and butyric acid are major products of microbial fermentation of non-digestible carbohydrates in the intestine. SCFAs contribute directly to intestinal homeostasis by strengthening the epithelial barrier, enhancing mucus secretion and regulating the expression of tight junction proteins (43). Indirectly, they influence host metabolic functions by serving as an energy source for the intestinal epithelial cells and regulating glucose and lipid metabolism (44). Additionally, SCFAs exert immunomodulatory effects by promoting the differentiation of regulatory T cells and suppressing pro-inflammatory cytokine production (45). However, the production of SCFAs rely on a complex interaction with the host, as their levels depend on factors such as the available substrate, gut microbiota composition and intestinal transit time (46).

Advancements in sequencing technologies have enabled the identification of microbiome-produced metabolites and their strong correlation with gut microbiome composition, providing insight into health and disease associations. For instance, imidazole propionate, a histidine-derived metabolite, is elevated in individuals with low microbiome diversity and is linked to severe obesity (47). On the other hand, indolepropionic acid (IPA), a tryptophan-derived metabolite, is produced by gut microbiota and regulates gastrointestinal barrier function. Higher IPA levels, which are associated with a more diverse microbiome, serve as a predictive marker for the development of type 2 diabetes (48). Although gut microbiota and their metabolites are known to affect metabolism and immunity, their specific roles in regulating reproductive function and stress-related behavior are still under investigation. Clarifying these mechanisms could reveal new therapeutic strategies targeting microbiome-host interactions.

1.5.1. Gut microbiome-brain axis

The interactions between the gut and the central nervous system rely on bidirectional communication mediated by neuronal, endocrine or immunological pathways. For instance, proper regulation of the hypothalamic-pituitary-adrenal (HPA) axis requires the presence of a functional gut microbiome (49). Studies have shown that the timing of microbial colonization, particularly during early life and the perinatal period, is critical to the normal development and activity of the HPA axis. Germ-free (GF) mice, lacking gut microbiota, exhibited altered HPA axis regulation, leading to elevated levels of

adrenocorticotropic hormone (ACTH) and corticosterone (50). Later in life, attempts at microbial colonisation fail to fully restore the regulation of the HPA axis. However, if GF mice were exposed to microbiota during early development stages, normal HPA axis activity could be restored (51). Furthermore, chronic stress induces highly elevated corticosterone levels, which alter the diversity of gut microbiota, particularly increasing the abundance of Proteobacteria and reducing *Akkermansia muciniphilia,* leading to impaired gut permeability (52). Altered microbiome composition and diversity have been reported in the context of various neurological and psychiatric disorders, such as anxiety, irritability, autism and certain forms of mental illness (53).

Besides hormonal regulation, neural pathways play a pivotal role in gut-brain communication. The vagus nerve, a key component of the parasympathetic nervous system, facilitates communication between the enteric nervous system (ENS) and the central nervous system (CNS). It senses microbiota metabolites through its afferents and transmits this information to the central arm of the autonomic nervous system, as well as influencing local intestinal permeability and immune responses (54).

Certain gut bacteria are capable of synthesising neurotransmitters, such as glutamate, gamma-aminobutyric acid (GABA), acetylcholine, dopamine and serotonin (55). In addition to neurotransmitters produced by gut microbes, bioactive metabolites, such as SCFAs play a significant role in gut-brain interactions. SCFAs regulate immune function via fatty acid receptors (FFARS) or G-protein coupled receptor 109A (GPR109A) by activating signalling pathways such as mitogen-activated protein kinases (MAPKS, nuclear factor-κB (NF-κB) (56). SCFAs may cross the blood-brain barrier (BBB) via monocarboxylate transporters, where they modulate neuroinflammation by affecting microglia morphology, thereby potentially influencing emotion and the pathophysiology of mental disorders (57). Additionally, SCFAs directly affect neutrophils by regulating the production of inflammatory cytokines and interleukins, further contributing to immune homeostasis (58).

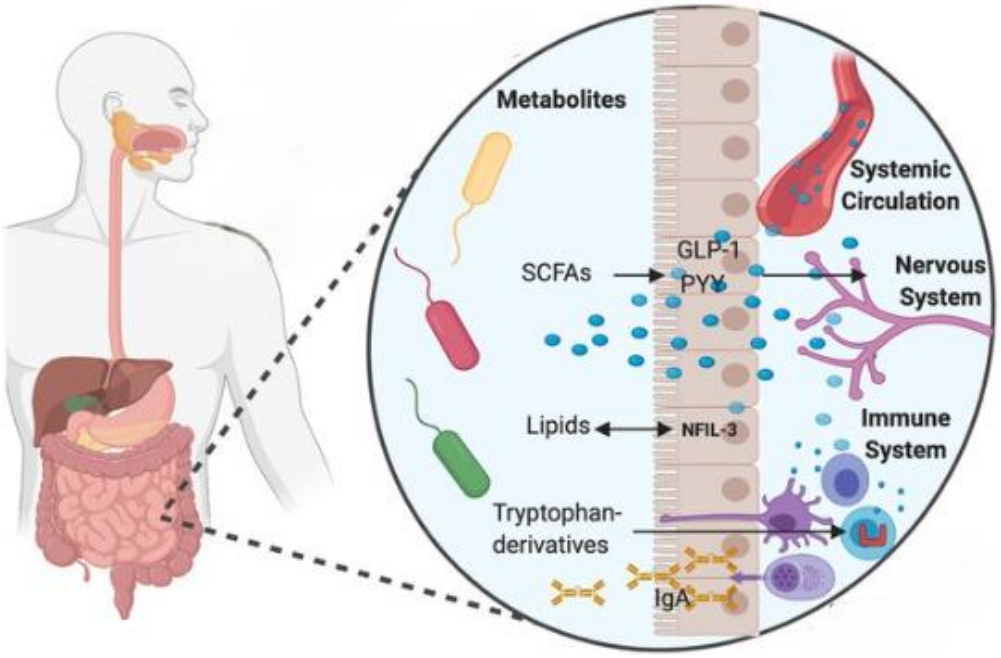


Figure 2. ***Gut-derived metabolites in host interactions (59)***

1.5.2. Gut microbiome-testis axis

Emerging evidence suggests that the gut microbiome extends its influence beyond metabolic and immune regulation and plays a key role in male reproductive health. This bidirectional communication involves complex interactions between gut-derived metabolites, hormonal regulation, immunomodulation and metabolic interactions.

Testes are immune-privileged organs, protecting male haploid germ cells. Due to their delayed production, these germ cells are particularly vulnerable to environmental challenges. To protect them, the seminiferous epithelium forms one of the body's most robust epithelial barriers, the blood-testis-barrier (BTB), which isolates this structure from the immune environment (60). Studies have shown that in the testes of germ-free (GF) mice, the BTB development was delayed, and its barrier function was reduced due to the downregulated zonula occludens-2 (ZO-2) and E-Cadherin expression. However, the GF mice that were administered with *Clostridium tyobutyricum* (CBUT), which secretes high levels of butyrate, restored the integrity of BTB and normalized the levels of cell adhesion proteins (61). Reproductive functions require high energy and robust immune regulation, both of which rely on essential metabolites provided by the gut microbiome. These include vitamins, nutrients and minerals, as Vitamin A, Folic acid,

Calcium and Vitamin K, that support immune and reproductive regulation in the testes (62).

However, the balance of the gut microbiome is critical for maintaining host and reproductive health. Dysbiosis, characterized by an imbalance in the gut microbiome composition, disrupts testicular homeostasis by promoting systemic inflammation, increasing intestinal permeability and allowing bacterial translocation that releases pro-inflammatory cytokines into systemic circulation (63). Dysbiosis also alters metabolic production, often generating harmful metabolites (64). Substantial evidence links metabolic disorders such as high-fat diet (HFD) -induced obesity and Type 2 Diabetes (T2D) to dysbiosis, which can negatively affect sperm production and motility (65, 66). For instance, in HFD mice, an imbalance in the gut microbial population was observed, characterized by an increased abundance of *Bacteroides* and *Prevotella* genera (67). Notably, the elevated levels of these genera were positively correlated with increased serum endotoxin levels, while showing a strong negative correlation with sperm motility. Ding et al. (68) demonstrated that the transplantation of the HFD-induced dysbiotic microbiome into normal fed (ND) mice resulted in impaired sperm production. Furthermore, their findings indicated that the dysbiotic microbiome induced by HFD contributed to metabolomic endotoxemia, which subsequently led to epididymal inflammation (68). This was observed by upregulated gene expression of pro-inflammatory cytokines in the epididymis, highlighting the interplay between gut microbiota, systemic inflammation and male reproductive health.

Castration has been shown to alter the composition of the gut microbiome, notably affecting beta diversity and increasing the *Firmicutes/Bacteroides* ratio (69). Interestingly, these changes shift the gut microbiome profile similar to that of females. However, reintroducing dihydrotestosterone (DHT) reversed these alterations, restoring the microbiome to its pre-castration state (70). In parallel, castration also improved glucose tolerance and insulin sensitivity in male mice. However, these effects on glucose metabolism were abolished when the gut microbiota was depleted by antibiotic treatment. These results indicated that androgen effects on glucose metabolism are highly dependent on the gut microbiome (70). Furthermore, the gut microbiota is implicated in the enterohepatic circulation of sex steroids. Bacterial enzymes, particularly β-glucuronidase, play a pivotal role in the deconjugation of steroid glucuronides, including testosterone

glucuronide (71). Colldén et al. reported that in GF mice, the deconjugation of testosterone glucuronide in the intestinal tract was negligible, whereas normal mice exhibited high concentrations of androgen glucuronides, highlighting the essential role of gut microbiota in steroid metabolism (72). Nevertheless, the mechanisms through which the gut microbiota impacts males' reproductive health are still unclear. Further research is needed to determine these pathways and their importance for reproductive outcomes.

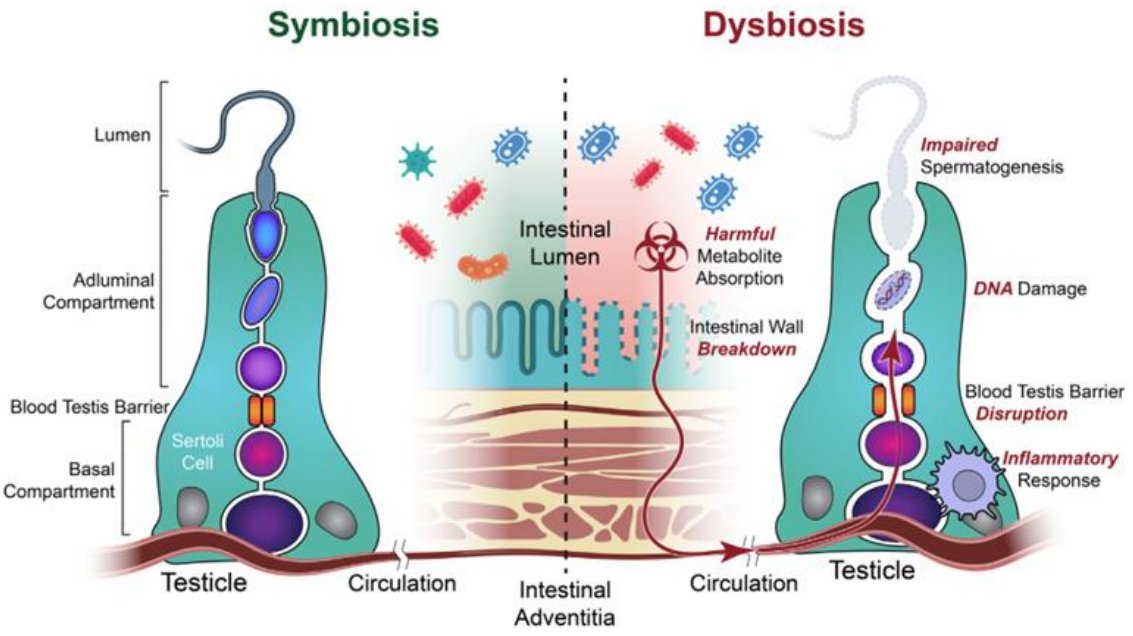


Figure 3. ***Gut-testis axis (65)***

1.6. Influencing the gut microbiome

The gut microbiome is a dynamic and constantly changing community, with its impact on the host largely influenced by the specific bacterial species colonizing the gastrointestinal tract. While the gut microbiota maintains a relatively stable composition under normal conditions, it is highly susceptible to modulation by various internal and external factors (73). In recent years, microbiota-based therapeutic strategies have gained increasing attention. Among these, probiotics, prebiotics and synbiotics have emerged as targeted dietary interventions aimed at restoring or enhancing beneficial microbial communities in the gastrointestinal tract (74).

1.6.1. Role of probiotics in health and disease

Probiotics are defined as live, non-pathogenic microorganisms that can positively influence the composition of the gut microbiome while providing health benefits to the host (75). They are primarily derived from fermented foods and dietary supplements. The most commonly used probiotic genera include *Lactobacillus* and *Bifidobacterium* (76).

A key characteristic of probiotics is their viability and ability to colonize in the gut, both of which are essential for exerting their beneficial effects. For effective oral administration, a minimum concentration of 10^6 CFU/ml is recommended to ensure sufficient delivery to the colon (77). However, gastrointestinal transit presents several challenges to probiotic viability, such as exposure to gastric acid, digestive enzymes, bile acids and colonization resistance by commensal bacteria (78). Therefore, the efficacy of probiotic colonisation depends on competition for resources and host-derived factors, including the production of antimicrobial peptides, maintenance of the epithelial barrier, and modulation of bile acid concentration through interaction with the host (79).

Adhesion to intestinal epithelial cells is another crucial requirement for successful colonisation. Probiotic strains with higher cell surface hydrophobicity have better adhesion capacity, which enhances colonisation efficiency (80). In addition, probiotic effects can be amplified through the cross-feeding mechanism, where one microbial species provides nutrients or metabolic byproducts that support the growth of others. For instance, short-chain fatty acids (SCFAs), produced by *Bifidobacterium* and *Lactobacillus* genera not only serve as energy sources for the epithelial cells of the gut but also create an environment that favors the proliferation of probiotics and beneficial microbes, while inhibiting pathogenic bacteria (81).

The combined use of prebiotics with probiotics, known as synbiotics, further improves the viability and colonization of probiotics. Prebiotics are non-digestible, fermentable food ingredients, such as inulin, fructo-oligosaccharides (FOS), and galacto-oligosaccharides (GOS), which act as metabolic fuel to promote the proliferation of probiotics and beneficial bacterial communities (82).

One of the primary mechanisms by which probiotics exert their beneficial effects is by restoring the microbial balance. While widely used for managing gastrointestinal disorders, the application of probiotics extends far beyond this area. Due to their anti-inflammatory properties, probiotics play a role in enhancing mucosal barrier function and

modulating immune responses (83). There is substantial evidence that probiotics influence the systemic immune response by promoting phagocytosis, increasing IgA secretion, modifying T-cell responses, enhancing Th1-mediated responses and attenuating Th2-driven responses (84). These immunomodulatory effects highlight their potential in the treatment of many diseases, including allergies, urogenital infections and other immune-related disorders (85). Although probiotics are widely recognized for supporting immune and metabolic health, their impact on male reproduction is not well understood. The effects of probiotics on reproductive function and stress responses remain unclear and the underlying mechanisms are not yet fully understood.

Recently, it has been recognized that probiotic bacteria produce a plethora of biologically active compounds known as postbiotics. Postbiotics are bacterial metabolic (by)products (metabolites) playing a crucial role in mediating the health-promoting effects of probiotics. On the other hand, the term parabiotics refers to inactivated, non-viable probiotics. Parabiotics include dead bacterial cells, cell lysates, cell fractions, and cell wall components that have health benefits and are stable and safe for use (86).

1.6.2. Impact of **Lactobacilli** *on host health and physiology*

Lactobacillus is the largest genus within the lactic acid bacteria group, comprising over 160 species. Many of these species are integral to the production of fermented foods such as dairy products, sourdough, meat and vegetables and are widely used as probiotics (87). Despite their importance, *Lactobacilli* represent only a minor part of the human gut microbiome. Taxonomically, *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Lactobacilli*. These bacteria are Gram-positive, catalase-negative, non-spore-forming, rod-shaped, and anaerobic (88).

One of the key functions of *Lactobacillus* species is their ability to produce lactic acid through the fermentation of carbohydrates. This acidification of the gut environment inhibits the growth of pathogenic bacteria and supports a balanced gut microbiome (89). In addition, *Lactobacilli* produce various bioactive compounds, collectively referred to as postbiotics, which influence host physiology. These metabolites include organic acids, amino acids, dipeptides, short-chain fatty acids (SCFAs), bacteriocins, conjugated linoleic acid, exopolysaccharides, and various vitamins (90).

Certain *Lactobacillus* strains exhibit anti-inflammatory and antioxidant properties. For instance, *Lactobacillus* NK318.1 has been shown to maintain adipose tissue homeostasis in obesity by modulating the differentiation of anti-inflammatory gut macrophages via STAT3 signalling (91). *Lactobacilli* may exert immunomodulatory effects through mechanisms such as binding directly to host cell surface receptors, including C-type lectin receptors (CLRs) or Toll-like receptors (TLRs) (92).

Moreover, *Lactobacillus* plays a pivotal role in gut mucosal immune and barrier function. A study by Miranda et al. demonstrated that a high-salt diet (HSD), a key mediator of experimental colitis, reduced the abundance of Lactobacillus spp., leading to diminished levels of the SCFA butyrate in the gut. However, supplementation with *Lactobacillus reuteri* restored butyrate levels, increased the thickness of the colon mucus layer, and enhanced protection against pathogens, thereby contributing to gut homeostasis (93).

Although Lactobacillus metabolites are under extensive investigation (94, 95), *Lactobacillus*-induced metabolomic changes in the host remain largely unexplored. In particular, certain species of *Lactobacillus* have been linked to reproductive health. For instance, in females, *Lactobacilli* in the vaginal microbiota prevent infections, clear pathogenic microbes and modulate inflammation (96). However, the impact of the gut microbiome, particularly *Lactobacilli*, on reproductive health is far less understood. Further research is essential to elucidate these complex host-microbiome interactions.

1.6.3. Impact of antibiotics on gut microbiome and on the host

Antibiotics are antimicrobial agents designed to inhibit bacterial growth or eliminate bacteria as the primary pharmacological defense against infections. Although they are effective against pathogenic bacteria, their administration can also have harmful consequences on the gut microbiome (97). Antibiotics disrupt the balance of commensal bacteria, significantly reducing microbiome abundance and diversity. Following antibiotic exposure, key phyla such as the *Firmicutes, Bacteroidetes* and *Verrucomicrobia* were largely replaced by *Proteobacteria* in mouse fecal samples (98). Besides microbial depletion, antibiotic treatment can impact gut barrier integrity and alter the gut metabolome, leading to immune dysregulation and an increased susceptibility to disease (99, 100).

For instance, early in life, antibiotic use has been shown to alter gut microbiota composition, affecting microbial lipid metabolism and downregulating gene expression of intestinal Th17 and Treg cells. These effects accelerated the development of Type 1 diabetes in non-obese-diabetic (NON) mice, underscoring the critical role of microbiome health in metabolic regulation (101). The impacts of antibiotics on the gut microbiota are influenced by key variables such as the type of antibiotic, dosage and timing of administration.

Antibiotics employ diverse mechanisms to inhibit or eliminate bacteria, significantly affecting the composition and diversity of the gut microbiome. For instance, it has been shown that the prolonged use of therapeutic doses of ampicillin in mouse models induced dysbiosis and enhanced NF-κβ activation in the colon, contributing to the inflammatory process (102).

Broad-spectrum antibiotic cocktails, such as combinations of vancomycin, neomycin, ampicillin and metronidazole, can induce substantial alterations in the diversity of the gut microbiome. This treatment also decreased effector T cell populations and elevated regulatory T cell (Treg) populations in the gut (103). Recovery from such treatment often takes several days and microbiome composition may remain altered for weeks or longer (104). Broad-spectrum antibiotics are effective against both Gram-negative and Gram-positive bacteria, whereas mono-antibiotic treatments are specifically designed to target either Gram-negative or Gram-positive species (105). Non-systemic antibiotics, such as rifaximin, are specifically designed to target pathogenic gut bacteria, while preserving beneficial gut microbiota (106). This selective action makes it valuable for treating localized infections, where maintaining the integrity of the gut microbiome is essential. Although antibiotics are known to disrupt gut microbial composition, the extent to which different antibiotic treatments affect male reproductive physiology, behavior and hormonal balance has not been systematically investigated.

2. Aims

The gut microbiota plays a crucial role in modulating host physiology, including metabolism, immune function, behavior and reproductive health. Recent research has highlighted the bidirectional relationship between gut microbiota composition and host stress responses, suggesting that alterations in the gut microbiome can influence behavioral outcomes and reproductive parameters. However, the underlying mechanisms, as well as the specific contributions of microbiota composition and microbial metabolites, remain unclear.

The central aim of this thesis is to investigate how modulation of the gut microbiota affects stress behavior and reproductive physiology in male mice. In particular, the thesis focuses on the effects of antibiotic-induced microbiota depletion and probiotic *L. plantarum* SNI3 treatment. The following specific aims were addressed:

1. To establish and compare the effects of a broad-spectrum antibiotic mix and gut-selective antibiotic on the colon microbiome and on the behavior of host mice.
2. To characterize how the probiotic *Lactiplantibacillus plantarum* SNI3 affects behavior, HPA axis activity, reproductive physiology and hormonal responses.
3. To provide evidence that the physiological effects of *Lactiplantibacillus plantarum* SNI3 are mediated by gut microbiota and can be transferred via fecal microbiota transplantation (FMT).
4. To find specific microbial metabolites, which mediate the beneficial effects of *L. plantarum* SNI3 on the host.

3. Materials and methods

3.1. Experimental animals

Adult (age 150-200 days) male C57BL/6J mice were used. Animals were bred at the Specific Pathogen Free (SPF) level in the animal facility of the Institute of Experimental Medicine, Budapest Hungary. Experiments were carried out at the Minimal Disease (MD) level of the same facility under standard controlled conditions with a 12 h light/dark cycle (lights on from 6 a.m. to 6 p.m.) at 21–22 °C and 65% humidity. Animals received standard pelleted rodent chow (VRF1, Special Diets Services (SDS), Witham, Essex, UK) containing 19.1 g% protein, 55.3 g% carbohydrate and 4.8 g% fat. Chow was provided *ad libitum*. Body weight was measured weekly and at the end of the experiment. Whole body composition of the mice was measured before starting *L. plantarum* SNI3 treatment and at the end of the experiment using EchoMRI™ (Whole body composition analyzer; E26-233RM, Echo Medical Systems, Houston, TX, USA).

For testing the sexual behavior, 130-day-old female C57BL/6J mice in the estrus cycle were used.

Experiments in this study were approved by the Animal Care and Use Committee of the Institute of Experimental Medicine, Hungarian Research Network (permit number: PEI/001/29-4/2013). All procedures complied with the ARRIVE guidelines and were performed in accordance with the guidelines of the European Communities Council Directive (86/609 EEC), EU Directive (2010/63/EU) and the Hungarian Act of Animal Care and Experimentation (1998; XXVIII, Sect. 243/1998).

3.2. *Lactiplantibacillus plantarum* SNI3

L. plantarum SNI3, used in these experiments, was isolated from feces of a breeding boar in Sajóbábony, Hungary by J. Kukolya (107). The strain has been deposited in the National Collection of Agricultural and Industrial Microorganisms, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences (NCAIM (P) B 001482).

L. plantarum SNI3 cultures were grown in sterile deMan, Rogosa and Sharpe (MRS) broth (VWR Chemicals) anaerobically at 37 ^{o}C for two days to reach bacterial density of 10^8 CFU/mL. Cultures (50 mL) were then centrifuged, the pellet was washed 2x in autoclaved tap water and re-suspended in 100 ml autoclaved tap water.

L. plantarum SNI3-treated mice received bacterial suspension as the sole drinking solution for four weeks. Based on the fluid consumption data (10ml/day/animal), the average *L. plantarum* SNI3 consumption was ~5x10^8 CFU/mouse/day. Control animals drank autoclaved tap water. Fluid and chow consumption were measured daily during the whole 4-week treatment. All drinking bottles were changed every second day at the beginning of the dark (active) phase.

3.2.1. Enzymatic lysis and heat inactivation of Lactiplantibacillus plantarum *SNI3*

The *L. plantarum* SNI3 was cultured as previously described. The bacteria pellet was collected by centrifuging at 14,000 rpm for 10 minutes, then resuspended and incubated in Lysozyme STET buffer at 37 ºC for 4 hours. After enzymatic lysis, the bacterial buffer was centrifuged for 10 min. at 7500 rpm to obtain the pellet. Then washed twice and resuspended in autoclaved tap water. For cell lysis, sonication was carried out in an ice-water bath at 20 kHz with one-minute on/off intervals for a total of 25 minutes. The lysed bacteria were then centrifuged for 15 min. at 4000 rpm and filtered through a 0.2 µm filter to remove large fragments. The resulting soluble supernatant was collected as the final product, aliquoted into 1ml portions and stored at -70 ºC until use.

The cultured *L. plantarum* SNI3 were transferred to a 50 ml Falcon tube, washed twice by centrifugation at 5000 rpm for 5 minutes, and then heat-inactivated in a water bath at 80 ºC for 1 hour. Following inactivation, the suspension was centrifuged again at 4500 rpm for 5 minutes and stored at -20 ºC until use.

To verify the effectiveness of enzymatic and heat inactivation, 10 µl of bacterial suspension was placed on MRS/LB agar plates and incubated overnight at 37 ºC. No bacterial growth was observed.

3.3. Chronic variable stress procedure

To reveal the effects of *L. plantarum* SNI3 on chronic stress-induced behavioral adaptations, four groups of mice were studied (n=20). Half of the mice were exposed to a chronic variable stress (CVS) paradigm, and the other half (controls) were left undisturbed. Half of the stressed and half of the control group received *L. plantarum* SNI3 to drink for 4 weeks, while the others received tap water. In the CVS paradigm, experimental mice were exposed to psychogenic stressors in a semi-random order, twice

a day for 4 weeks. Each stressor contributes to the chronic variable stress (CVS) environment, generating unpredictable conditions that prevent the mice from adapting to the stress. The following stressors were used:

- *Social Defeat*: Experimental mice (intruders) were placed into the home cage of a dominant CD1 male mouse (aggressor) for 8 minutes.
- *Water avoidance Stress (WAS):* Mice were positioned on a glass cylinder platform (8 cm diameter) in the center of a plastic tank (40 cm x 40cm x 30cm) filled with water to 1 cm below the platform's surface and left for 1 hour.
- *Forced Swum:* Mice were placed into 18 cm high and 14 cm diameter glass cylinders filled with clean tap water (23 ºC) for 5 minutes.
- *Disturbed circadian rhythm*: The natural circadian rhythm of the mice was disrupted by reversing or altering the light/dark phase.
- *Foot shock:* Mice were administered 0.5 mA electric shocks at 20-second intervals for 12 minutes, using a DC Shocker Controller (Supertech Instruments).
- *Slanted cage*: The home cage of mice was tilted to a 45º angle.
- *Soaked Bedding*: The bedding in the home cage was mixed with tap water to create a wet environment.
- *Crowding:* To induce social stress, two cages of mice were co-housed together in a restricted 10 cm x 5 cm x 10 cm plastic cage, limiting their movement area.
- *Shaking*: Mice cages were placed on an orbital shaker, with rotation speeds ranging from 30 to 50 rpm for 1 hour. The speed was adjusted randomly throughout the session.
- *Isolation*: Mice were isolated in a clean cage for 4 hours.
- *Rat feces odor*: A perforated Falcon tube containing feces from adult male rats was placed in the home cage, introducing a novel olfactory stressor.
- *Restraint*: For the procedure, 50 ml Falcon tubes were used with small holes on the side and one at the end for breathing and ventilating. Tubes were filled with paper towels from the opening of the tube and closed with their own cap to prevent mice from turning around. This setup safely immobilized the mice, creating a stressful event without causing harm. During the 1-hour period, the tubes were stabilized externally.

More detailed information about the stressors used can be found in Table 1.

***Table 1**. Chronic variable stress (CVS) protocol.*

CVS protocol		
day	***a.m.***	***p.m.***
1. day	social defeat (8 min)	foot shock (12min)
2. day	forced swim (5min)	soaked bedding + slanted cages (4h)
3. day	crowding + shaking (30min)	isolation (12h)
4. day	restraint (1h)	crowding + dark for 18h
5. day	social defeat (8 min)	overnight light (12h)
6. day	crowding + shaking (30 min)	soaked bedding + slanted cages (4h)
7. day	forced swim (5 min)	crowding +soaked bedding + slanted cages (30min)
8. day	water avoidance (1h)	overnight light (12h)
9. day	social defeat (8 min)	foot shock (12min)
10. day	isolation + rat feces odor (4h)	isolation + rat feces odour + dark for (18h)
11. day	crowding + shaking (30 min)	crowding + soaked bedding + slanted cages (30min)
12. day	forced swim (5min)	overnight light (12h)
13. day	social defeat (8 min)	restraint (1h)
14. day	rat feces odor + dark for 18h	crowding + shaking (30 min), overnight light (12h)
15. day	isolation + rat feces odor (4h)	footshock (12min)
16. day	forced swimming (5min)	crowding + soaked bedding
17. day	social defeat (8 min)	isolation + rat feces odour + dark for 18h
18. day	dark for 18h	crowding +soaked bedding + slanted cages (30min)
19. day	water avoidance (1h)	footshock (12min)
20. day	social defeat (8 min)	forced swimming (5min)
21. day	footshock (12min)	isolation + rat feces odour (4h)

22. day	water avoidance (1h)	overnight light (12h)
23. day	crowding + shaking (30 min)	isolation + rat feces odor + dark for 18h
24. day	footshock (12min)	soaked bedding + slanted cages (4h)
25. day	social defeat	forced swimming (5 min)
26. day	footshock (12min)	restraint + shaking

3.3.1. Two-hit stress protocol

This protocol is a widely used method to induce anxiety or depressive-like symptoms in experimental animals (108). The procedure began on postnatal day 1, when pups were subjected to maternal separation (MS) for 3 h per day for 12 days (early life stress, called first hit) (109). During separation, mothers were placed in individual cages, while pups were transferred to a small box kept at 30-33 °C using a heating pad. Mothers and pups were kept in separate rooms during this time. Control litters were not disturbed except for weekly bedding changes.

Pups were weaned on postnatal day 21 and housed in groups of 2-3 per cage. At 50 days of age, mice were exposed to the chronic variable stress (CVS) paradigm for a second time. The CVS protocol followed the schedule detailed in Table 1.

3.3.2. Experimental procedures for rifaximin treatment

During the chronic variable stress (CVS) protocol, half of the animals were treated with rifaximin, a non-absorbable antibiotic (300 mg/kg/body weight/day; Sigma). Rifaximin was dissolved in a 5% Hypromellose solution and administered via drinking water. Control mice received drinking water containing 5% Hypromellose as a vehicle.

3.4. Short-term antibiotic administration procedure

Mice were treated with a broad-spectrum antibiotic cocktail administered via oral gavage (0.2mL/animal). The cocktail consisted of ampicillin (1 g/L; semicilin 250 mg), gentamicin (0.5 g/L; Gentamicin Sandoz, 80mg/2ml), vancomycin (0.5 g/L; Vankocin) and metronidazole (1 g/L; Klion, 250 mg), all dissolved in sterile saline. Before each administration, mice were fasted for 6 hours.

3.5. Fecal microbiota transplantation

To establish whether gut microbiome mediates pro-reproductive effects of *L. plantarum* SNI3, fecal microbiota transplantation (FMT) was done in a separate cohort of adult male mice. The FMT procedure was done as described by Stebegg et al. (110). For donor stool preparation, ~150 mg of fecal pellets from control or *L. plantarum* SNI3-treated mice were collected into sterile Eppendorf tubes and soaked in 1 ml ice-cold saline, homogenized via vortexing for 1 min and centrifuged (500 rpm, 5 min). The bacterial suspension was then washed twice with sterile saline and the concentration was set to ~10^9 CFU/ml by measuring the OD of the solution at 600nm.

Recipient mice were treated with an antibiotic mixture (ampicillin, 1g/L; gentamycin, 0.5 g/L; vancomycin, 0.5 g/L; metronidazole, 1g/L) through oral gavage (200 µl/animal) for three consecutive days. 24h after the last antibiotic treatment, mice were transplanted with donor bacterial samples via oral gavage (200 µl/animal, 5x10^8 CFU/animal. Recipient animals received a donor bacterial solution daily for 5 consecutive days, followed by weekly administration until decapitation on day 36 post-treatment. Recipient mice were divided into two groups: one group was transplanted with microbiota from control animals (Control FMT, n=6), and the other group received microbiota from *L. plantarum* SNI3-treated mice (SNI3 FMT, n=13). An additional group of mice received antibiotic pretreatment but did not receive any microbiome (No FMT, n=5).

3.6. γ-GluGlu administration

Separate cohorts of adult male C57BL/6 mice (n=78) were used for testing the effects of γ-GluGlu. γ-GluGlu (Acros Organics) was administered orally (n=15), injected intraperitoneally (n=41) or directly given into the testis (n=22). For oral administration, γ-GluGlu (1 mg/kg bw) was delivered via oral gavage (200 µL/ mouse), daily, for 4 weeks. Controls received 200 µL autoclaved phosphate-buffered saline (PBS) via gavage. For intraperitoneal injection, γ-GluGlu was dissolved in PBS and used in the 1-100 mg/kg dose range. 300 µL/mouse γ-GluGlu solution or PBS was injected daily. For intratesticular injections, animals were anaesthetized with a ketamine/xylazine cocktail (16.6 mg/ml ketamine and 0.6 mg/mL xylazine-hydrochloride in 0.9% NaCl, 10 ml/kg body weight i.p.). An incision was made in the midline of the scrotum to expose the testes, which were carefully placed on sterile saline-moistened gauze. Each testis was injected

with γ-GluGlu, 100pg-10ng in 25µL PBS, or with PBS vehicle (25µL/testis) using a Hamilton syringe. The abdominal muscles were sutured with absorbable yarn and the skin was closed with surgical clamps. Operated animals were checked daily for signs of behavioral discomfort (changes in home cage locomotor activity, grooming, writhe, appearance of fur) (111). Mice receiving intratesticular injections were sacrificed one week after injection, blood was collected for testosterone measurement and sperm were collected from the cauda epididymis and counted as described above.

3.7. Behavior tests

3.7.1. Elevated plus maze

The elevated plus maze consisted of a platform made of dark-grey plexiglass with open arms and two closed arms (30cm in length, 7cm in width) elevated 80 cm above the ground. The walls of the closed arms were 30 cm high, while the open arms were bordered by 0.3 mm ledges for minimal edge guidance. Each mouse was placed in the central area facing an open arm and allowed to explore for 5 minutes. An entry into a compartment was defined as all four paws crossing the line separating the compartments. Video recordings were analyzed using Noldus Observer software, and the percentage of time spent in the closed arms was recorded as an indicator of anxiety-like behavior. The apparatus was cleaned with tap water and paper towels between subjects

3.7.2. Open field test

The open field was a white, non-transparent plastic box measuring 40x40x30cm. The mice were placed in the middle of the field and allowed to explore freely for 10 minutes and then analyzed by the Noldus EthoVision XT 10 program. Locomotor activity was quantified by recording the passage along the lines of an invisible 4 x 4 grid (each square 10 x 10cm), which was displayed on the video monitor. Time spent in the central area, defined as the four central squares, served as an index of anxiety-like behavior. Between each trial, the apparatus was cleaned with tap water and paper towels to prevent odor-based influences.

3.7.3. Marble burying test

The marble burying test was performed to evaluate the compulsive-like, anxiety-related behaviors. Mice were individually placed in cages containing 5cm deep bedding with 15 evenly spaced glass marbles placed on the surface. Mice were allowed to explore and interact with the marbles for 30 minutes. At the end of the test, the number of marbles buried (defined as at least two-thirds covered by bedding) was recorded. The percentage of buried marbles was calculated to quantify the extent of burying behavior, reflecting repetitive digging and defensive responses (112). Between trials, bedding was changed to ensure consistent conditions.

3.7.4. Social interaction test

A separate cohort of mice was used to assess social interactions between male mice. One day before testing, male mice were placed into individual cages and received the same *L. plantarum* SNI3 solution or water as in their group housing. For the social interaction test, C57BL/6 test animals (black fur) were placed into the cage of a CD1 male mouse (resident, white fur) for 5 min and videotaped. The appearance of escape, defense, and aggressive fighting was evaluated. Nose-to-nose, nose-to-anogenital sniffing, following and crawling were summarized as pro-social behavioral elements. The frequency and duration of each behavior element were analyzed by a Solomon coder. One CD1 mouse was used only once/day.

3.7.5. Sexual behavior

A separate cohort of mice (n=17) was used in this experiment. The estrus cycle of C57BL/6J female mice (age between 60 and 90 days) was followed by daily vaginal lavage and microscopic analysis of the smears for at least 2 consecutive cycles. Only females displaying normal 4-day cycles were included in the study. Both experimental males and females were separately habituated to a rectangular open field box (40 cm x 40 cm x 40 cm) for 30 min on two consecutive days prior to the experiment. In the test, the male mouse was placed first into the arena, allowed to freely explore for 5 minutes, and then an estrus female was introduced. All tests were performed in the first hour of the dark phase of the circadian cycle. Sexual interactions were videotaped for 30 min with a Noldus Media recorder camera. Duration and frequency of behavioral elements were analyzed by a Solomon coder.

3.8. Decapitation, autopsy and tissue collection

Animals were decapitated, trunk blood was collected, centrifuged, and serum samples were stored at -20ºC until hormone assay. The caudal part of the epididymis was dissected for sperm collection. Testes were collected and measured and normalized organ weights were calculated as mg/g body weight. The samples were then frozen in liquid N_2 for 30 min and kept at -70ºC until MALDI-MSI analysis. Colon content (approx. 200 mg) from the proximal part was collected, frozen in dry ice then kept at -70ºC until assay. Epididymis, thymus, adrenals and cecum were also dissected, measured. Normalized organ weights were calculated.

Whole brains and the whole pituitaries were also collected. The hypothalamus was dissected along the optic chiasm rostrally, the mammillary bodies caudally and the optic tract laterally. Tissues were collected on dry ice and stored at -70ºC.

3.9. Extraction and quantification of total DNA from colon content samples

Total DNA was extracted from 200 mg of colon content using the QIAamp Fast DNA Stool Mini Kit (Qiagen), following the manufacturer's protocol. The QIAamp DNA Stool Mini Kit provides silica membrane-based purification of up to 30 µg genomic, bacterial, viral and parasitic DNA from fresh or frozen human stool or other sample types with high concentrations of PCR inhibitors. The combined action of InhibitEX, a unique adsorption resin and an optimized buffer leads to the removal of PCR inhibitors. The concentration and quality of the extracted genomic DNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher).

3.10. Microbiome analysis

For microbiome analysis, DNA was extracted from the colon contents using NucleoMag DNA Microbiome Kit (Machery-Nagel, Germany). Library preparation for the V3-V4 regions of the bacterial 16S rRNA gene followed the Illumina 16S Metagenomic Sequencing Library Preparation protocol (15044223 Rev.B.), producing amplicons of ~460 bp. Libraries were dual-indexed with the Illumina Nextera XT Index Kit (FC-131-1001/2), and PCR cleanups and amplicon size selection were performed using KAPA Pure Beads, resulting in final libraries of ~ 580-630 bp. The 16S amplicon

libraries of each sample were quantified by qPCR, normalized based on amplicon sizes and pooled into a single library at equal molar quantities. The pooled library was denatured and diluted to a final concentration of 8 pM. Sequencing was carried out with MiSeq Reagent Kit v3 - 618 cycle (MS-102-3003) following the manufacturer's protocols (Illumina, Inc., San Diego, CA, USA).

Paired-end reads were demultiplexed by the integrated software of the Illumina MiSeq sequencing machine. The FastQ files were imported into the Qiime 2 pipeline (https://qiime2.org/) according to the „Atacama Soil microbiome" tutorial. Residual adapter sequences (CTGTCTCTTATACACATCT) were trimmed from the 3' end of the reads with the Cutadapt software integrated into the Qiime 2 pipeline. Quality trimming and denoising were applied using DADA2 software (113), with forward and reverse read lengths set to 299 and 249 bases, without trimming at the start of the reads.

3.10.1. Operation Taxonomic Unit (OTU) generation

Aligned sequences were taxonomically classified using the Naïve Bayesian classifier with the Greengenes reference database, clustering into operational taxonomic units (OTUs) at 99% sequence identity while excluding singletons to minimize artifacts. A phylogenetic tree was generated with FastTree (114), and alpha diversity indices were calculated in R.

3.11. Gene expression analysis

Frozen testis, hypothalamic and pituitary tissue samples were homogenized in TRI reagent for RNA extraction. Then, total mRNA was isolated from the homogenate using a Total mRNA Mini Kit (Geneaid) according to the manufacturer's instructions. To prevent genomic DNA contamination, samples were treated with DNase I (Fermentas). The concentration and purity of the extracted RNA were assessed using the NanoDrop spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-Time PCR was carried out in an ABI StepOnePlus instrument (Applied Biosystems) with Fast EvaGreen quantitative PCR master mix (Biotium) and gene-specific primers. Primers (Microsynth) were designed in our laboratory using Primer-BLAST software of

the National Center for Biotechnology Information (NCBI). Forward and reverse primers used to quantify mRNA are listed in Table 2. Gene expression levels were quantified using the 2-ΔΔCT method with the ABI StepOne Software v2.3 (Applied Biosystems). Melt curve analysis was conducted to confirm product specificity on the ABI StepOnePlus instrument (Applied Biosystems). Relative changes in gene expression were normalized against GAPDH mRNA expression.

Table 2. **Forward and reverse primers for the selected genes**

Genes	Forward primer	Reverse primer
Gapdh	TGACGTGCCGCCTGGAGAAA	AGTGTAGCCCAAGATGCCCTTCAG
Gnrh	GCACTGGTCCTATGGGTTG	CATTTGATCCACCTCCTTGCC
Fsh-β	TGGAGACTCTGGCATGATTG C	GTTGAGCAGCCTAACCTTGTG
Lh-β	GTCCTAGCATGGTCCGAGTA	AGGGCTACAGGAAAGGAGACTA
Inha	CTGTCATCAGGGCAAGTGAA CTAT	CCACATCCCGTAGGGTCAA
Inhba	CGTGGAGTTGGAGCTTTGTG	CTAGCGGAGGCGTTCTGATT
Inhbb	GGGACTTCCTCCTGGTGTTAT G	TCACCCTCGGGAACAACTTC

3.12. Serum hormone measurements

Serum testosterone was measured by ELISA (NovaTec, Immunodiagnostics GmBH, Dietzenbach, Germany) according to the manufacturer's instructions. Serum corticosterone concentration was measured by direct radioimmunoassay as previously described (115).

3.13. Sperm counting and staining

Groups of control and *L. plantarum* SNI3-treated mice were sacrificed for spermatozoa preparation. An incision was made in the scrotum to expose the left cauda of epididymis, which was then expelled and placed into 100 µl Card & Fertiup medium (CosmoBio Co, USA). The tissue was gently minced and incubated at 37ºC for 5 min. to release the spermatozoa. Solution was diluted 1:5 with saline and the number of spermatozoa was counted in a Makler chamber and on smears made on defatted, uncoated slides (116).

Additional smears were stained with aniline-eosin (5% aniline and 0.5% eosin in 4% acetic acid). Slides were scanned with the Pannoramic® MIDI II Slide Scanner. Images were analyzed with Caseviewer 2.3 software. Counting and quality analysis were performed by two independent investigators, who were blinded to treatments. Sperm smears were briefly fixed with 4% paraformaldehyde, permeabilized with 0.1% TritonX 100 then incubated with AlexaFluor568-Phalloidin (1.200, ThermoFisher) and PNA (peanut agglutinin)–FITC (1:400, Sigma-Aldrich). Slides were then counterstained with DAPI-Fluoromount-G (1:1000, Southern Biotech). Smears were scanned with the Pannoramic® MIDI II Slide Scanner. Images were analyzed with Caseviewer 2.3 software.

For computer-assisted sperm analysis (CASA Microptic SCA), samples were prepared as above and transferred into a pre-warmed counting chamber, where twenty images from each sample were obtained for evaluation of sperm count, motility and velocity.

3.14. Statistics

Data are expressed as mean ± SEM and analyzed using GraphPad Prism software (version 10.3.1; San Diego, CA, USA). A two-tailed unpaired Student's t-test was used to compare the control and *L. plantarum* SNI3-treated groups. For comparisons involving two independent factors, a two-way ANOVA followed by Sidak's multiple comparisons post hoc test was conducted to determine mean differences between groups. For FMT results and sperm counts after intraperitoneal and intratesticular γ-GluGlu injections, one-way ANOVA with Dunnett's post hoc test, followed by multiple comparisons was performed. The correlation between the abundance of different microbiota and sperm number was identified by Spearman's rank correlation coefficient method. In all cases, statistically significant results were considered as P-value, $p<0.05$.

3.15. MALDI-MSI experiments

Animals treated with *L. plantarum* SNI3 strain and showing phenotypic changes were selected for MALDI-MSI analysis. Frozen testis samples (7 control and 7 *L. plantarum* SNI3-treated) were sectioned at 12µm on a microtome (HM 355S, Micron, ThermoScientific) and mounted onto indium-tin-oxide coated glass slides. The 9-

aminoacridine (9-AA) matrix solution (10mg/ml in a 30:70 water/methanol ratio) was applied using a SunCollect^{TM} automatic sprayer. Flow rates were 10, 20, 30 and 40 µL/min for the first four layers. The remaining four layers were performed at 40 µL/min. The MALDI-MSI measurement was performed on a Bruker Solarix 7T FT-ICR-MS (Bruker Daltonik, Bremen, Germany) in negative ion mode using 100 laser shots per spot at a frequency of 1000 Hz. The MALDI-MSI data were acquired over a mass range of m/z 75-1000 with 50µm lateral resolution. After MALDI-MSI measurements, acquired data underwent spectra processing in FlexImaging v. 5.0 (Bruker Daltonics, Bremen, Germany) and SCiLS Lab v. 2019 (Bruker Daltonics, Bremen, Germany). MALDI-MSI data were normalized to the root mean square of all data points.

3.15.1 Metabolite annotation and bioinformatics analysis

MATLAB® R2014b (v.7.10.0, Mathworks, Inc., Natick, MA) was used for pre-processing of MALDI spectra as described previously (117, 118). Mass spectra underwent resampling, smoothing and baseline subtraction to decrease the data dimensionality and to remove noise-level peaks and artefacts. Peak picking was performed using an adapted version of the LIMPIC algorithm (119) with *m/z* 0.0005 minimum peak width. The signal-to-noise and intensity threshold were set to 2 and 0.01%, respectively. Isotopes were automatically identified and excluded. Peaks in the mass range of *m/z* 75 to 1000 were resolved and annotated by accurate mass matching in the Human Metabolome Database HMDB (120) and METASPACE (121). Pathway analysis was performed with MetaboAnalyst 4.0 (122) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (123). Metabolomic statistical analyses were performed using MetaboAnalyst 4.0, which offers a variety of methods commonly used in metabolomic data analyses (124). The volcano plot is a combination of fold change and t-tests. The x-axis is $\log_2$ (FC). The y-axis is log10 (p value) based on p values. The discriminative metabolites were selected by a volcano plot with fold change >2 or <0.5, $p < 0.05$. Box plots were created with GraphPad PRISM v. 5.00 (GraphPad Software, Inc, La Jolla, USA). Statistical significance testing was performed using the unpaired t-test (two-tailed).

3.15.2 Metabolic interaction network

A metabolite correlation network was created with Cytoscape (v.3.7.2) (125). Nodes represent metabolites with a node size and color corresponding to the intensity of $\log_2$ fold changes between control and *L. plantarum* SNI3 treatment. Edges represent functional correlations. Metabolites with significant differences between control and *L. plantarum* SNI3 treatment ($P<0.05$) are shown. The network was visualized using the Compound Spring Embedder layout.

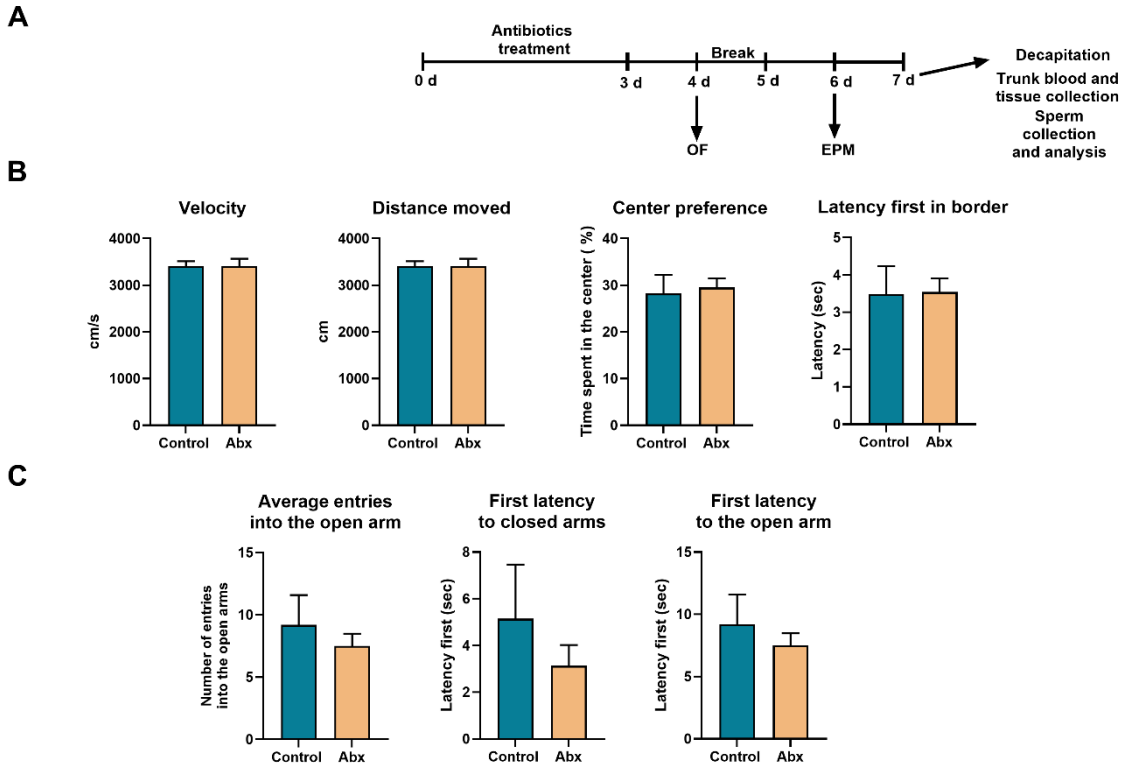
4. Results

4.1. Effects of broad-spectrum and non-absorbable (rifaximin) antibiotic treatments on behavior and gut microbial DNA level in mice

4.1.1. Impact of broad-spectrum antibiotics mix on mouse behavior

A broad-spectrum antibiotic cocktail comprising ampicillin, gentamicin, vancomycin and metronidazole was employed to evaluate the influence of gut microbiome modulation on behavioral activity. Mice received the antibiotic treatment (Abx) for 3 consecutive days followed by a 1-day initial break. Open field (OF) and elevated plus maze (EPM) tests were performed on experimental mice on days 4 and 6, respectively as shown in *Fig. 4 (A)*.

The open-field parameters showed no significant differences between the groups (Fig. 4B). Furthermore, the broad-spectrum antibiotic cocktail did not affect anxiety-like behavior, as the average entries and latencies into the open or closed and open arms remained unchanged between the groups (Fig. 4C).



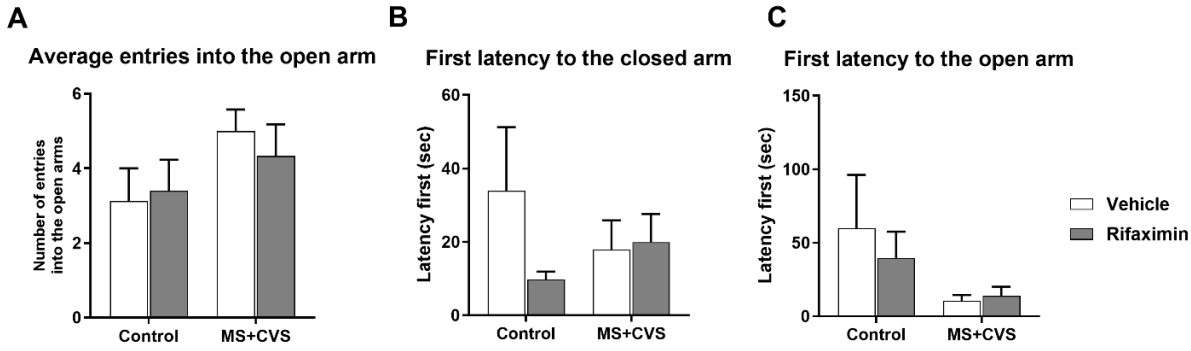
<u>Figure 4.</u> Effects of broad-spectrum antibiotics treatment on open field and elevated plus maze parameters

Schematic representation of the experimental design (A) Open field test results: velocity, distance moved, center preference and latency first in border (B). Elevated Plus Maze parameters: open arms preference, average entries into the open arm, first latency to closed or open arms (C). All data are expressed Mean±SEM values and were analyzed using an unpaired two-tailed Student t-test (n=14) (not published).

4.1.2. The impact of non-absorbable antibiotic rifaximin on anxiety-like behavior in mice exposed to chronic stress

To investigate whether modulation of gut microbiota by non-systemic antibiotics affects stress-related behavior, the non-absorbable antibiotic rifaximin was used. The two-hit stress protocol is designed to induce anxiety and depressive-like symptoms in experimental animals. This procedure began on postnatal day 1, when pups were subjected to early life stress through maternal separation (MS). This initial stressor was followed by chronic variable stress (CVS) on postnatal day 21. Early-life stress is known to significantly impact the gut microbiota composition, which plays a crucial role in behavioral outcomes. To evaluate whether modulation of the gut microbiota by non-systemic antibiotics affects stress-related behavior, the selective non-absorbable antibiotic rifaximin was administered throughout the experimental period.

Behavioral assessment using the elevated plus maze (EPM) demonstrated that rifaximin treatment had no significant impact on anxiety-like behavior, neither in mice exposed to the two-hit stress protocol nor under a non-stressed control condition. Specifically, the average entries into the open arms did not differ between rifaximin-treated and untreated groups, regardless of stress exposure (Fig. 5A). Similarly, the first latency to enter the closed arms, as an indicator of anxiety-like behavior, showed a decreasing tendency in MS+CVS mice and in rifaximin treated controls, however, these differences were not significant (Fig. 5B). The first latency to enter the open arms, parameter less associated with anxiety also remained unchanged between groups (Fig. 5C). This suggests that the non-absorbable antibiotic is insufficient to influence anxiety-related behavior.



<u>Figure 5.</u> No significant changes in anxiety-like parameters following rifaximin treatment

Average entries into the open arms (A) First latency to the closed arms (B) and first latency to the open arms (C). All data are presented as Mean ± SEM and analyzed by two-way ANOVA followed by Sidak's multiple comparison test (n=9-14 per group) (126).

4.1.3. Differential effects of broad-spectrum antibiotics and rifaximin treatment on total microbial DNA content of the colon

To investigate how different antibiotics affect gut microbial load, we measured the total bacterial DNA content from colon content samples after broad-spectrum antibiotic mix or rifaximin administration. Mice treated with a broad-spectrum antibiotic cocktail (Abx) for 3 consecutive days showed a significant reduction in total DNA level in colon content samples compared to control animals (Fig. 6A), indicating a substantial depletion of gut microbiota. In contrast, rifaximin treatment did not result in any significant change in total DNA content compared to control mice (Fig. 6B). These results suggest that broad-spectrum antibiotics strongly reduce the overall microbial DNA level in the colon, whereas rifaximin, which affects mostly pathogenic bacteria does not cause significant changes in the total bacterial load.

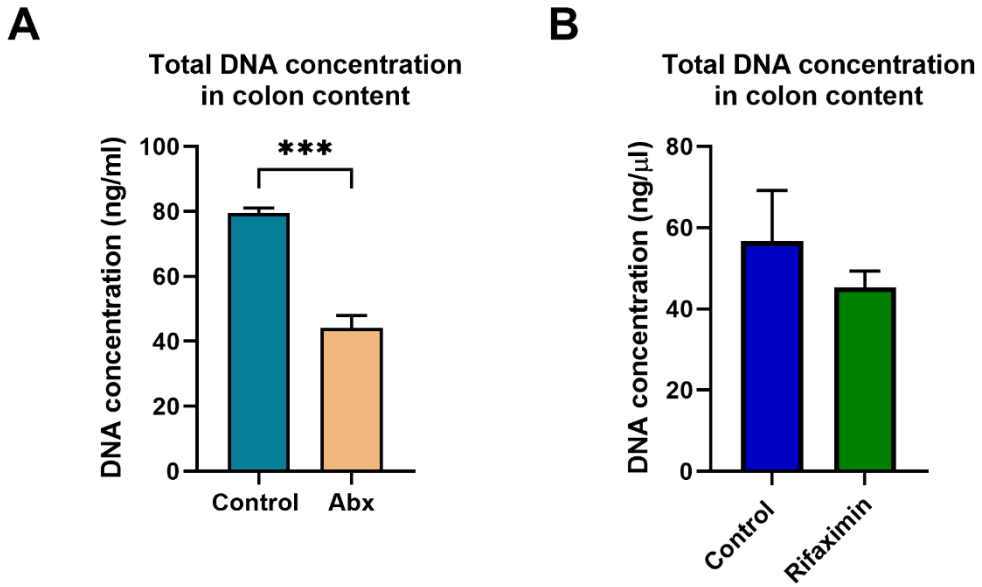


Figure 6. **Impact of different antibiotics treatment on total DNA content in colon samples**

*Total bacterial DNA concentration in colon content (A-B) All graphs are presented as Mean ± SEM. Statistical significance is indicated as ***p<0.001 vs control, determined by unpaired, two-tailed Student t-test (n=14) (not published).*

4.2. Effects of the probiotic *Lactiplantibacillus plantarum* SNI3

Adult male C57Bl6 mice were administered with a suspension of *Lactiplantibacillus plantarum* SNI3 ($5x10^7$CFU/ml) in autoclaved tap water for 4 weeks, control animals received autoclaved tap water. The bacterial suspension was well-tolerated, with no observed changes in fecal color or consistency, nor any signs of gastrointestinal discomfort or sickness behavior in the treated animals.

4.2.1. Impact of L. plantarum *SNI3 treatment on behavior*

4.2.1.1. Open field test

The open field test was performed to assess whether *L. plantarum* SNI3 treatment influences locomotor activity, general exploratory and potential anxiety-like behavior. No significant differences were observed in total distance moved or velocity during the open field test, between *L. plantarum* SNI3-treated and control mice (Fig. 7A-B), indicating that probiotic treatment did not affect overall activity levels. In addition, analysis of center preference revealed that *L. plantarum* SNI3-treated animals spent slightly more time in center zone compared to controls (Fig. 7C), but it was not significant.

Furthermore, *L. plantarum* SNI3 had no significant effect on the latency to first enter the periphery zone (Fig. 7D), while the frequency of peripheral zone entries was slightly reduced in *L. plantarum* SNI3-treated mice, without any significant differences (Fig. 7E).

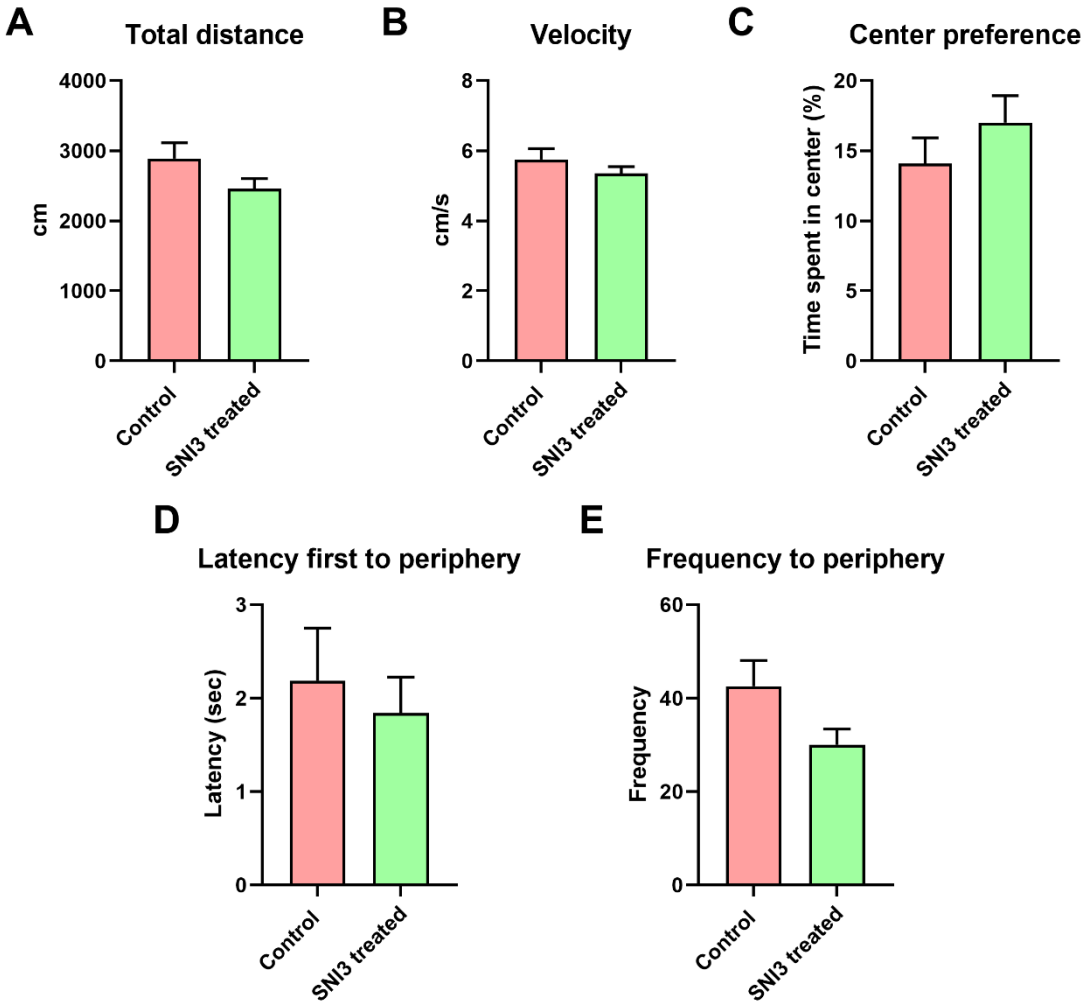


Figure 7. **Effects of L. plantarum *SNI3 on open field test behavior parameters***

Total distance during open field test (A), velocity (B), center preference (C), first latency to periphery (D) and frequency to the periphery (E). Data are present Mean ± SEM values and were analyzed by unpaired, two-tailed Student t-test (n=24) (not published).

4.2.1.2. Marble burying test

To examine the effect of *L. plantarum* SNI3 on obsessive/compulsive-like behavior and anxiety-related behavior, the marble burying test was performed. The *L. plantarum* SNI3-treated mice buried significantly fewer marbles than the control mice (Fig. 8). This reduction suggests decreased repetitive or defensive digging behaviors following

probiotic treatment. These findings are consistent with an anti-compulsive effect of *L. plantarum* SNI3 treatment.

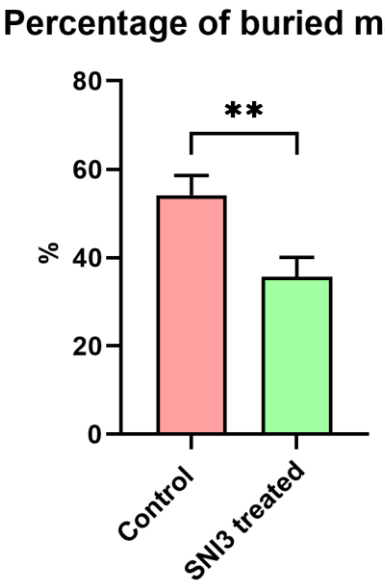


Figure 8. **Effects of L. plantarum SNI3 on the marble burying test**

*Percentage of buried marbles. Data were analyzed by unpaired, two-tailed Student t-test (n=24). Mean ± SEM values **p<0.01 vs control (not published).*

4.2.1.3. Social interaction test

To investigate whether *L. plantarum* SNI3 treatment affects social behavior between males, a social interaction test was performed. The results show that the pro-social behavior elements, which include nose-to-nose interactions, anogenital sniffing, and following were more frequent in *L. plantarum* SNI3-treated mice compared to controls (Fig. 9). Conversely, aggressive, defensive and escape behaviors were rarely observed in the treated groups, highlighting a shift toward more affiliative social interactions.

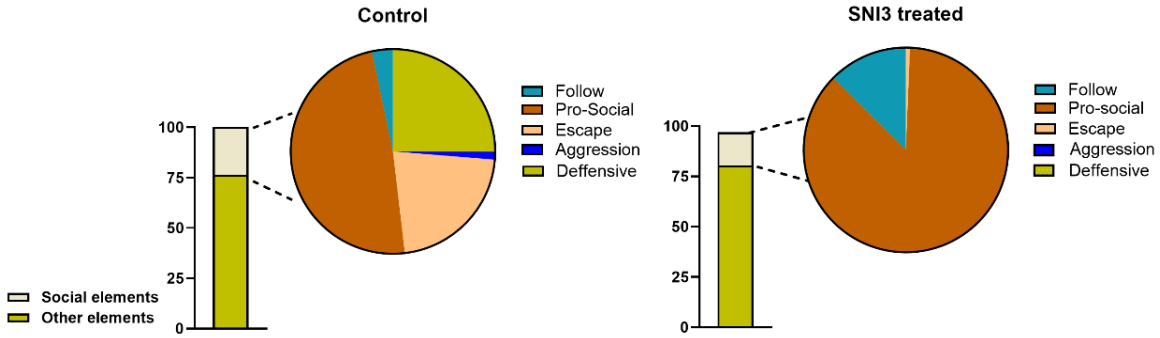


Figure 9. ***Effects of* L. plantarum SNI3 *treatment on social interaction test***

Pie charts illustrate the distribution of representative social behavior elements in social interactions of L. plantarum SNI3-treated and control male mice (n=12) (not published).

4.2.1.4. Sexual behavior

To explore the potential impact of *L. plantarum* SNI3 treatment on male sexual behavior, we determined key elements of sexual behavior. The typical sequence of male mouse sexual behavior consists of (1) following the female, (2) anogenital sniffing, (3) mounting, (4) intromission, (5) ejaculation, and (6) post-ejaculation grooming (127) (Fig. 10A). In *L. plantarum* SNI3-treated mice, the frequency and duration of nose-to-nose interactions, anogenital sniffing, and mounting were significantly increased compared to control animals (Fig. 10B and C). However, no significant differences were observed in other aspects of sexual behavior between the treated and control groups.

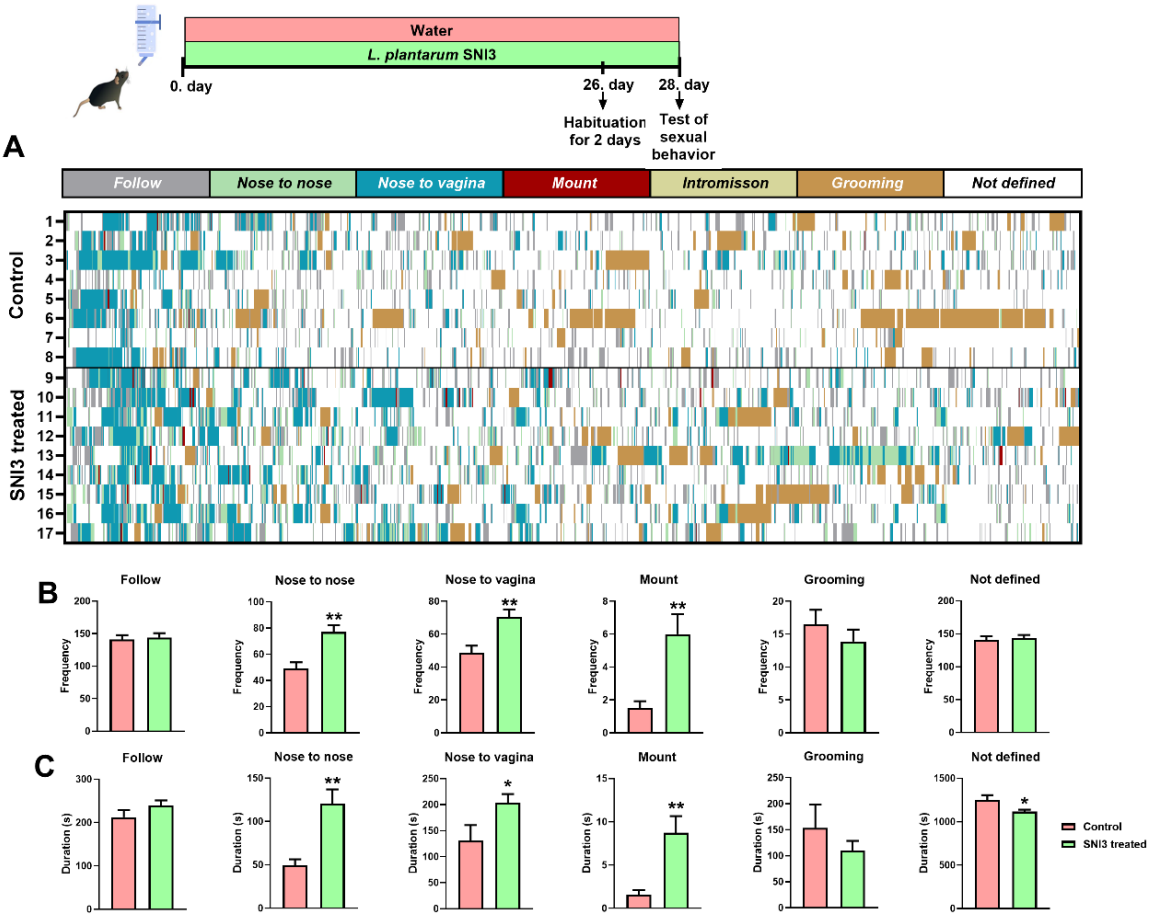


Figure 10. **L. plantarum SNI3** ***administration impacts sexual behavior***

*Top: Schematic representation of experimental groups and study design. Color-coded ethograms illustrating sequences of sexual behavior in control and L. plantarum SNI3-treated mice. Six distinct sexual interaction-related behaviors were analysed. Each row represents one mouse (A). Summary graphs showing the frequency (B) and duration (C) of specific sexual behavior elements in control and L. plantarum SNI3-treated mice. Data are expressed as Mean ± SEM (n=17), and the statistical difference was determined by unpaired two-tailed Student t-test *p<0.05; **p<0.01 (128).*

4.2.2. Effects of L. plantarum *SNI3 treatment on gut microbiome composition*

Next-generation sequencing (NGS) analysis of the V3-V4 region of the 16S rRNA gene on colon content samples revealed that *L. plantarum* SNI3 administration did not significantly alter the composition of the colon microbiome at the phylum level (Fig. 11A). Although *Firmicutes* levels increased and *Bacteroidetes* levels decreased in treated animals, the F/B ratio did not show a statistically significant difference. At the family level, the abundance of *Lactobacillaceae* and *Monoglobaceae* was significantly elevated, while *Eubacterium coprostanoligenes group* and *Akkermansiaceae* were significantly less abundant in *L. plantarum* SNI3-treated mice compared to controls (Fig. 11B). The top 20 taxa at the genus level included *Akkermansia*, *Lachnospiraceae* NK4A136 group, *Lactiplantibacillus* and the *Prevotellaceae* NK3B31 and UCG001 groups. In the microbiome of *Lactiplantibacillus plantarum* SNI3-treated animals, the abundance of *Lactiplantibacillus* and *Lachnospiraceae* NK4A136 group increased, while *Akkermansia* significantly decreased (Fig. 11C).

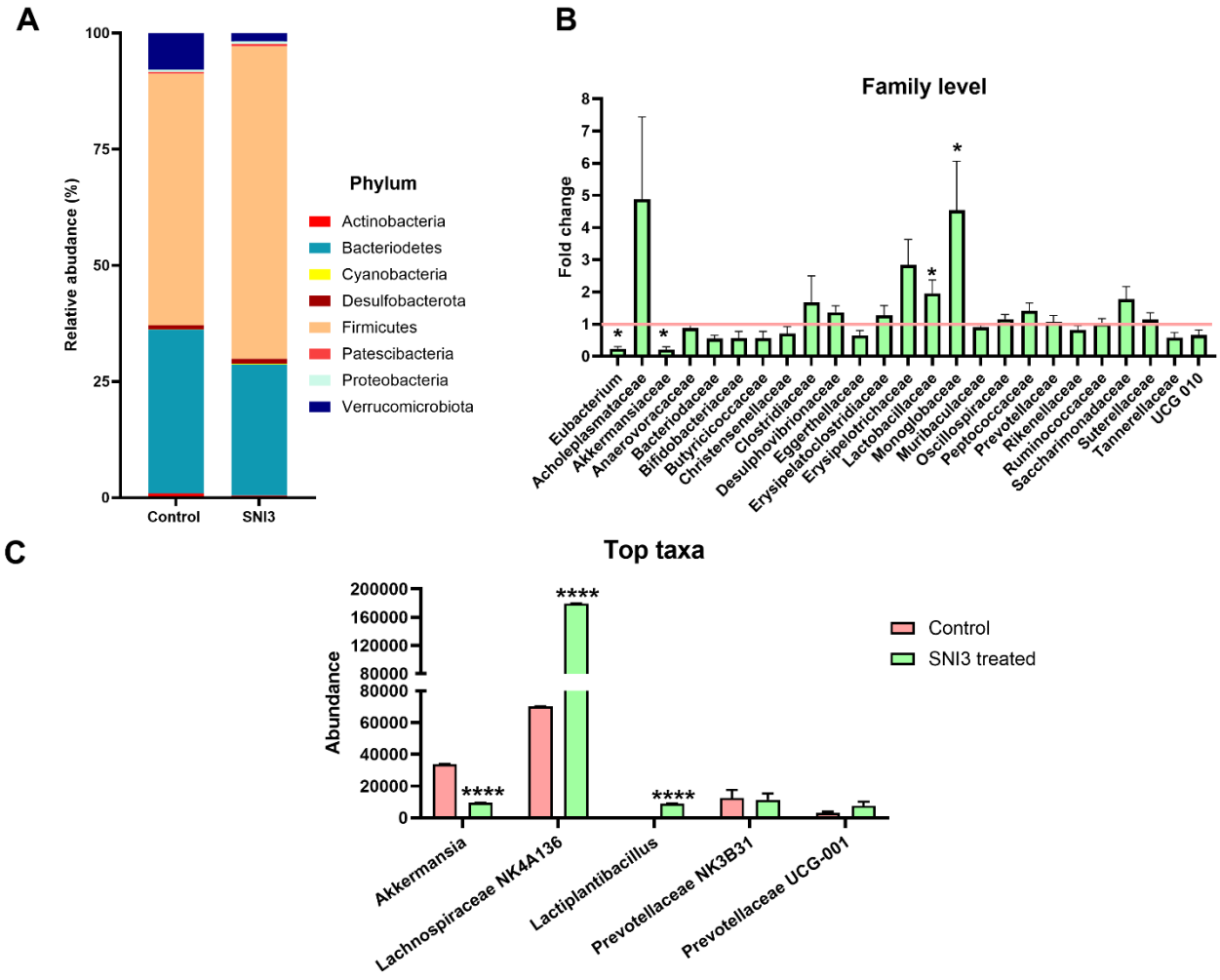


Figure 11. ***Effects of*** **Lactiplantibacillus plantarum** ***SNI3 on colon microbiome composition at phylum, family and genus levels***

*The abundance of main phyla and the top 20 taxa at genus level in colon microbiome (A, C) and fold change in the abundance at the family level (B) Data are presented as Mean ± SEM, with statistical significance indicated as *p<0.05; ****p<0.0001 vs control (n=14) (128).*

4.2.3. Effects of L. plantarum *SNI3 treatment on body composition, dietary intake and organ weights*

The body weight gain, food consumption and fluid intake did not differ between the treatment groups (Fig. 12A and B). However, the body composition analysis using EchoMRI revealed a significant increase in lean mass and a corresponding significant decrease in fat mass in treated animals (Fig. 12C). Administration of *L. plantarum* SNI3 significantly affected the testis weight (Fig. 12D). In contrast, the normalized epididymis weight showed no difference between control and treated animals (Fig. 12F).

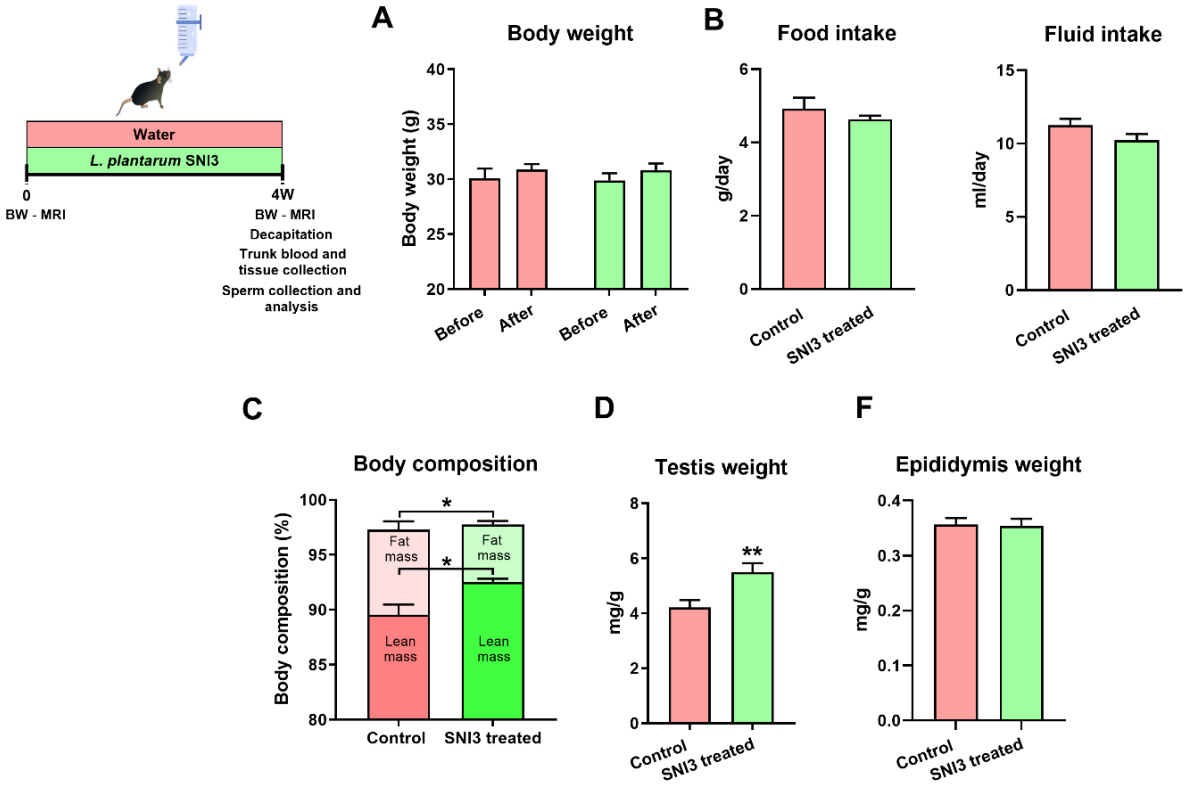


Figure 12. ***Effects of* L. plantarum SNI3 *on body composition, dietary intake and organ weights***

*Body weight (A), average daily food and fluid intake (B), body composition of mice (C), and weight of testis and epididymis (D and F). All graphs are presented as Mean ± Standard Error of Mean (SEM). Statistical significance is indicated as *p<0.05, **p<0.01 vs control, determined by unpaired, two-tailed Student t-test (n=20) (128).*

4.2.4. Effects of L. plantarum *SNI3 treatment on hypothalamo-pituitary-adrenal (HPA) axis in acute and chronic stress*

4.2.4.1. Impact of *L. plantarum* SNI3 on acute stress induced corticosterone

Since the social interaction test represents an acute stress for mice, we investigated whether *L. plantarum* SNI3 probiotic treatment can modulate the HPA axis response. Serum corticosterone (CORT) levels were measured under basal conditions and following the social interaction in both control and *L. plantarum* SNI3-treated mice. Baseline CORT concentrations did not differ significantly between the groups. However, exposure to the acute social stressor led to a significant increase in CORT levels in both control and treated mice (Fig. 13). However, the corticosterone response in *L. plantarum* SNI3-treated mice was significantly reduced compared to controls, indicating the probiotic treatment attenuates HPA axis activation under acute stress conditions.

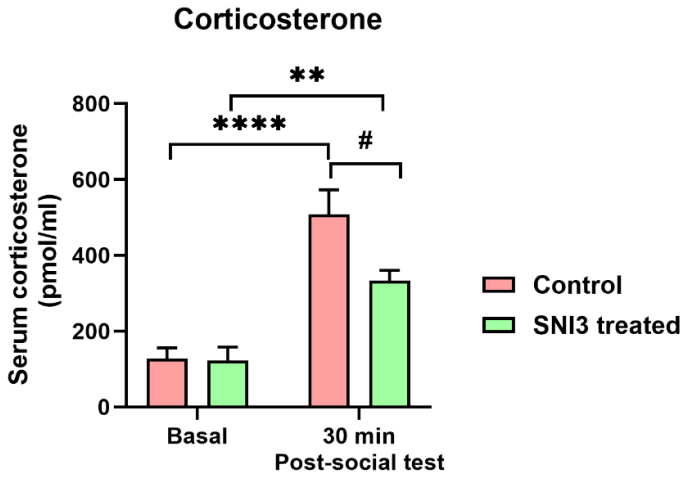


Figure 13. Effect of* L. plantarum SNI3 *on acute stress response

*Stressed blood samples were collected 30 min after social interaction test (as ana cute stress). Data are presented as. Mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Sidak's multiple comparison post hoc test (n=12), significance indicated as **p<0.01; ****p<0.0001 vs baseline; #p<0.05 vs control (not published).*

4.2.4.2. Impact of *L. plantarum* SNI3 treatment on chronic stress-induced hormonal and physiological changes

As *L. plantarum* SNI3 treatment attenuated CORT levels following social interactions, we investigated whether these effects extend to chronic variable stress conditions. Adult mice were either exposed to chronic variable stress (CVS) or not disturbed as controls. Half of the animals in each of the CVS or control groups were treated with *L. plantarum* SNI3, the other half received sterilized tap water. Body weight decreased in both groups exposed to chronic variable stress (CVS), however, a significant reduction was observed only in the control CVS group (Fig. 14A). Changes in adrenal and thymus weight, alongside corticosterone levels, are well-established markers of chronic stress. The normalized weight of the adrenals was significantly increased in CVS-exposed animals, with no differences between *L. plantarum* SNI3-treated and control animals (Fig. 14B). While thymus weight showed a decreasing trend following CVS exposure, the reduction was not statistically significant (Fig. 14C).

The CVS procedure resulted in a significant elevation of serum corticosterone concentration in control CVS mice (Fig. 14D). Remarkably, *L. plantarum* SNI3 treatment prevented CVS-induced corticosterone response. Serum testosterone concentrations were also analyzed. *L. plantarum* SNI3 treatment increased testosterone levels in control (non-stressed) mice, but not in CVS-exposed mice (Fig 14E).

We examined the effect of chronic stress on colonic microbial changes by measuring total DNA concentration from colon content samples. Neither the CVS exposure nor the *L. plantarum* treatment had a significant effect on the total DNA levels in colon content (Fig 14F).

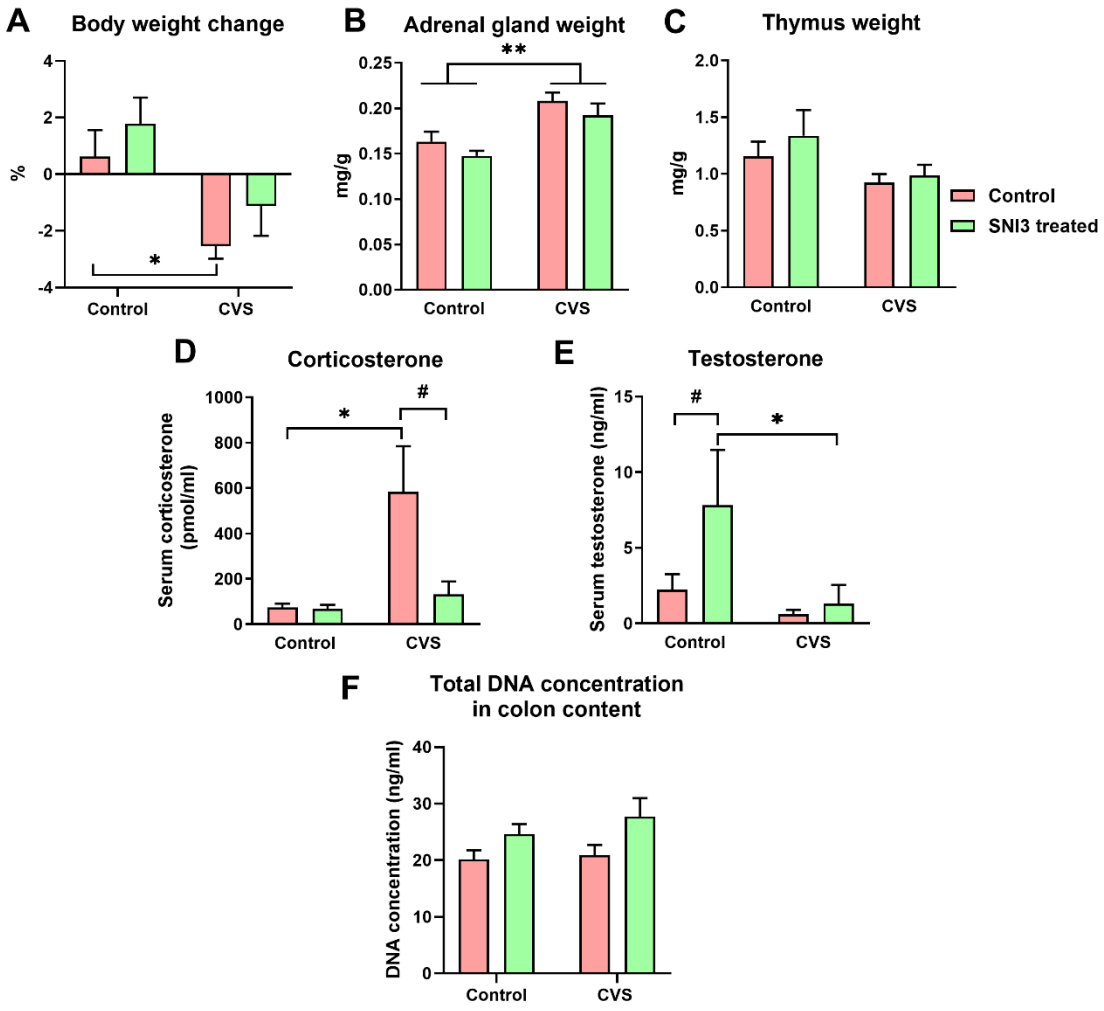


Figure 14. ***Effects of*** **L. plantarum SNI3** ***treatment on chronic stress-induced changes in body weight, organ weights, hormones and colonic bacterial DNA level***

*Percentage change in body weight (A) Normalized weights of adrenal gland and thymus (B and C.) Serum corticosterone and testosterone concentration (D and E). Total DNA concentration in colon content samples (F) Data are presented as. Mean ± SEM (n=20). Statistical analysis was performed with two-way ANOVA followed by Sidak's multiple comparison post hoc test, significance indicated as *p<0.05; **p<0.01; vs CVS; #p<0.05 vs control (not published).*

4.2.4.3. Impact of *L. plantarum* SNI3 treatment on the behavior of mice exposed to chronic variable stress

To assess anxiety-related behavior in response to chronic stress, an open field test was performed following confirmation of the CVS procedure's effectiveness through chronic stress markers. Despite significant changes in corticosterone levels and other physiological indicators of chronic stress, no differences were observed between experimental groups in the parameters of the open-field test (Fig. 15A - D).

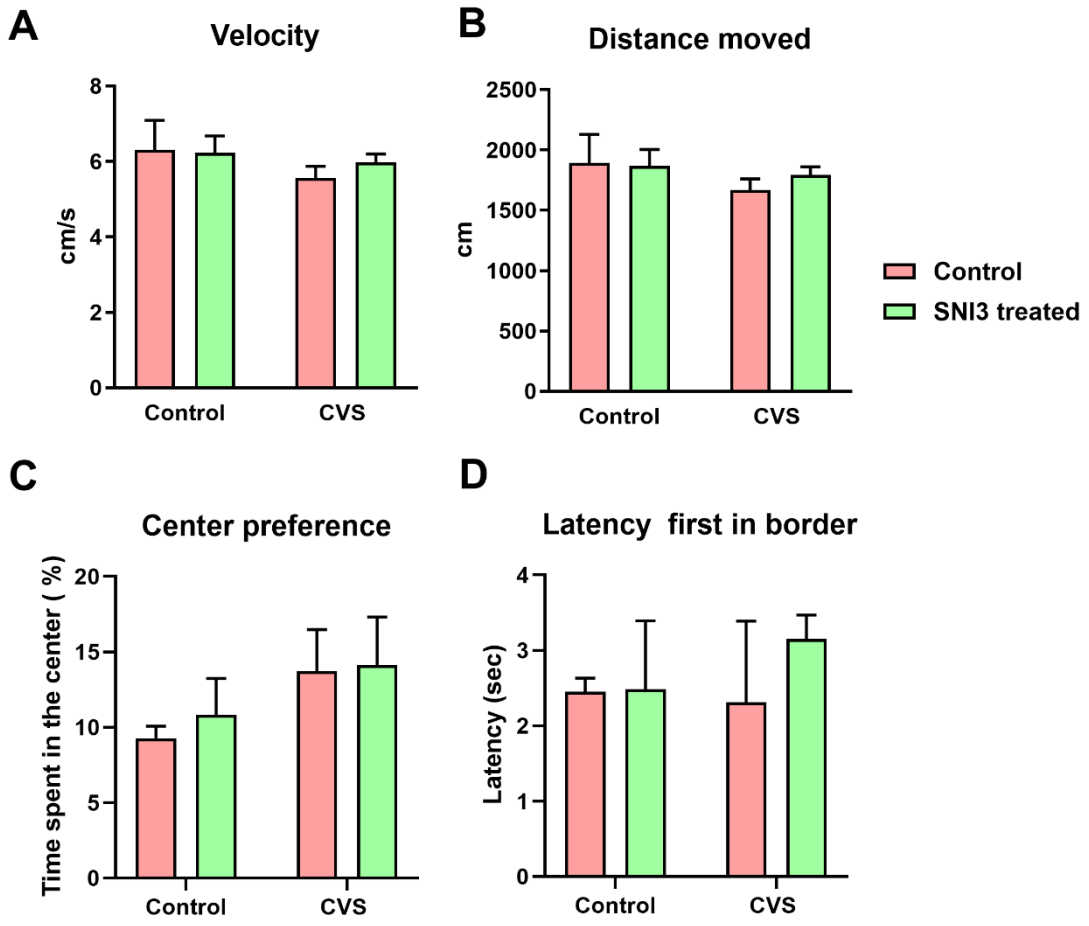


Figure 15. ***Effects of* L. plantarum SNI3 *treatment on behavioral response to chronic variable stress in mice***

Velocity during open field test (A), distance moved (B), center preference (C) and first latency in border zone (D) All data are expressed Mean ± SEM and analyzed by two-way ANOVA followed by Sidak's multiple comparison tests (n=20) (not published).

4.2.5. Effects of L. plantarum *SNI3 treatment on hypothalamo-pituitary-gonad (HPG) axis*

4.2.5.1. Impact of *L. plantarum* SNI3 on hormonal responses in male reproductive physiology

Reproductive functions are primarily regulated by the hypothalamic-pituitary-gonadal (HPG) axis. To investigate whether *L. plantarum* SNI3 administration influences the expression of key hormonal regulators, quantitative real-time PCR (qRT-PCR) was performed on hypothalamic and pituitary samples. The analysis revealed no significant changes in the gene expression of hypothalamic gonadotropin-releasing hormone (*Gnrh*) or the β-subunits of follicle-stimulating hormone (*Fsh β*) and luteinizing hormone (*Lh β*) between the control and treated groups (Fig. 16A).

However, treatment with *L. plantarum* SNI3 resulted in significant increases in hypothalamic oxytocin (*Oxt*) mRNA expression (Fig. 16C), suggesting potential modulation of neuroendocrine pathways involved in social and reproductive behaviors. To investigate potential effects on the gonads, we analyzed the expression of inhibin alpha (*Inha*), inhibin beta A (*Inhba*) and inhibin beta B (*Inhbb*) in the testis (Fig. 16B). No significant differences were detected in the expression of these genes, indicating that inhibin signaling pathways were not affected by the *L. plantarum* SNI3 treatment.

Additionally, serum testosterone levels were measured to assess changes in reproductive hormone profiles. *L. plantarum* SNI3-treated mice displayed a significantly higher testosterone concentration than the control mice (Fig. 16D).

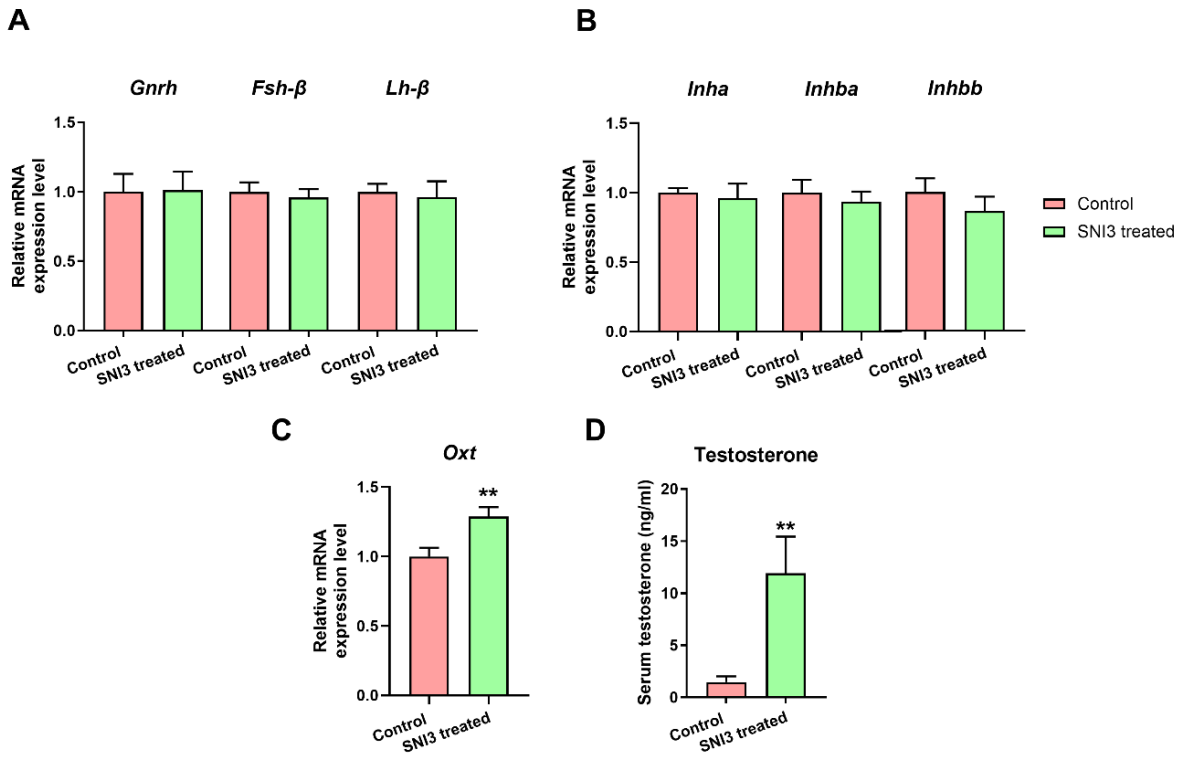


Figure 16. ***Effects of*** **L. plantarum SNI3** ***on gene expression and hormonal responses in male reproductive physiology***

*Expression of Gonadotropin-releasing hormone (GnRH), Follicle-stimulating hormone (FSH)- and Luteinizing hormone (LH)-β subunits in control and L. plantarum SNI3-treated mice (A). Expression of inhibin (Inha) and activin (Inhba and Inhbb) related genes in the testis (B) and hypothalamic oxytocin expression (C). Serum testosterone (TESTO) levels in control and L. plantarum SNI3-treated mice. Data are expressed as Mean ± SEM **p<0.01 using the unpaired, two-tailed Student t-test (n=14) (128).*

4.2.5.2. Effect of *L. plantarum* SNI3 treatment on seminiferous tubule morphology in mouse testes

To reveal histomorphological changes associated with the increased testis size observed in *L.plantarum* SNI3-treated mice, hematoxylin-eosin-stained testis sections were analyzed. Specifically, the seminiferous tubule area and the thickness of the epithelium were measured in control and treated mice. Both parameters were significantly increased in the *L. plantarum* SNI3-treated group compared to controls (Fig. 17A and B), indicating enhanced spermatogenesis activity following probiotic treatment.

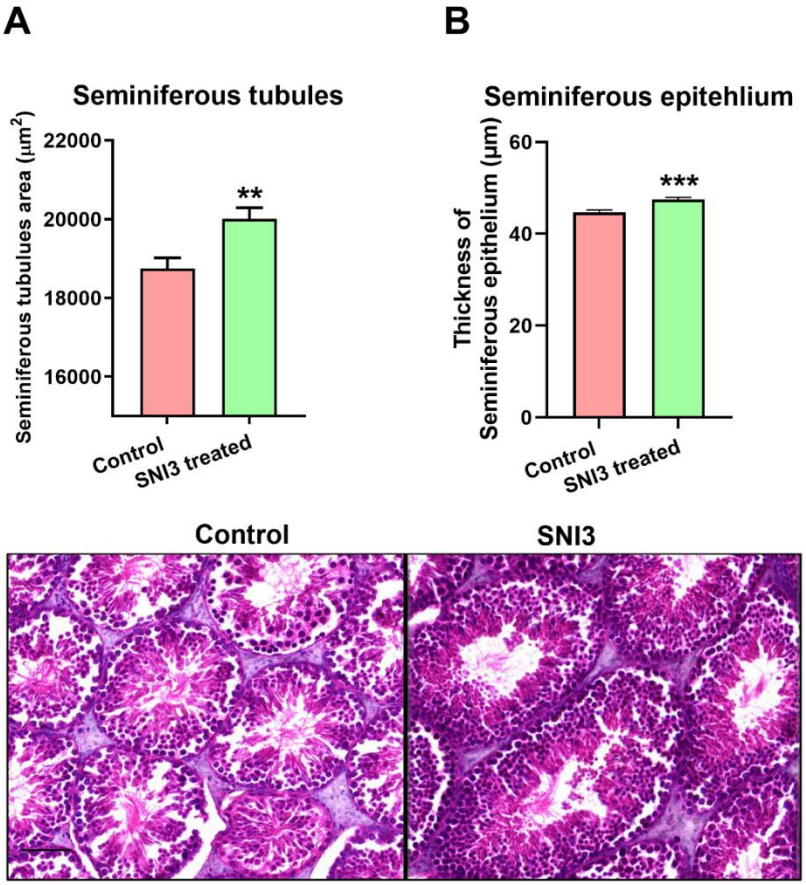


Figure 17. Effect of* L. plantarum SNI3 *on seminiferous tubule morphology in mouse testes

*Area of seminiferous tubule and thickness of seminiferous epithelium in control and treated mice (A-B). Below: representative haematoxylin-eosin-stained sections from testes of control and L. plantarum SNI3-treated mice. All data in the graphs are expressed as Mean ± SEM. An unpaired two-tailed Student t-test was used to evaluate significant differences between L. plantarum SNI3-treated and control groups (n=20). Statistical significance is indicated as **p<0.01, ***p<0.001 vs control (128).*

4.2.5.3. Effect of *L. plantarum* SNI3 treatment on sperm parameters

We evaluated the impact of *L. plantarum* SNI3 treatment on sperm count. The number of sperm collected from the cauda epididymis was significantly higher in treated animals compared to controls (Fig. 18A). To assess potential morphological abnormalities, sperm smears were stained with aniline-eosin, revealing a low frequency of defects such as immature acrosomes, crooked midpieces, and hairpin tails, with no significant differences between groups. Triple fluorescence labeling (peanut lectin + phalloidin + DAPI) of sperm smears did not indicate noticeable morphological differences in sperm heads (acrosome) between control and *L. plantarum* SNI3-treated samples (Fig. 18B). Additionally, computer-assisted sperm analysis (CASA) revealed a significantly higher percentage of motile sperm in L. plantarum SNI3-treated mice compared to controls (Fig. 18C).

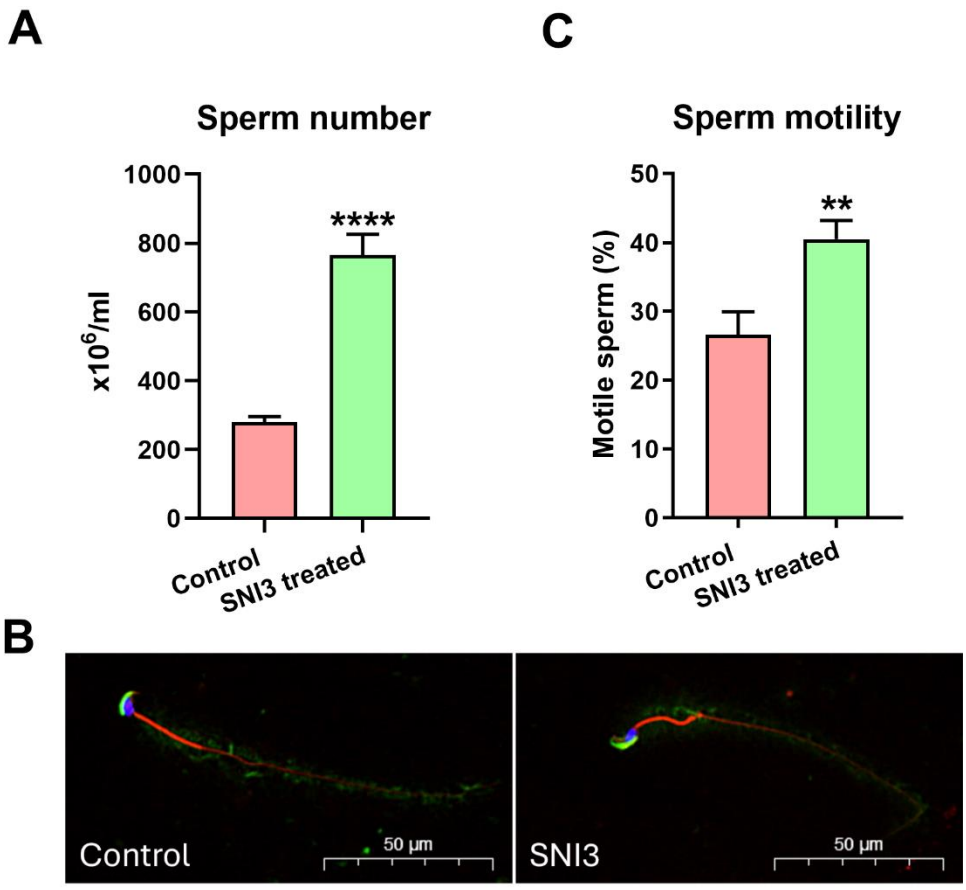


Figure 18. ***Effect of*** **L. plantarum SNI3** ***on spermatozoa***

Sperm count in control and L plantarum SNI3-treated mice was determined using a Makler chamber (A). Fluorescent labelling of individual sperm collected from control and

*L. plantarum SNI3-treated mice, showing peanut lectin-FITC (green, acrosome), phalloidin-Alexaflour595 (red, actin cytoskeleton) and DAPI (blue, DNA) labelling (B). The percentage of motile sperm in the control and treated group was assessed by CASA (computer-assisted sperm analysis) system (C). All data in the graphs are expressed as Mean ± SEM. An unpaired two-tailed Student t-test was used to evaluate significant differences between L. plantarum SNI3-treated and control groups (n=20). Statistical significance is indicated as **p<0.01, ****p<0.0001 vs control (128).*

4.2.5.4. Effects of *L. plantarum* SNI3 on sperm number depend on the bacterial viability

To determine the role of bacterial viability in the observed effects on the sperm number, we compared live *L. plantarum* SNI3 treatment with its lysed and heat-inactivated forms (Fig. 19). Only mice administered with live *L. plantarum* SNI3 showed a significant increase in sperm number. In contrast, mice treated with the enzymatically lysed or heat-inactivated bacteria showed no effect. This indicates that an increase in sperm count depends on the presence of metabolically active bacteria.

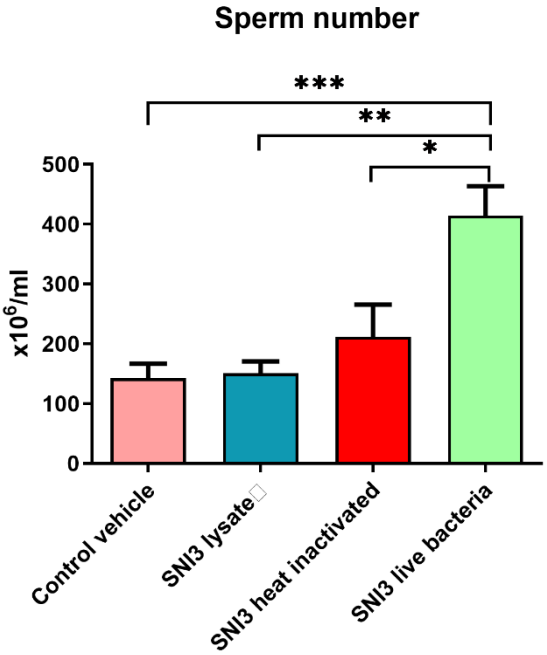


Figure 19. Effect of* L. plantarum SNI3 *on sperm count

*Sperm number was determined in Makler chamber. Data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison post hoc test (n=20). Mean ± SEM values, *p<0.05; **p<0.01; ***p<0.001 vs control vehicle (not published).*

4.2.5.5. Correlations between colon microbiome composition and sperm count

Significant positive correlations were identified between sperm count and the abundance of *Lactiplantibacillus plantarum* and *Lachnospiraceae* in colon content samples from *L. plantarum* SNI-treated mice (Fig. 20A and B). In contrast, the abundance of *Akkermansia* in the colon microbiome exhibited a significant negative correlation with sperm count (Fig. 20C).

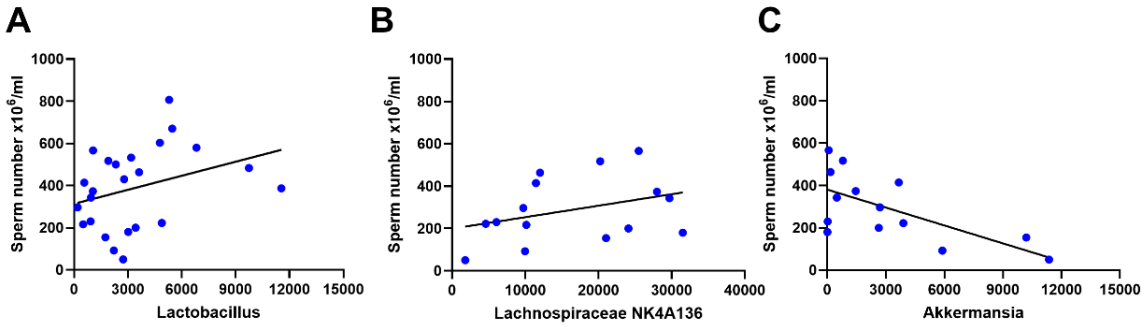


Figure 20. Correlation between colon microbiome composition and sperm count in L. plantarum* SNI3-*treated mice

Correlation between the abundance of different microbial populations in the colon and sperm count (A, B and C). Scatter plots illustrate the relationship between microbial population abundance and sperm number, with each point representing an individual sample (n=14). Correlations between microbiota abundance and sperm count were analyzed using Spearman's correlation, with significance set at p<0.05 (128).

4.2.5.6. Fecal microbiota transplantation (FMT) confirms the role of gut microbiota in improving reproductive health following *L. plantarum* SNI3 treatment

Building on the reproductive improvements observed following *Lactiplantibacillus plantarum* SNI3 treatment, fecal microbiota transplantation (FMT) was conducted to investigate whether these effects are mediated by changes in the gut microbiome. The microbiome of all recipient mice was attenuated by a 3-day antibiotic treatment prior to FMT. Fecal microbiota isolated from donor mice treated with *L. plantarum* SNI3 was administered to recipient mice, while control recipient mice received fecal microbiota from untreated donor mice. A separate group of mice (No FMT group) did not receive any fecal microbiota.

No change in total DNA concentration of colon contents was observed in mice receiving control fecal microbiota (Fig. 21A). In contrast, FMT from *L. plantarum* SNI3-treated donors led to a significant increase in total DNA level in colon content compared to both the No FMT and control FMT groups. Furthermore, fecal microbiota from *L. plantarum* SNI3-treated donors increased the sperm number in recipient mice (Fig. 21B). In addition, mice receiving SNI3 FMT also showed a significant increase in serum testosterone levels (Fig. 21C). Conversely, FMT from control donor mice did not affect any reproductive markers.

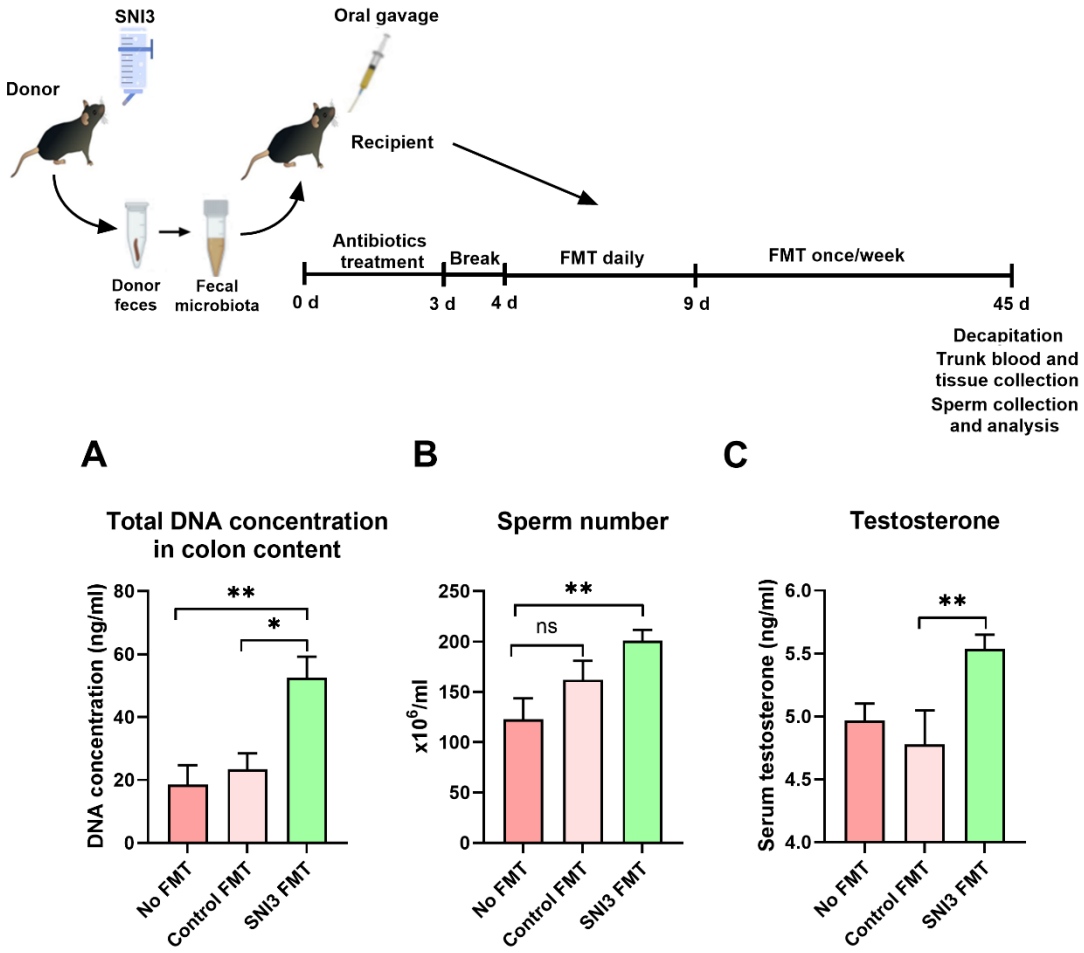


Figure 21. ***Impact of fecal microbiota transfer (FMT) on colon content DNA concentration and reproductive markers in recipient mice***

*Total DNA concentration in colon content (A) sperm number was determined by Makler chamber (B) serum testosterone concentration (C) Data were analyzed by one-way ANOVA Dunnett's followed by multiple comparison post hoc test. Mean ± SEM values, *p<0.05; **p<0.01; vs control mice (n=6-13 per group) (128).*

4.2.5.7. Broad-spectrum antibiotic-induced gut microbiome depletion alters sperm count

Exposure to the broad-spectrum antibiotic cocktail resulted in notable changes in reproductive parameters. Although serum testosterone level showed a slight reduction, this decrease was not significant (Fig. 22A). However, a significant decline in sperm number was observed in antibiotic-treated mice compared to control mice (Fig. 22B). Additionally, antibiotic treatment led to a significant enlargement of the cecum (Fig. 22C), a well-established indicator of gut microbiota depletion. Importantly, a negative

correlation between sperm number and cecum weight was identified in the antibiotic-treated group (Fig. 22D), suggesting a potential link between microbiome depletion and impaired sperm number.

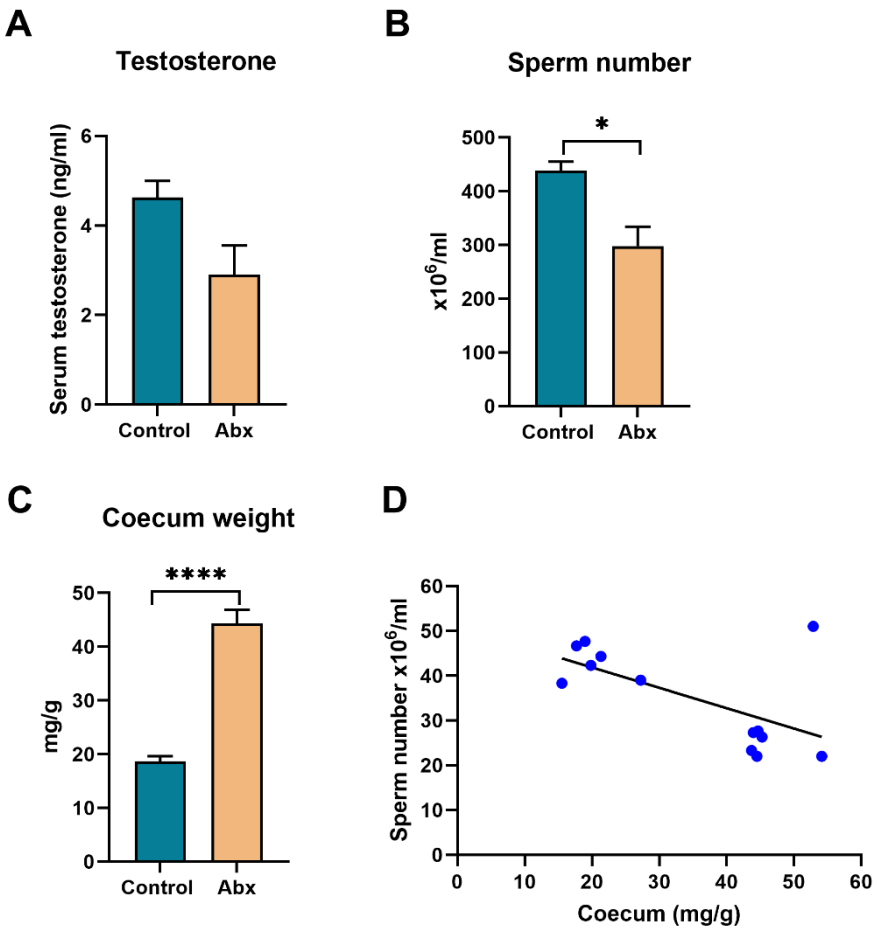


Figure 22. ***Impact of broad-spectrum antibiotic treatment-induced gut microbiome depletion on sperm number***

*Serum testosterone concentration (A) sperm number was measured by Makler chamber (B) normalized coecum weight (C) correlation between sperm number and coecum weight were analyzed using Sperman's correlation, with significance set at $p<0.05$ (D) Data were analyzed using an unpaired two-tailed Student t-test (n=14). All values are expressed as Mean±SEM *$p<0.05$, ****$p<0.0001$ vs control mice (not published).*

4.2.5.8. Impact of *L. plantarum* SNI3 on testicular metabolism in male mice

To investigate the metabolic impact of *Lactiplantibacillus plantarun* SNI3 treatment on testicular function, a correlation network was constructed using untargeted metabolomics data obtained through mass spectrometry imaging (Fig. 23). This network revealed functionally linked metabolites and highlighted key pathways affected by the treatment.

In the testis of *L. plantarum* SNI3-treated mice, metabolites associated with central metabolic pathways, such as glucose, galactose, fructose, palmitoyl carnitine, phosphoenolpyruvate, succinate, and fumarate were significantly reduced. Despite these changes, ATP levels remained unchanged, suggesting that the energy balance of the testis was maintained. Lipid metabolism pathways, including arachidonic acid, linoleic acid, and glycerophospholipid metabolism, were also suppressed in response to the treatment.

Conversely, specific fatty acids, such as palmitic acid, palmitoleic acid, stearic acid and oleic acid, were elevated in treated animals compared to controls. Notably, palmitic glucuronide was among the most highly upregulated metabolites. Although its precise function in the testis is not well understood, glucuronidation is commonly associated with metabolite excretion.

Additionally, non-proteogenic amino acids, including dimethylglycine (DMG) and gamma-aminobutyric acid (GABA), as well as peptides such as glutamyl peptides (GluGlu, GluGln) and oxidized glutathione (GSSG), showed significant enrichment in the testes of treated animals.

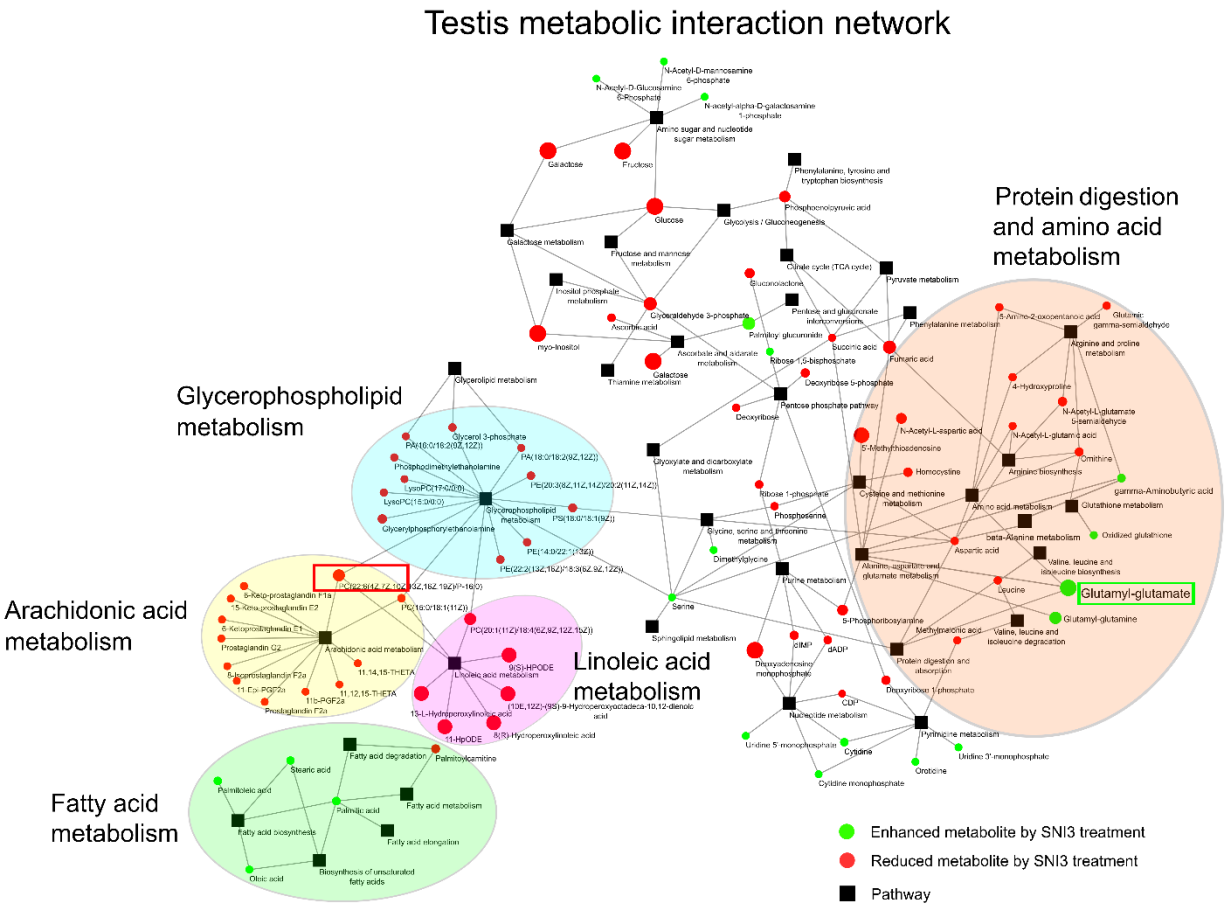


Figure 23. ***Metabolic interaction network in testis treated with*** **Lactiplantibacillus plantarum SNI3**

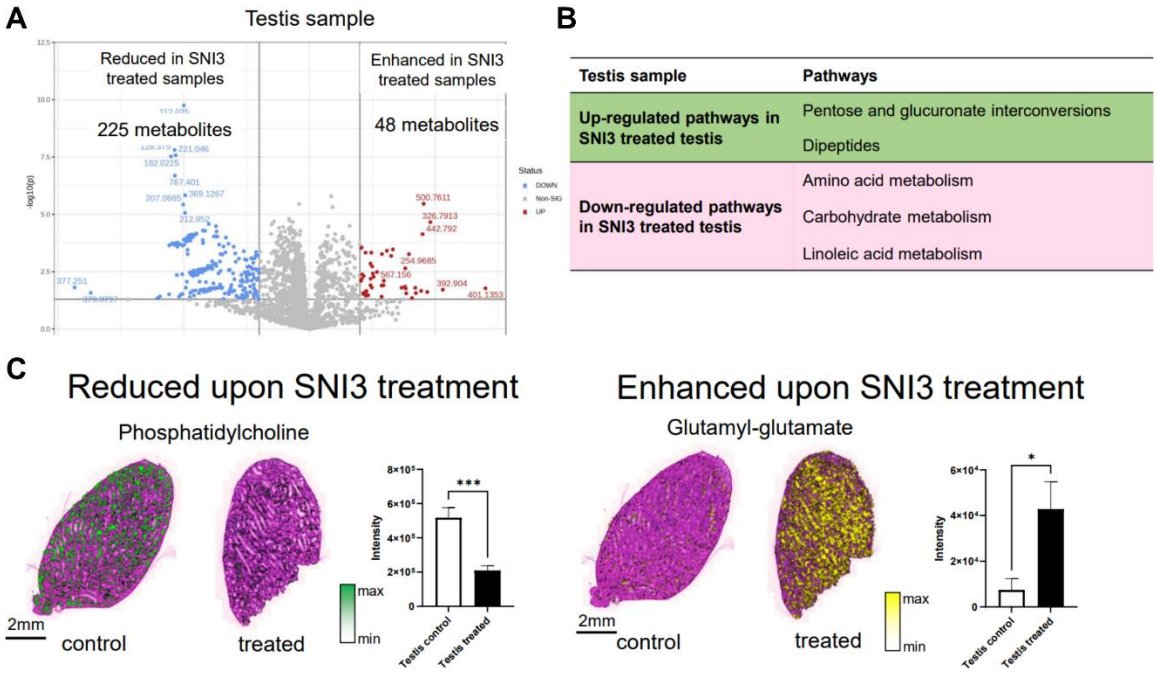
The network nodes represent metabolites, node size indicates log2 fold change between control and L. plantarum SNI3-treated testis samples. Larger node size corresponds to greater log2 fold changes. Nodes are labelled based on a log2 fold change threshold, with green indicating upregulated metabolites (log2 fold change > 0) and red indicating downregulated metabolites (log2 fold change <0). The edges represent common KEGG pathways, the pathway categories are color-coded as follows: yellow for arachidonic acid metabolism, green for fatty acid metabolism and orange for protein digestion and amino acid metabolism. The most upregulated metabolite (glutamyl-glutamate) and the most downregulated metabolite (phosphatidylcholine, PC (22:6(4Z,7Z,13Z,16Z,19Z)/16:0) are highlighted within green and red squares respectively (128).

4.2.5.9. Impact of *L. plantarum* SNI3 treatment on glutamyl glutamate (GluGlu) levels and testicular metabolism

To explore the metabolic pathways influenced by *L. plantarum* SNI3 treatment in the testis, volcano plots (Fig. 24A) and pathway enrichment analysis (Fig. 24B) were utilized. Discriminative metabolites were identified based on a fold change > 2 or < 0.5 with a p-value <0.05 and subsequently annotated using the HMDB database. Identified metabolites were subjected to pathway analysis via KEGG.

The pathway enrichment analysis (Fig. 24B) highlights metabolic changes in response to *L. plantarum* SNI3 treatment. Upregulated pathways included pentose and glucuronate interconversion and dipeptides associated with amino acid degradation. Conversely, amino acid, carbohydrate and linoleic acid metabolism pathways were found to be downregulated.

Figure 24 (C) provides mass spectrometry imaging (MSI) representations of selected downregulated (phosphatidylcholine) and upregulated (glutamyl-glutamate, GluGlu) metabolites in the testis. GluGlu MSI signals appear to be localized within the seminiferous tubules. Notably, GluGlu was the most significantly upregulated metabolite in the testis of *L. plantarum* SNI3-treated mice.



Testis sample	Pathways
Up-regulated pathways in SNI3 treated testis	Pentose and glucuronate interconversions
	Dipeptides
Down-regulated pathways in SNI3 treated testis	Amino acid metabolism
	Carbohydrate metabolism
	Linoleic acid metabolism

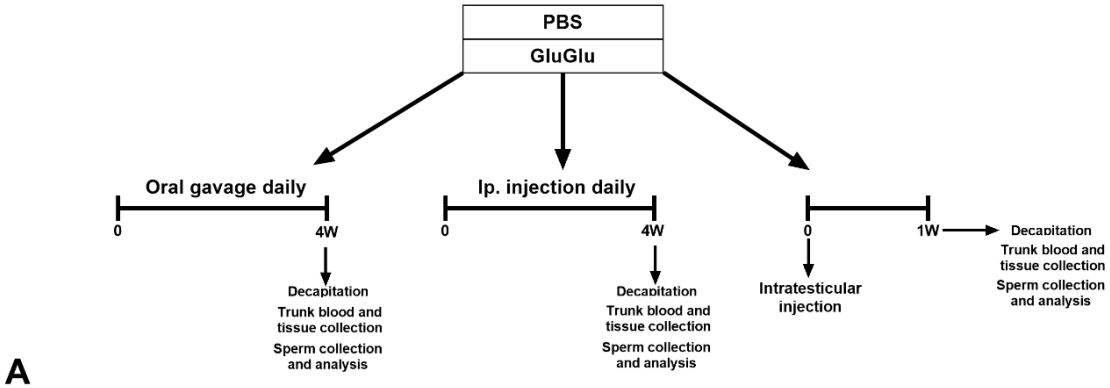
Figure 24. ***Identification of differential metabolites and pathway alterations in the testis of control and*** **L. plantarum** ***SNI3-treated mice***

*Vulcano plot illustrating discriminative metabolites based on fold change (FC) and statistical significance. The x-axis represents log2(FC), while the y-axis displays the -log10(p-value) adjusted by false discovery rate (FDR). Metabolites with a fold change >2 or <0.5 and p-value <0.05 were considered significant (A). Pathway enrichment analysis was performed using KEGG pathways. The figure summarizes the pathways significantly impacted by L. plantarum SNI3 treatment in testis samples (B). Mass spectrometry imaging (MSI) of representative metabolites affected by L. plantarum SNI3 treatment. The left panel shows MSI of phosphatidylcholine, a downregulated metabolite in the treated group, while the right panel illustrates glutamyl-glutamate (GluGlu), an upregulated metabolite. MSI images highlight the localization of these metabolites within the testis. Statistical significance was determined using an unpaired t-test, *p<0.05, ***p<0.001 (128).*

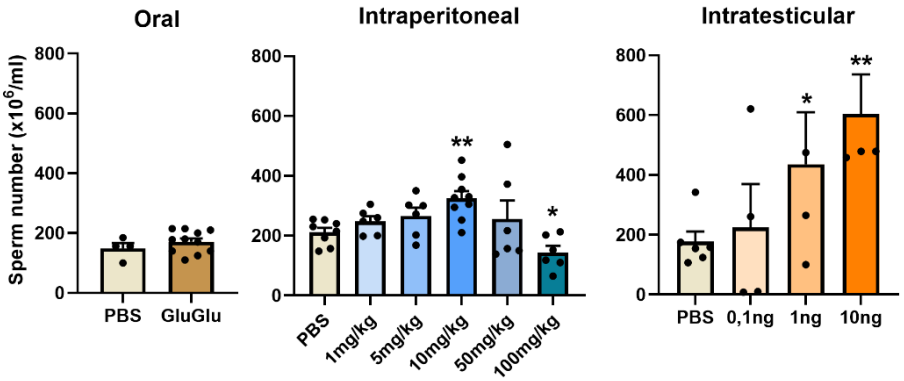
4.2.5.10. Effects of γ-glutamyl-glutamate (γ-GluGlu) administration on reproductive parameters in mice

To investigate whether the significantly elevated γ-GluGlu metabolite in the testis of mice treated with *L. plantarum* SNI3 could replicate the observed effects of bacterial treatment on reproductive and hormonal parameters, different administration methods were tested. Oral administration of γ-GluGlu had no effect on sperm number. In contrast, intraperitoneal injection of the dipeptide over a 4-week period led to a dose-dependent increase in sperm count. To further evaluate the localized effects of the dipeptide metabolite, direct intratesticular injections of γ-GluGlu were administered to mice. Dose-ranging from 0.1 to 1 ng significantly increased sperm numbers isolated from the cauda epididymis compared to vehicle (PBS)-injected controls, demonstrating the potent and specific impact of γ-GluGlu on sperm production (Fig. 25A).

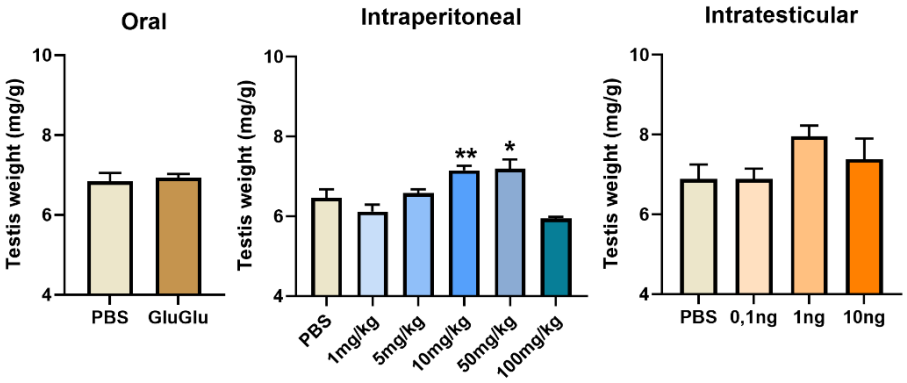
Systemic intraperitoneal administration of γ-GluGlu at doses 10 mg/kg and 50 mg/kg resulted in a significant increase in relative testis weight compared to controls (Fig. 25B). This effect was dose-dependent and was not observed at lower doses or following oral or local intratesticular injections. Despite these significant increases in sperm count, neither oral, systemic, nor local administration of γ-GluGlu induced any changes in serum testosterone levels (Fig. 25C).


PBS
GluGlu
Oral gavage daily
0
4W
Decapitation
Trunk blood and tissue collection
Sperm collection and analysis
Ip. injection daily
0
4W
Decapitation
Trunk blood and tissue collection
Sperm collection and analysis
0
Intratesticular injection
1W
Decapitation
Trunk blood and tissue collection
Sperm collection and analysis

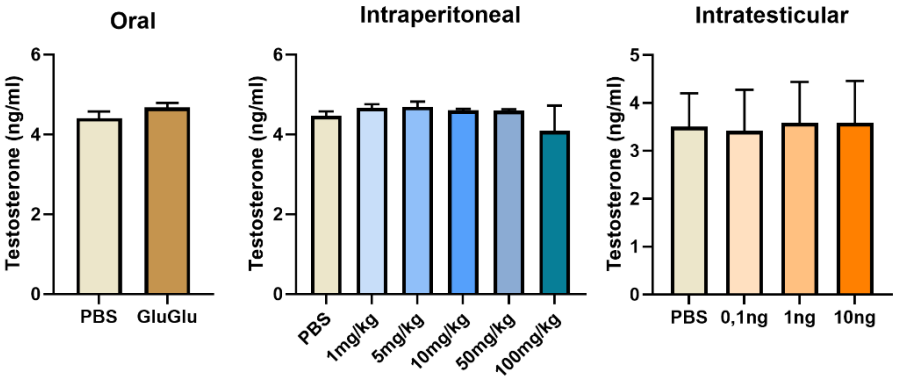
A


Oral
Intraperitoneal
Intratesticular
Sperm number (x10^6/ml)
PBS
GluGlu
1mg/kg
5mg/kg
10mg/kg
50mg/kg
100mg/kg
0,1ng
1ng
10ng

B


Oral
Intraperitoneal
Intratesticular
Testis weight (mg/g)
PBS
GluGlu
1mg/kg
5mg/kg
10mg/kg
50mg/kg
100mg/kg
0,1ng
1ng
10ng

C


Oral
Intraperitoneal
Intratesticular
Testosterone (ng/ml)
PBS
GluGlu
1mg/kg
5mg/kg
10mg/kg
50mg/kg
100mg/kg
0,1ng
1ng
10ng

<u>Figure 25.</u> Impacts of γ-glutamyl-glutamate (γ-GluGlu) administration on reproductive parameters in mice

*Dose-dependent evaluation of sperm counts isolated from cauda epididymis following oral, intraperitoneal and local administration of γ-GluGlu (A) relative testis weight following oral, intraperitoneal and local administration of γ-GluGlu (B) and serum testosterone levels after oral, intraperitoneal and local administration of γ-GluGlu (C) Data were analyzed by one-way ANOVA Dunnett's followed by multiple comparison post hoc test. Mean ± SEM values, *p<0.05; **p<0.01; vs control mice (n=78) (128).*

5. Discussion

Given the challenges of modern life, emerging evidence underscores the important role of the gut microbiome in human health and diseases. The gut microbiome is particularly susceptible to many undesirable lifestyle factors such as unhealthy diet, lack of sleep, circadian rhythm disturbance, sedentary behavior and other lifestyle-related stressors. For example, an epidemiological study highlighted that the rise in Alzheimer's disease prevalence in Japan and other developing countries is linked to changes in national dietary patterns (129). Furthermore, *Ternák et al*. demonstrated a negative correlation between the consumption of broad-spectrum penicillin (J01 CA) and combination penicillin (J01 CR) and Parkinson's disease (PD) prevalence across various European countries. This association was further supported by preclinical studies and detailed retrospective analysis of antibiotic exposure data in PD patients (130). It is more likely that changes in the gut microbiome affect not only the gut-brain axis but also the reproductive functions. Recent studies have linked exposures to pesticides and antibiotics to declines in sperm quality, contributing to the global trend of decreasing sperm counts observed over the past decades (131). Consequently, the use of pre- and probiotic treatments is gaining increased attention as a strategy to maintain and restore gut health and overall homeostasis.

Our findings revealed that *Lactobacillus plantarum* SNI3 probiotic treatment significantly enhanced reproductive parameters in healthy mice by influencing the gut microbiome diversity and affecting testis metabolism. One key finding was the elevated level of gamma-glutamyl glutamate, which mediated increased sperm production. In addition to enhancing reproductive parameters, *L. plantarum* SNI3 treatment was associated with reduced stress-induced corticosterone, highlighting its role in regulating the gut microbiome-brain axis under both acute and chronic stress conditions. These results suggest that targeted probiotic interventions have the potential not only to improve reproductive health but also to mitigate the effects of environmental stressors on gut and systemic homeostasis.

5.1. Gut microbiome-testis axis

The dynamic nature of the gut microbiome plays a crucial regulatory role in reproductive physiology (132). Our findings provide new insight into reproductive health, demonstrating that orally administered *Lactiplantibacillus plantarum* SNI3 can enhance sperm number, motility and serum testosterone, thereby improving overall reproductive function even in healthy, non-stressed mice. This is particularly significant, as most existing literature on the gut-testis axis primarily focuses on restoring reproductive health in disease models, such as obesity and several pesticide exposures (133-135). Clinical studies have demonstrated that a 10-week probiotic supplementation containing different *Lactobacillus* strains, *Bifidobacteria* and *Streptococcus thermophiles* significantly improved sperm count and motility in men with idiopathic oligoasthenoteratozoospermia (136). This further supports a potential link between the gut microbiome and testis, suggesting that probiotic-driven microbiome modulation may influence male reproductive health across species. Our study highlights the potential of probiotic interventions in optimizing reproductive function beyond pathological conditions, expanding the scope of microbiome research in reproductive health.

Our probiotic treatment lasted for 4 weeks, covering almost a full spermatogenetic cycle, which takes approximately 35 days (137). The effectiveness of probiotic treatments depends on several factors, including colonization capacity and metabolic activity. While non-viable bacterial components, such as cell wall fragments (parabiotics) and secreted metabolites (postbiotics), have been reported to exert beneficial effects on gut microbiota (138), our findings demonstrate that elevated sperm numbers were only observed in response to intact, metabolically active *L. plantarum* SNI3. By contrast, heat and enzymatically inactivated bacteria did not significantly affect sperm production. These results imply that the presence of live *L. plantarum* SNI3 and its metabolite production play a role in the gut microbiome, potentially influencing sperm kinetics and increasing testis weight. Furthermore, hematoxylin-eosin (H&E) staining of the testicular sections revealed a significant increase in seminiferous tubule area and epithelial thickness, further supporting an improvement in spermatogenesis.

The 16S rRNA sequencing from colonic samples confirmed the successful colonization of *L. plantarum* SNI3 within the gut microbiota, accompanied by distinct compositional shifts. Notably, the *Lachnospiraceae* NK4A136 group increased by

approximately 2.5-fold, whereas *Akkermansia* showed the most pronounced reduction in *L. plantarum* SNI3-treated mice. The *Lachnospiraceae* NK4A136 group is known for its ability to produce short-chain fatty acids (SCFAs), such as butyrate (139), which play a critical role in gut homeostasis, immune modulation, and oxidative stress regulation (140, 141). The observed increase in *Lachnospiraceae* NK4A136, following *L. plantarum* SNI3 treatment, may support gut and systemic metabolic health (142), potentially contributing to improved sperm quality. However, direct effects on reproductive health remain to be elucidated. Our analysis revealed a significant correlation between microbiome composition and sperm parameters. Specifically, sperm count showed a positive correlation with the abundance of *Lactoplantibacillus* and *Lachnospiraceae* NK4A136, while a negative correlation was observed between the abundance of *Akkermansia*. Although *Akkermansia muciniphila* has been recognized for its beneficial role in metabolic health, including its efficacy in improving obesity and inflammatory bowel disease (IBD) (143, 144), its broader physiological implications remain complex. Currently, no studies have explored the relationship between *Akkermansia muciniphila* and reproductive function in healthy mice.

Because the specific pathways linking gut microbiota to sperm production remain unclear, non-targeted metabolomic analysis of testicular tissue was performed to identify potential microbial-derived mediators. Our metabolomic analysis revealed downregulation of central metabolic intermediates such as glucose, galactose, fructose, palmitoyl carnitine, phosphoenolpyruvate, succinate and fumarate without affecting ATP levels. In contrast, specific fatty acids like palmitic, palmitoleic, stearic and oleic acids were elevated. These findings indicate a shift toward fatty acid metabolism, potentially supporting membrane remodelling and sperm production. This observation may align with evidence suggesting that distinct stages of spermatogenesis prefer different energy substrates (145). For instance, primary spermatocytes show a higher preference for fatty acid metabolism compared to round spermatids, which is observed in both rodents and humans (146). While our results suggest a shift toward fatty acid metabolism, further investigations are required to fully elucidate the mechanistic role of *L. plantarum* SNI3 treatment in modulating testicular metabolic pathways.

In addition, we have observed that 4 weeks of *L. plantarum* SNI3 treatment impacts the body composition, resulting in a significant reduction in fat mass and an increase in

lean mass, despite unchanged food intake. This metabolic modulation might be attributed to the diversity of the gut microbiome and SCFA-producing bacteria, such as *Lachnospiraceae* NK4A136, which was enriched following the *L. plantarum SNI3* treatment. SCFAs are known to regulate host metabolism, with previous studies demonstrating that SCFA supplementation prevents high-fat diet obesity by decreasing body weight and promoting fatty acid oxidation (147).

Spermatogenesis is a highly energy-demanding process, and a huge number of spermatozoa undergo apoptosis, while a limited number can continue the maturation process(148). These factors increase the production of reactive oxygen species (ROS) and, thus, the oxidative stress in the testis (149). The high serum testosterone levels following 4 weeks of *L*. plantarum SNI3 treatment may indicate that the antioxidant capacity of the testes was enhanced, protecting them from oxidative stress (150).

One of the most interesting findings from our testicular metabolomic analysis was the upregulation of several non-proteinogenic amino acids and peptides, such as dimethylglycine, GABA, γ-GluGlu, γ-GluGln and oxidized glutathione (GSSG). Among these, dipeptide glutamyl-glutamate (GluGlu) was the most upregulated metabolite in the testes of *L. plantarum* SNI3-treated animals. Because of its role in the γ-glutamyl cycle, γ-GluGlu may boost antioxidant capacity and support sperm production by facilitating the transport and availability of amino acids required for glutathione biosynthesis (151). Previous studies have shown that certain lactic acid bacteria can synthesize various dipeptides, including γ-GluGlu (152). In bacteria, γ-glutamyl transferase (GGT) and γ-glutamyl cysteine ligase (GCL) mediate γ-GluGlu synthesis (153). The presence of GCL (SNI3-14110) proteins in the L. plantarum SNI3 genome (107) further supports the hypothesis that the *L. plantarum* SNI3-derived metabolites may contribute to sperm production.

To elucidate this potential mechanism, we administered γ-GluGlu directly into the testis. Intratesticular injection of this metabolite significantly increased the sperm number. Moreover, systemic intraperitoneal (ip) injection of γ-GluGlu for four weeks increased sperm counts in a dose-dependent manner. However, administration of the highest dose of γ-GluGlu (100mg/kg) significantly decreased the sperm number, indicating that an excess of this metabolite in the testis may negatively affect sperm production. Interestingly, testis weight was only elevated at 10 and 50 mg/kg of systemic

intraperitoneal administration. Despite its pronounced effect on sperm count, γ-GluGlu did not alter serum testosterone levels in any case. Our findings provide strong evidence that microbiome composition and its derived metabolites actively influence male reproductive health, with potential applications for fertility optimization.

The gut microbiome-testis interaction was further supported by short-term 3-day broad-spectrum antibiotics treatment, resulting in a significantly reduced sperm count, while having no effect on serum testosterone. Previous findings have shown that antibiotic-induced gut microbiota disruption, depending on the type and spectrum of antibiotics used, can adversely affect spermatogenesis by increasing oxidative stress and hormonal imbalance (154, 155). Notably, antibiotic treatment also resulted in coecum enlargement, which is a well-established marker of microbiota depletion, as the absence of normal microbiota fermentation and dietary breakdown leads to the accumulation of undigested materials in the cecum (156). These findings imply that even short-term broad-spectrum antibiotic treatment can substantially alter gut microbiota composition, with adverse consequences on sperm production. This association is further strengthened by a negative correlation observed between sperm count and enlarged coecum weight, which underlines the importance of the gut microbiota in maintaining reproductive function.

Given the observed decline in sperm count following antibiotic-induced microbiota depletion, we investigated whether fecal microbiota transplantation (FMT) from *L. plantarum* SNI3-treated mice could influence the reproductive parameters. In recipient mice whose gut microbiome was depleted by antibiotic treatment (Abx) prior to FMT, transplantation of the microbiome from *L. plantarum* SNI3-treated mice significantly increased sperm number and serum testosterone levels. In contrast, the control microbiome (Control FMT) had no effect on sperm count and testosterone levels. These observations are in accordance with previous studies demonstrating the ability of gut microbiota to influence male reproductive function. For instance, the microbiome from young mice treated with *Lactobacillus reuteri* enlarged testis size and restored testosterone levels in aged mice (157). Furthermore, our findings suggest that restoring microbiome composition following antibiotic exposure is essential for reproductive health. The observed reduction in total DNA concentration in colon content after a 3-day broad-spectrum antibiotic treatment highlights the significant impact of microbiome depletion. In contrast, in SNI3 FMT mice, total DNA levels were significantly increased

by day 45, indicating successful gut microbiota re-establishment after antibiotic depletion. While SNI3 FMT partially improved sperm number and testosterone levels compared to the control groups, these parameters remained lower than in *L. plantarum* SNI3-treated mice that did not undergo Abx exposure. This may suggest that the full reproductive benefits of *L. plantarum* SNI3 treatment depend on the duration of FMT treatment (158). Overall, these results confirm that microbiome composition and its derived metabolites play a critical role in maintaining sperm production and hormonal balance.

Regarding the regulation of testosterone levels, our results suggest a complex microbiome-mediated mechanism. Elevated levels were observed exclusively in mice treated with L. plantarum SNI3 and mice receiving the SNI3 microbiome, while administration of γ-GluGlu had no effect, indicating that this metabolite is not solely responsible for the observed hormonal changes. Shin et al., previously reported a strong correlation between testosterone levels and gut microbiota diversity, suggesting that microbial composition plays a crucial role in steroid hormone regulation (159). This peripheral regulation is further supported by the unchanged expression of hypothalamic GnRH and pituitary FSH and LH genes in *L. plantarum* SNI3-treated mice. Similarly, the expression of the testicular inhibin gene, which influences the regulation of testosterone feedback, was not altered. Our results imply that the observed hormonal changes were likely mediated through microbiome-driven metabolic pathways rather than direct endocrine modulation. Previous findings reported that 7 days of colistin, as a narrow spectrum antibiotic treatment, significantly reduced serum testosterone levels, primarily through the selective depletion of Akkermansia muciniphila (160). This microbial shift was associated with the downregulation of fatty acid metabolism, pyruvate metabolism and purine metabolism pathways critical for steroidogenesis. Interestingly, our findings contrast with this model. Despite observing a marked reduction in Akkermansia abundance, the serum testosterone level was significantly increased in *L. plantarum* SNI3-treated mice, suggesting an alternative mechanism at play. These findings indicate that testosterone regulation is likely influenced by a more intricate microbiome-mediated mechanism beyond the direct or indirect effects of individual microbial species. One possible mechanism explaining this difference is microbiota-driven testosterone deglucuronidation, a process that may enhance hormone bioavailability and systemic

balance (161, 162). In general, Lactobacilli, including *L. plantarum* SNI3, display low glucuronidase activity (163). Therefore, the elevation of testosterone levels is likely influenced by other bacterial species that possess higher glucuronidase activity, facilitating testosterone deglucuronidation and increasing its bioavailability

5.2. Gut microbiome-brain axis

The dynamic changes in the gut microbiome provide new insight into its regulatory role in reproductive health, despite having no direct impact on hypothalamic-pituitary-gonad-gonad (HPG) axis activity or its feedback mechanism. Although the concept of "reprobiotic" is not yet established in the literature, probiotic treatments have been extensively studied in the context of psychobiotics (164) due to their influence on central nervous system function and behavior. Building on this, we investigated the effects of *L. plantarum* SNI3 on behavior. The test of sexual behavior revealed that *L. plantarum* SNI3-treated male mice showed increased reproductive activity, spending more time mounting and vaginal sniffing of female mice, compared to controls. These behavioral parameters align with previous findings as indicators of enhanced sexual motivation and mating drive (127). In high-fat diet (HFD) + streptozotocin (STZ) models, Edem et al. (165) demonstrated that hyperinsulinemia reduced the frequency of mounting and intromissions. However, probiotic treatment with *L. plantarum* was able to restore sexual activity. Notably, in that experiment, the control probiotics group had no effect on sexual activity and reproductive function. One possible regulatory mechanism underlying these effects is the elevated serum testosterone levels observed in *L. plantarum* SNI3-treated mice, which may contribute to increased sexual activity. However, the precise mechanism linking the gut microbiome, endocrine function and sexual behavior remains unclear. Studies have suggested that oxytocin (OXT) release from the paraventricular hypothalamus (PVN) to extrahypothalamic regions plays an important role in erectile function and overall sexual behavior (166). In support of this, we have detected increased hypothalamic OXT gene expression levels in mice with *L. plantarum SNI3*, but further research is required to elucidate the specific pathways through which the gut microbiome modulates neuroendocrine regulation of sexual activity.

Previous studies reported that certain strains of Lactobacillus can attenuate anxiety-like behavior in rodents (167, 168). Consistent with these results, our marble burying test

showed that *L. plantarum* SNI3 treatment reduced compulsive and anxiety-like behavior as evidenced by a significant reduction in the number of buried marbles compared to controls. However, no significant differences were observed in locomotor activity or exploratory behavior assessed by the open field test. This indicates that the anxiolytic effect of *L. plantarum* SNI3 was not associated with changes in activity levels. Interestingly, these behavioral effects were further supported by the social interaction test, which served as an acute stressor in our study. *L. plantarum* SNI3-treated mice displayed enhanced social behavior rather than aggression with CD1 male mice, which was accompanied by reduced serum corticosterone (CORT) levels compared to controls. Together, these findings suggest a potential role of *L. plantarum* SNI3 in promoting stress resilience during acute stress through modulation of the gut microbiome-brain axis.

A previous study reported that the gut microbiome influences corticosterone regulation, particularly in response to social interactions (169). Notably, antibiotic-treated (Abx) mice displayed impaired social activity and significantly elevated CORT levels following one hour of social interaction, despite showing no differences in c-Fos density in the PVN compared to control-stressed mice (170). This indicates that microbiome depletion alters HPA axis response to social stimuli without directly affecting PVN neuronal activation patterns. Moreover, microbiota modulation through different antibiotic combinations resulted in variable corticosterone responses, highlighting the strain-specific and functional diversity of gut microbiota in stress regulation. Treatment with ampicillin, vancomycin, and metronidazole (AVM) led to a greater reduction in CORT level following social interactions than AVM combined with neomycin (AVM+N) (170). These findings suggest that certain microbial taxa targeted by neomycin may contribute to corticosterone regulation, potentially influencing HPA axis sensitivity to social experiences.

Additionally, exposure to a single 2-hour social stressor altered the colonic mucosa-associated microbiota communities, particularly by inducing a significant reduction in the abundance of *Lactobacillus* (171). This underlines the sensitivity of *Lactobacillus* to stress-induced changes, supporting a bidirectional interaction between the microbiota and stress regulation. Given this susceptibility of *Lactobacillus*, we further investigated how *L. plantarum* SNI3-treated mice responded to chronic variable stress (CVS), a paradigm known to induce HPA axis dysregulation and neuroendocrine adaptations (172).

Following 4 weeks of CVS exposure, we observed a significant elevation in serum corticosterone (CORT) levels and increased adrenal gland weight in control CVS-stressed animals, consistent with previous findings that chronic stress activates the HPA axis and increases systemic glucocorticoid responses (173). However, while the increased weight of the adrenal gland was also observed in *L. plantarum* SNI3-treated CVS mice, their CORT levels remained comparable to non-stressed control mice. This suggests that L. plantarum SNI3 treatment mitigated stress-induced HPA axis overactivation, potentially through microbial metabolite modulation of glucocorticoid receptor signalling. These findings follow previous studies, which showed that increased *Lactobacillus* abundance was associated with enhanced stress resilience in mice exposed to chronic defeat stress, as oral *Lactobacillus* murinus attenuated the CORT level in stressed animals (174). For instance, one key pathway is short chain fatty acids (SCFAs) such as butyrate, acetate and propionate, which enhance glucocorticoid receptor (GR) sensitivity, suppress neuroinflammation and stabilize corticosterone feedback mechanism, thereby preventing prolonged activation of the HPA axis (175). On the other hand, Lactobacillus may also affect local glucocorticoid metabolism by downregulating 11β-HSD1 expression in the gut, thus mitigating systemic glucocorticoid overproduction and attenuating HPA axis hyperactivity (176).

Our analysis revealed no detectable alterations in the total DNA level from colon content samples of *L. plantarum* SNI3, suggesting that bacterial treatment may have influenced stress resilience through functional alterations in microbiota composition or microbial metabolite production. In addition, chronic variable stress (CVS) exposure resulted in reduced serum testosterone levels in *L. plantarum* SNI3 + CVS mice compared to non-stressed *L. plantarum* SNI3-treated mice, highlighting the well-established inhibitory effects of chronic stress on the hypothalamic-pituitary-gonadal axis-mediated steroidogenesis (177). Despite these CVS-induced physiological alterations, no significant anxiety-like or depressive-like behavioral differences were observed in the open field test between groups. One possible explanation may be that animals exhibited stress-resilient behavior or adaptation due to the chronic stress protocol (178). Using a two-hit stress protocol, where early life stress (maternal separation (MS) combined with chronic variable stress (CVS) exacerbated anxiety-like behavior (126). Kuti et al. also found that MS+CVS exposure resulted in gut permeability dysfunction and microbiota

dysbiosis (126), further linking gut microbial alterations with behavioral phenotypes. Previous studies reported a strong association between microbiota alterations and stress-related behavior outcomes (179), confirming the hypothesis that gut microbiome-brain axis dysfunction contributes to the long-term neurobiological effects of chronic stress (180, 181).

Interestingly, a short-term 3-day broad-spectrum antibiotic (Abx) treatment resulted in reduced bacterial DNA levels in colon content. However, despite these alterations, no significant changes in anxiety-like behavior were observed in the open field and elevated plus maze (EPM) test. Similarly, rifaximin, a non-absorbable antibiotic that selectively targets pathogenic bacteria, neither altered total DNA levels in colon content nor affected anxiety-like behaviors under non-stressed and combined maternal separation with chronic variable stress (MS+CVS) conditions. In contrast, previous studies demonstrated that long-term antibiotic exposure induced profound gut dysbiosis, leading to alterations in serotonergic synapses, neuroactive ligand receptor interaction and methionine metabolism, which collectively contribute to anxiety-like behavior (182).

These findings emphasize the therapeutic potential of targeted microbiota-based interventions in mitigating stress-related disorders and optimizing reproductive health. Future research should focus on the precise identification of microbial metabolites and host pathways involved, which may pave the way for novel microbiome-targeted therapies in human health and disease.

6. Conclusions

The main conclusions and novel scientific findings of my dissertation are:

1. Broad-spectrum antibiotic treatment disrupts gut microbiota and reduces sperm count, while having no impact on serum testosterone level and behavioral changes

2. Non-absorbable antibiotic rifaximin treatment did not affect colonic microbial DNA level or anxiety-like behavior, even under chronic stress conditions.

3. Probiotic *L plantarum* SNI3 enhances reproductive health through increased sperm count, improved testicular morphology, elevated serum testosterone and promoting prosocial and sexual behavior. It also improves stress resilience as evidenced by attenuated corticosterone responses to acute and chronic stress.

4. Gut microbiota mediates the reproductive benefits of *L. plantarum* SNI3 treatment. Fecal microbiota transplantation (FMT) from Lbsni3-treated mice restored sperm number and testosterone levels in antibiotic-depleted recipients, confirming the role of microbiota.

5. The gut microbiome-derived metabolites are essential in mediating the pro-reproductive effect of *L. plantarum* SNI3. Elevated levels of γ-glutamyl-glutamate (γ-GluGlu) in the testis contributed to increased sperm production, as confirmed by the direct administration of the metabolite.

In conclusion, the findings provide novel insights into the gut microbiome-brain and gut microbiome-testis axis, highlighting microbiome-targeting strategies as promising tools for improving men's reproductive and behavioral health.

7. Summary

The role of the gut microbiome in host physiology is receiving growing attention. Antibiotics, pro-, pre-, post-, and parabiotics are valid means with which to manipulate the gut microbiome, however specific mechanisms, particularly along the gut-brain and gut-testis axis, are still unclear.

In my dissertation, first, I provided evidence that a broad-spectrum antibiotic mix and a non-absorbable gut-specific antibiotic had no effect on the basal and stress-induced anxiety behavior of mice, although these antibiotics have a differential effect on the gut microbiome. Next, we investigated the effect of a novel probiotic lactobacillus strain, *Lactiplantibacillus plantarum* SNI3 (LbSNI3) on the microbiome composition and aligned these changes to behavioral and bodily parameters in male mice. LbSNI3 treatment increased the abundance of the *Lactiplantibacillus* and *Lachnospiraceae NK4A136* group while reducing *Akkermansia muciniphila*. These microbiota changes were accompanied by reduced anxiety-like behavior, enhanced pro-social behavior, and reduced corticosterone response to both acute and chronic stress. In addition, the LbSNI3-treated mice displayed more active sexual behavior.

The most striking finding of our study is that LbSNI3 treatment significantly improved reproductive parameters in mice, including increased testis size, sperm count and motility, elevated serum testosterone levels and morphological changes in seminiferous tubules, all indicating enhanced fertility. Fecal microbiota transplantation from LbSNI3-treated mice highlighted that the pro-reproductive effect is dependent on the gut microbiome. In search for compounds that mediate effects of LbSNI3, using in situ metabolomics, we have identified and validated a dipeptide metabolite, gamma-glutamyl glutamate as a potent postbiotic.

Since current data are limited on the microbiome-reproduction relationship, our studies provide new insights into the role of the gut microbiome in regulating host reproductive function, supporting the future therapeutic potential of probiotic-based interventions.

8. Összefoglalás

A bélmikrobiom szerepe a gazdaszervezet élettani folyamataiban egyre nagyobb figyelmet kap. Az antibiotikumok, valamint a pro., pre-, poszt-, és parabiotikumok hatékony eszközei a bélmikrobióta befolyásolásának, azonban a bél-agy és bél-here tengely mentén működő mechanizmusok még nem teljesen ismertek.

Disszertációmban elsőként bemutattam, hogy egy széles spektrumú antibiotikumkeverék, illetve egy nem felszívódó, bél-specifikus antibiotikum nem befolyásolta az egerek alap- és stressz helyzetben megnyilvánuló szorongásos viselkedést habár eltérő módon módosították a bélmikrobióta összetételét. Ezután egy új probiotikus törzs, a *Lactiplantibacillus plantarum* SNI3 (LbSNI3) hatásait vizsgáltuk a bélmikrobióta összetételére, valamint az ezzel összefüggő viselkedéses és testi paraméterekre hím egerekben. Az LbSNI3 kezelés fokozta a *Lactiplantibacillus* és a *Lachnospiraceae* NK4A136 arányát, miközben csökkent szorongásszerű viselkedés, fokozott szociális aktivitás és csökkent kortikoszteron-válasz kísérte akut és krónikus stressz hatására. Emellett az LbSNI3 kezelés fokozta a szexuális aktivitást.

A legkiemelkedőbb eredményünk, hogy az LbSNI3 kezelés jelentősen fokozta az egerek reprodukciós paramétereit, beleértve a herék tömegének, a spermiumok számának és mozgékonyságának növekedését, a szérum tesztoszteron szintjének emelkedését, valamint a herecsatornák morfológiai változásait, ami mind a fokozott termékenységre utal. Az LbSNI3-mal kezelt egerekből származó székletmikrobióta átültetés rávilágított arra, hogy a reprodukciót elősegítő hatás a bélmikrobiomtól függ. Az LbSNI3 hatását közvetítő vegyületek keresése során in situ metabolomika segítségével azonosítottunk és validáltunk egy dipeptid metabolitot, a gamma-glutamil-glutamátot, mint hatásos posztbiotikum.

Mivel a mikrobiom-reprodukció kapcsolatáról jelenleg kevés adat áll rendelkezésre, vizsgálataink új ismerettekkel szolgálnak, a bélmikrobiom és a gazdaszervezet reproduktív szabályozásában, elősegítve a probiotikus alapú beavatkozások jövőbeli terápiás potenciálját.

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10. Publications of the author

Publications that form the basis of the Ph.D. dissertation:

Juhász B, Horváth K, Kuti D, Shen J, Feuchtinger A, Zhang C, Bata-Vidács I, Nagy I, Kukolya J, Witting M, Baranyi M, Ferenczi Sz, Walch A, Sun N, Kovács KJ.
Dipeptide metabolite, glutamyl-glutamate mediates microbe-host interaction to boost spermatogenesis. ***SCIENTIFIC REPORTS*** 14:1 pp: 21864, 18 p. (2024)

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Gastrointestinal (non-systemic) antibiotic rifaximin differentially affects chronic stress-induced changes in colon microbiome and gut permeability without effect on behavior. ***BRAIN, BEHAVIOR, AND IMMUNITY*** 84pp. 218-228, 11p. (2020)

Other publications:

Horváth K, Vági P, **Juhász B**, Kuti D, Ferenczi S, Kovács KJ. Sex Differences in the Neuroendocrine Stress Response: A View from a CRH-Reporting Mouse Line. ***INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES*** 25(22):12004. (2024)

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