CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Biological and Medical Sciences

DIPLOMA THESIS

SEXUAL DIMORPHISM OF RAT GUT MICROBIOTA COMPOSITION AND INTESTINAL IMMUNITY

Supervisor: PharmDr. Miroslav Kovařík, Ph.D.

Consultants: Dr. Francisco J. Pérez Cano, Dr. Malen Massot-Cladera

HRADEC KRÁLOVÉ 2019 Pavla Kalousová

KARLOVA UNIVERZITA

FARMACEUTICKÁ FAKULTA HRADEC KRÁLOVÉ

Katedra biologických a lékařských věd

DIPLOMOVÁ PRÁCE

SEXUÁLNÍ DIMORFISMUS VE SLOŽENÍ STŘEVNÍ MIKROFLÓRY A STŘEVNÍ IMUNITY U POTKANA

Vedoucí práce: PharmDr. Miroslav Kovařík, Ph.D.

Konzultanti: Dr. Francisco J. Pérez Cano, Dr. Malen Massot-Cladera

HRADEC KRÁLOVÉ 2019

Pavla Kalousová

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Hradec Králové ……..

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ABSTRACT

Charles University
Faculty of Pharmacy in Hradec Králové
Department of Biological and Medical Sciences
Candidate: Pavla Kalousová
Supervisor: PharmDr. Miroslav Kovařík, Ph.D.
Title: Sexual dimorphism of rat gut microbiota composition and intestinal immunity

Background and Aim: Many factors can influence the composition of gut microbiota and the immune system. It is well-known that one of those factors is sex. This sexual dimorphism can lead to a specifically adjusted treatment of diseases for different gender and nutritional interventions. This study focuses on analysing sexual differences in gut microbiota and intestinal immunity in adult rats.

Methods: Caecal content from 12-week-old female and male Wistar rats were collected and analysed by DNA-sequencing technique to characterize microbiota composition. ELISA test was performed to quantify the concentration of IgA in faeces and gut wash. Flow cytometry determined the concentration of IgA-coating bacteria in faecal samples.

Results: Metagenomic analysis revealed that female gender presents 1 phylum, 4 families, 13 genera, and 13 species which are not present in male rats. Only one male-specific colonization was observed at the species level. Quantitative analysis showed a higher proportion of *Firmicutes* phylum in males which was associated with higher *Lactobacillaceae* and *Lactobacillus animalis.* Female rats showed higher occurrence in *Verrucomicrobia* phylum associated with *Akkermansiaceae* and *Akkermansia muciniphila,* and *Bacteroidetes* phylum which was increased due to a higher proportion of *Prevotellaceae – Prevotella shahii*, *P. stercorea* and *Porphyromonadaceae.* Moreover, female rats displayed a significantly higher proportion of individual species *Roseburia faecis* and families *Ruminococcaceae, Oscillospiraceae.* No sexual differences in intestinal IgA nor in IgA-coated bacteria were observed.

Conclusion: Sexual dimorphism does occur in microbiota composition and diversity in rats. This fact should be considered in the treatment of diseases associated with a disruption in the intestine and also in designing preclinical intervention studies related to the intestinal examination.

Keywords: gut microbiota, intestine, immune system, rat, sexual dimorphism

ABSTRAKT

Univerzita Karlova
Farmaceutická fakulta v Hradci Králové
Katedra biologických a lékařských věd
Kandidát: Pavla Kalousová
Školitel: PharmDr. Miroslav Kovařík, Ph.D.
Název práce: Sexuální dimorfismus ve složení střevní mikroflóry a střevní imunity u potkana

Pozadí a cíl: Mnoho faktorů může ovlivnit složení střevní mikroflóry a imunitního systému. Je dobře známo, že jedním z těchto faktorů je pohlaví. Tento pohlavní dimorfismus může vést k speciálně upravené léčbě nemocí pro jednotlivé pohlaví a také při nutričních opatřeních. Tato práce cílila na analýzu pohlavních rozdílů u střevní mikroflóry a imunity u dospělých potkanů.

Metody: Jako testovací skupina byli použiti 12 týdnů staří potkani mužského a ženského pohlaví kmene Wistar. Střevní obsah byl zanalyzován DNA-sekvenční metodou k identifikaci složení střevní mikrobů. Pomocí metody ELISA byla provedena detekce koncentrace IgA ze vzorku exkrementů a střevního obsahu. Průtoková cytometrie při analýze vzorku exkrementů stanovila hladinu IgA-obalených bakterií.

Výsledky: Metagenomická analýza objevila 1 specificky samičí řád, 4 rodiny, 13 rodů a 13 druhů, které nejsou přítomné u samčích potkanů. Jen jeden druh mikroba byl identifikován jako specificky samčí. Kvantitativní analýza ukázala vyšší poměr řádu *Firmicutes* u mužského pohlaví, který je dále spojen s vyšším výskytem rodiny *Lactobacillaceae* a *Lactobacillus animalis.* Potkani ženského pohlaví se projevili vyšším poměrem řádu *Verrucomicrobia*, spojené s *Akkermansiaceae* a *Akkermansia muciniphila* a řádu *Bacteroidetes*, který byl zvýšen v důsledku vyššího výskytu *Prevotellaceae – Prevotella shahii, P. stercorea* a *Porphyromonadaceae.* Potkani samičího pohlaví měly také zvýšený počet osamoceně postaveného druhu *Roseburia faecis* a rodin *Ruminococcaceae, Oscillospiraceae.* Při analýze střevní IgA a IgA-obalených bakterií nebyly nalezeny žádné rozdíly v pohlaví.

Závěr: Existence pohlavního dimorfismu byla prokázána při analýze rozmanitosti a složení střevní mikroflóry u potkanů. Tento fakt by měl být brán v úvahu při léčbě nemocí spojených s narušením střevního traktu a také při projektování preklinických studií, které mají, co dočinění se zkoumáním střevních mechanismů a funkcí.

Klíčová slova: střevní mikrobiota, střevo, imunitní systém, potkan, sexuální dimorfismus

1 INTRODUCTION

The intestinal tract is encrusted by trillions of microbial organisms forming together the gut microbiota (GM). This term represents all bacteria, viruses, and fungi, which pass through the intestine. The total number of GM cells existing in the intestine surpasses ~ 10 times the number of human body cells. The long evolution shaped the ideal composition of the GM and created a beneficial relationship between host organism and present microorganisms (Thursby & Juge, 2017). The GM disposes of abilities crucial for the proper function of the intestine and gut homeostasis such as digesting, energy harvesting, the creation of vitamins, and cooperation on the maturation of gut immunity. Many factors influence the composition of microbiota in the intestine. Genetics, sex, age, and primarily diet affect the structure and abundance of the microbiota living in the intestine and ensure unique composition for every individual.

The intestinal tract encounters a great number of pathogens that try to enter into the body and cause harm. For this reason, immunity in the intestine needs to be in high quality and well-regulated for recognition of self-antigens and harmful invaders. Studies with germ-free animals[1] (GF-animals) have demonstrated the essential role of GM in shaping gut immunity. In the state of microbiotic deficiency, both innate and adaptive immunity are altered and malfunctioning, which causes dysbiosis, persistent inflammations and disruption in gut homeostasis (Min & Rhee, 2015). In present days many studies are unravelling the importance of properly functioning intestine and beneficial and crucial asset of intestinal microbiota. Studies suggest that malfunctioning intestine immunity or disturbance in GM are connected with multiple severe diseases such as functional dyspepsia, severe diarrhoea, inflammatory bowel disease (IBD), colorectal cancer, celiac disease, and irritable bowel syndrome (IBS) (Nagao-Kitamoto, Kitamoto, Kuffa, & Kamada, 2016), liver diseases (Tilg, Cani, & Mayer, 2016), obesity, and several psychiatric and neurologic disorders (Martin, Osadchiy, Kalani, & Mayer, 2018).

To successfully manage or cure these diseases we need to understand the mechanisms of cooperation between the host organism and the GM. One of these complex mechanisms important for treatment is an alteration in immune responses based on sexual dimorphism. It is widely known that females have a stronger immune system (IS) but are more inclined to autoimmune diseases (Fransen et al., 2017). In this study, we compare the composition of GM in male and female rats to see if there is any sex difference in the present GM and gut immunity.

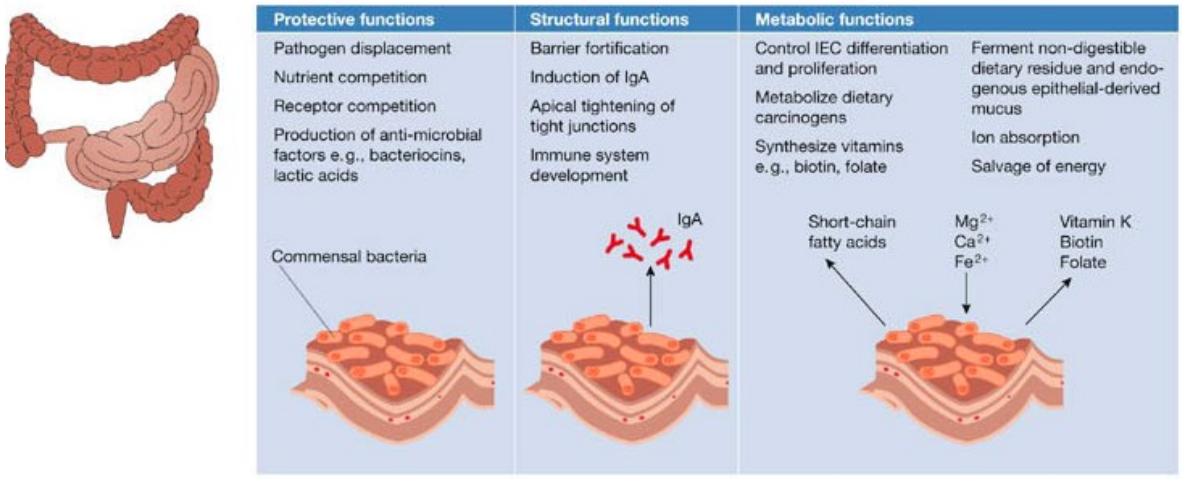
[1] Germ-free animals are animals raised in the absence of live microbes (usually rodents)

2 THEORETICAL PART

2.1 GUT MICROBIOTA

GM refers to over trillions of microbes harbored in the gastrointestinal tract (Ley, Peterson, & Gordon, 2006). This complex symbiotic relationship was co-evolved over centuries and has significant importance during homeostasis and in the state of disease. The function of GM varies and affects multiple structures and processes such as a protective barrier, nutrition, energy or mucosal IS (O'Hara & Shanahan, 2006) (Picture 1). One of the protective function of the GM is to compete with harmful pathogens over nutrients and intestinal niches, which eliminate the potential overgrowth of pathogens in the intestine, this phenomenon is called colonization resistance (Rolhion & Chassaing, 2016). The metabolic benefit includes anaerobic fermentation of non-digestible diet. The result of the fermentation is the production of short chain fatty acids (SCFAs) as an important part of cholesterol metabolism and source of energy (Valdes, Walter, Segal, & Spector, 2018). The GM is also responsible for the synthesis of vitamin K and elements of vitamin B (Ramakrishna, 2013). The effect of GM on the IS will be discussed in the next chapter.

If these mechanisms are altered, a condition called dysbiosis occurs. According to Petersen and Round (Petersen & Round, 2014) ,,*Dysbiosis is any change to the composition of resident commensal communities relative to the community found in healthy individuals"*. This modification in microbial composition and consequently the disruption in beneficial relationship contributes to development of gastrointestinal diseases such as IBD, IBS, functional dyspepsia, severe diarrhoea, celiac disease, colorectal cancer and can also be partly responsible for progress of other serious and complex diseases (diabetes, obesity, cardiovascular and central nervous system disorders, etc.) (Belizário & Faintuch, 2018; Nagao-Kitamoto et al., 2016). There are several factors that can cause microbial dysfunction – intrinsic factors include genetics, psychological and physical stress, altered peristalsis and aging. The extrinsic factors cover diet, radiation, appendectomy and antibiotic use, which is the most common and severe (Hawrelak & Myers, 2004; Nagao-Kitamoto et al., 2016).



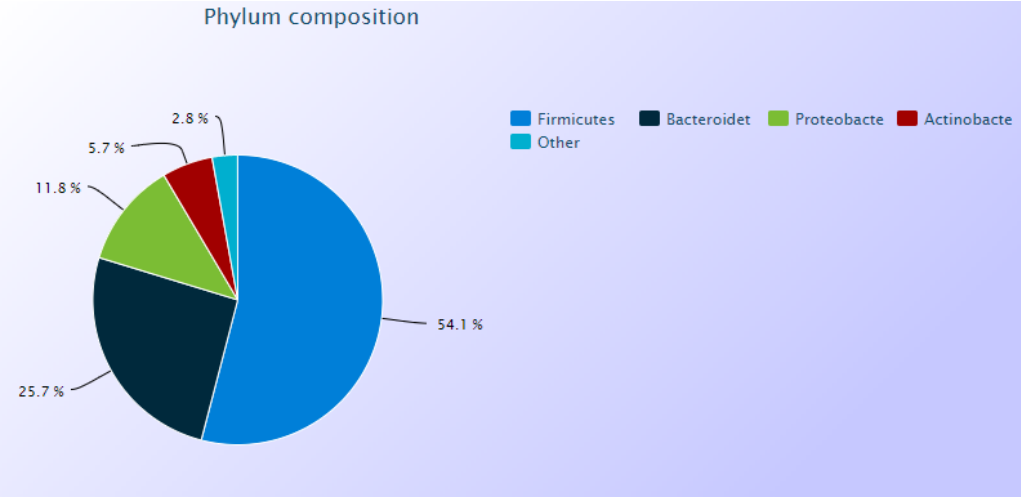
Picture 1 Summary of the function of gut microbiota – protective, structural, metabolic.
Source: (O'Hara & Shanahan, 2006)

2.1.1 Healthy gut microbiota composition

Until the beginning of the 21st century the methods to identify the scope of microbiota, inhabiting the gastrointestinal tract, were based on highly time-consuming and insensitive culture techniques. Nowadays this ability has improved due to more effective and also cheaper sequencing methods. These techniques implement the fact that 16S ribosomal RNA (rRNA) gene is part of every bacteria and archaeon. Furthermore, the gene includes 9 highly variable regions (V1-V9), which provide recognition of taxonomic species (Thursby & Juge, 2017).

To set a concrete composition of GM in human has proven to be quite difficult. Many environmental and biological factors – age, diet, genetics, etc. influence the abundance and diversity of microbiota between individuals (Kurokawa et al., 2007; Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012; Thursby & Juge, 2017). Many studies agree on elementary bacterial composition. The majority of bacteria is represented by phylum *Firmicutes* and *Bacteroidetes.* In lower percentage are presented *Proteobacteria, Actinobacteria,* and *Verrucomicrobia* (Picture 2). Species dominating intestinal flora taxonomically belongs under *Firmicutes* and are sub-grouped in *Clostridium coccoides* (Clostridium cluster XIVa) and *Clostridium leptum* (Clostridium cluster IV). *Bacteroidetes* phylum is mainly represented by *Prevotella* and *Porphyromonas* (Bibbo et al., 2016; Eckburg et al., 2005; Hold, Pryde, Russell, Furrie, & Flint, 2002; Lozupone et al., 2012; Tap et al., 2009).

Regarding GM composition, it is also important to mention less occurring organisms contributing to overall function. These include methanogenic archaea (*Methanobrevibacter smithii*), fungi (*Candida* genus) and viruses (mainly bacteriophages) (Gagliardi et al., 2018; Ianiro et al., 2014; Lozupone et al., 2012).

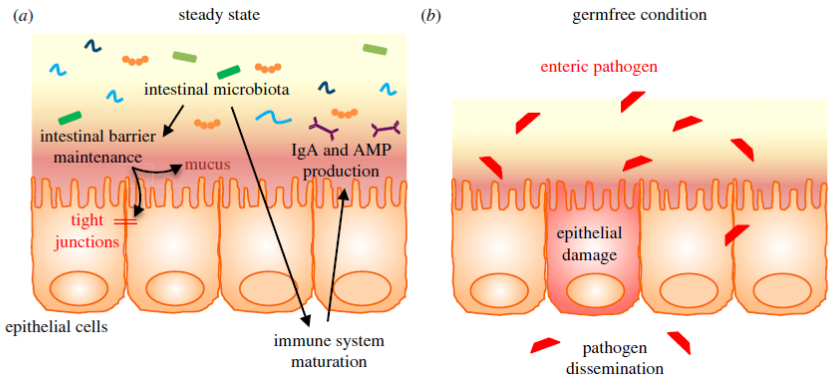


Picture 2 Graphic illustration of composition of phylum in human intestine.
Source: (Li et al., 2014)

2.2 IMMUNOLOGY

2.2.1 General mucosal immunity in the intestine

The general role of the gastrointestinal IS is to keep a balance between the eradication of harmful pathogens and tolerance of self-antigens, harmless food, and commensal bacteria (Picture 3). If this immune homeostasis is disturbed, altered conditions in host-microbiota symbiosis can lead to autoimmune inflammatory diseases or systematic disorders (Platt, n.d.).



Picture 3 Microbiota/host homeostasis in the intestine *(a) Intestine with healthy microbiota provides mature IS and effective immune responses (b) In the state of GM absence, the intestinal epithelium is damaged and pathogens can enter further into the host. AMP = antimicrobial peptide.*

Source: (Rolhion & Chassaing, 2016)

2.2.2 Innate immunity

Innate immunity is a first-line defense system that is composed of many components (Picture 4). One of the most important is the recognition and tolerance of normal host flora. The system depends on several families of pattern recognition receptors (PRRs) which are responsible for identifying pathogen-associated molecular patterns (PAMPs) and can trigger an innate immune response such as inflammatory response and phagocytosis. The family of PRRs includes Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like (NOD-like) receptors (Alberts B, Johnson A, Lewis J, 2002a; Lin & Zhang, 2017; McDonald & Levy, 2019; Porcelli, 2017).

Another part of immediate defense is gut epithelium consisted of intestinal epithelial cells (IECs). IECs represent physical and biochemical barrier separating host immune cells in lamina propria and GM. The separation is provided by mucosal activity (goblet cells), secretion of immunological mediators and bacterial antigens delivery (Chassaing, Kumar, Baker, Singh, & Vijay-Kumar, 2014; Peterson & Artis, 2014). The segregation is also supported by chemical substances for example acidity, detergents, proteolytic enzymes, lysozyme, defensins[2], and antibacterial proteins, which are produced mainly by Paneth cells in the small intestine. In case of failure of these barriers and penetration, there is the possibility of unregulated intestinal inflammation (Chassaing et al., 2014; Okumura & Takeda, 2017; Peterson & Artis, 2014).

[2] Defensins: A family of potent antibiotics made within the body; increase membrane permeability in bacteria, fungi and viruses

Mechanical Barrier	Epithelial cell monolayer, intestinal motility
Immunoglobulins	Secretory IgA*
Antimicrobial peptides and proteins	Defensins, lysozyme, secretory phospholipase A2, angiogenins
Microbial	Commensal intestinal flora
Others	Gastric acid, biliary and pancreatic secretion, mucins
* Although by definition belonging to adaptive immunity, S-IgA acts in first line mucosal defense. In this study, it is discussed in chapter 2.2.4. Secretory IgA	

Picture 4 Summary of mechanisms contributing to the innate immunity in the gut.

Source: (Yuan & Walker, 2004)

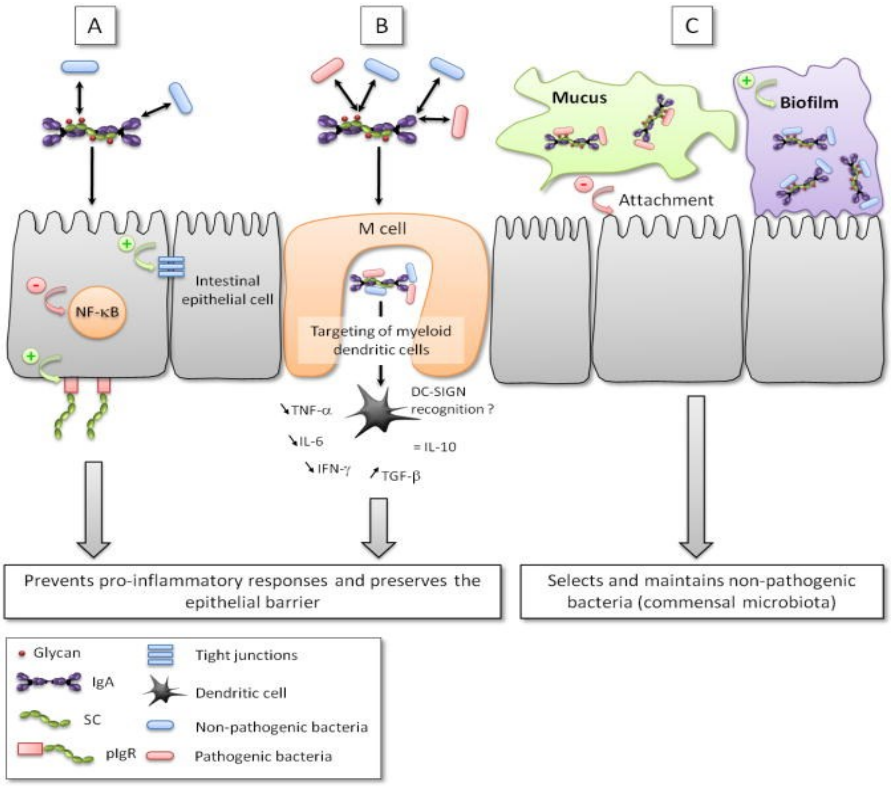
2.2.3 Adaptive immunity

Adaptive immunity is pathogen-specific targeted immunity response. It is activated by innate immunity and dendritic cells (DCs). Innate immunity, specifically an antigen-presenting cell (APC) such as phagocyte and macrophage, displays proteins characteristic for a pathogen (antigen) and this alerts the T-cells. The adaptive immune response can also be triggered by PRRs located, among others, on DCs. After recognition of PAMPs, the cells present those proteins to T-lymphocytes and at the same time start producing cytokines, which attract both innate and adaptive immunity to the infected location (Iwasaki & Medzhitov, 2015; Janeway CA Jr, Travers P, Walport M, 2001). The response is then carried out by 2 types of white blood cells – T and B lymphocytes. B cells, once activated and differentiated, are responsible for antibody production (immunoglobulins). The antibody inactivates pathogen and marks it for further destruction mostly by phagocytosis. T cells are composed of various subtypes and can fight with antigen directly (Alberts B, Johnson A, Lewis J, 2002b).

2.2.4 Secretory IgA

Secretory IgA (S-IgA) plays a role as first-line actors in antigen-specific immunity response in the gut (Picture 5). The secretion of S-IgA in healthy humans is up to 3g/day and up to 74% of intestinal bacteria are coated with S-IgA, which makes it the most abundant immunoglobulin in the gut lumen (Rogier, Frantz, Bruno, & Kaetzel, 2014). The S-IgA has several functions (Chassaing et al., 2014; Mantis, Rol, & Corthésy, 2011):

- prevent the penetration of commensal bacteria and pathogens in the intestinal epithelium by blocking their access to receptors, entrapping them in mucus, and facilitating their removal
- protection against enteropathogens
- promotion of the retro-transport of antigens across the intestinal epithelium to DCs through M-cells
- down-regulation of pro-inflammatory responses (usually associated with potential allergens and highly pathogenic bacteria)
- influence the composition of the intestinal microbiota

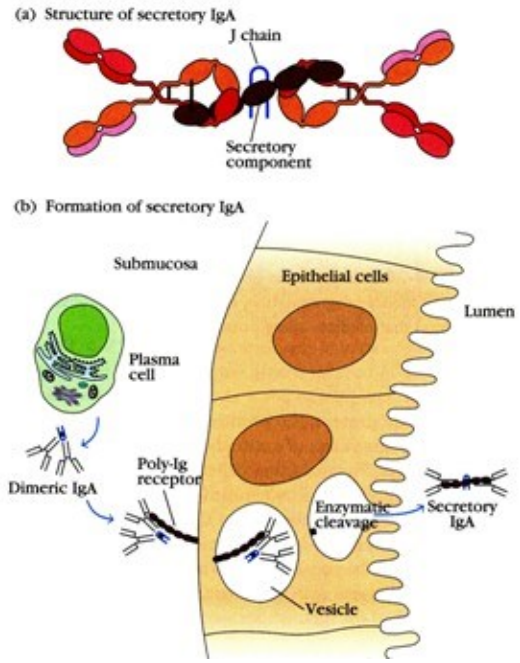


***Picture 5 Function of S-IgA in the intestinal mucosa**. (A) Non-pathogenic bacteria coated by S-IgA strengthen the intestinal epithelium e.g. improvement of tight junctions, higher production of pIgR and lower NF-κB nuclear translocation. (B) Complexes of S-IgA and non-pathogenic/pathogenic bacteria are carried through M cells to DCs*

*(binding location probably DC-SIGN). In DCs they reduce pro-inflammatory responses represented by ILs and other cytokines. (C) S-IgA keeps pathogenic bacteria in mucus and thus eliminates them from the epithelium. Close to the epithelium, S-IgA also promotes the creation of biofilm of non-pathogenic bacteria. **+**: activation effect; **-:** inhibitory effect; **NF-κB**: nuclear factor kappa-light-chain-enhancer of activated B cells (role in the regulation of the immune response to infection); **M cells**: located in GALT of PPs, initiate mucosal immune responses and allow transport across the epithelial cell layer.*

Source: (Mantis et al., 2011)

S-IgA production depends on the sampling process provided by DCs as antigen-presenting cells. DCs are located in Peyer´s patches (PPs) in the small intestine and in isolated lymphoid follicles in the large intestine. PPs are part of gut-associated lymphoid tissues (GALTs), which is the designation for all lymphoid structures and aggregates lining up the intestine (Min & Rhee, 2015). DCs activate naïve B cells by specialized molecular footprint and signals to differentiate into IgA producing plasma cells. This promotion is possible primarily by the presence of intestinal commensal bacteria (Massacand et al., 2008). Switched IgA B cells endure recirculation from the PPs via mesenteric lymph nodes, blood stream in thoracic duct and back to intestinal *lamina propria* as plasma cells secreting IgA. In this state, IgA has a dimeric structure, which is linked by a J chain. Dimeric IgA binds to basolaterally located polymeric Ig receptor (pIgR) on epithelial cell and is transferred into the intestinal lumen (Macpherson, Geuking, Slack, Hapfelmeier, & McCoy, 2012; Massacand et al., 2008). After IgA is captured by pIgR and is being transported to the luminal surface, extracellular part of pIgR is degraded and covalently bound to IgA. This part is known as the secretory component (SC) and with IgA creates a complex of S-IgA (Picture 6). The secretory component has an important function for adaptive and innate immunity. It protects S-IgA from degradation by the microbial proteases and other enzymes present in the gut, which prolongs the lifetime of the antibody. Additionally supports innate immunity by glycan-dependent adherence to bacteria and neutralization of pro-inflammatory host factors (Mathias, Pais, Favre, Benyacoub, & Corthésy, 2014; Rogier et al., 2014).



Picture 6 Structure and formation of secretory IgA *(a) S-IgA is a structure comprised of at least two IgA molecules. They are covalently linked with J-chain and secretory component (SC). (b) On the basolateral membrane dimeric IgA binds to a poly-Ig receptor (pIgR) then it is transported by endocytosis to the luminal site. pIgR is then split and forms a complex of S-IgA with released SC.*

Source: (Fleischmann W. Robert, 2016)

2.2.5 Regulation of the mucosal immune system and immune responses

Gut mucosa consists of several immune cells and proteins which all contribute to the maintenance of immune homeostasis, protection against over-inflammation and tolerance to non-pathogenic bacteria and food. DCs play a key role as APC underlaying M-cells in *lamina propria* together with micro-environmental cytokines. Their presentation of antigen to T cells can result in differentiation to T regulatory cells (Tregs), Th1-type cell-mediated immunity or Th2-type antibody-mediated immunity (Bio-Rad Laboratories Inc., 2016; Gray & Letarte, n.d.) (Picture 7).

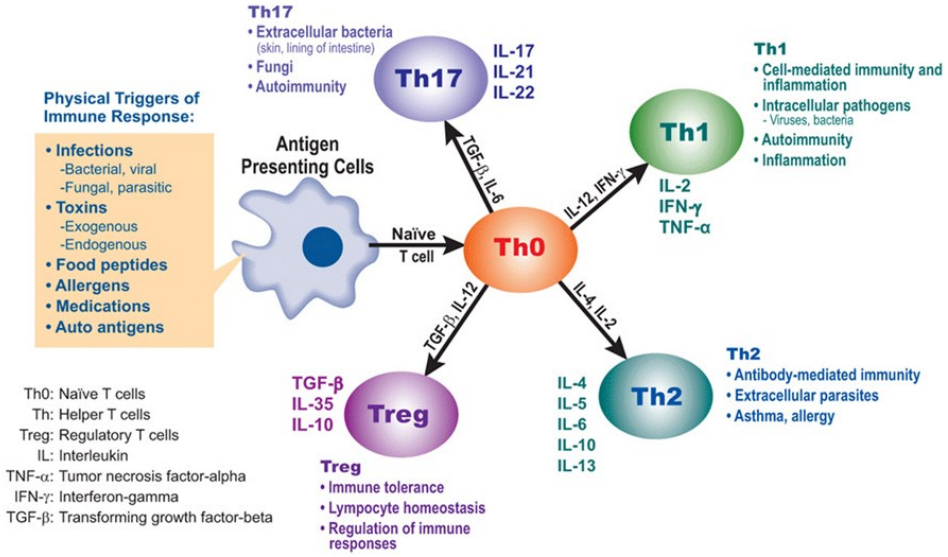
Treg cells are $CD4^+$ T-cell subset, which controls, suppresses and inhibits the inflammatory response. Induction of Treg cells depends mainly on Foxp3 (Forkhead box P3) transcription factor. Mutation in this factor can lead to altered Treg cells and cause allergy, infectious diseases, severe autoimmune disorders and disbalance in intestinal homeostasis (Harrison & Powrie, n.d.). Treg generation and expansion can also be promoted by DCs (Lin & Zhang, 2017). Treg cells produce regulatory cytokines such as IL-10 and transforming growth factor (TGF-β). Cytokine IL-10 decreases the production of Th1 cytokines (IL-1, IL-2, IFNγ, IL-12,

etc.) and regulates intestinal myeloid cell activity (Okumura & Takeda, 2016). TGF-β is secreted by many cell types and has various functions. Regarding immune regulation, it contributes to the promotion of Treg conversion and down-regulates Th1 and Th2 cell differentiation. Interestingly, TGF-β is also a key factor in the differentiation of pro-inflammatory Th-17 cells, which produce inflammatory cytokines (IL-17, IL-22). A possible explanation could be in the level of concentration of TGF-β (low concentration – pro-inflammatory effect; high concentration – anti-inflammatory effect) (Worthington, Fenton, Czajkowska, Klementowicz, & Travis, 2012).

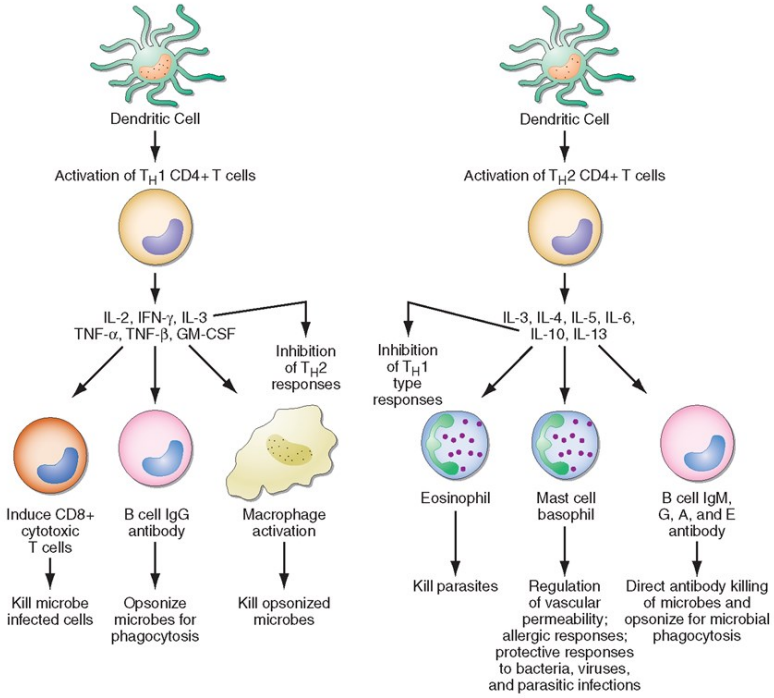
Recognition of harmful antigen presented by DCs to $CD4^+$ T-helper cell leads to the initiation of the immune response. Th0 cell (naïve T-helper cell) can induce transcription of its subsets Th1, Th2, and Th17. The characterization of differentiation is based on genetic sensitivity, type of presented antigen and types of released cytokines (Doyle, 2017).

Th1 transcription is triggered by interferons (IFN α, β, γ) and IL-12. Th1 cells further produce IFN-γ, IL-2 and TNF-β and are responsible for cell-mediated immunity[3] and inflammation targeting intracellular parasites such as bacteria and viruses. Th2 develop in response to the production of IL-4. They produce multiple cytokines IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and mostly fight against helminths and other extracellular parasites (Picture 8). These cytokines induce B-cell proliferation, antibody production, mast cell activation, and production of eosinophils (Doyle, 2017; Romagnani, 2000; Spellberg & Edwards Jr., 2001). For all subsets it is known, that production of their cytokines stimulate further production of own subset and at the same time inhibit the production of the other Th subset (Perkel, 2001; Romagnani, 2000).

[3] Cell-mediated immunity include phagocytosis, cytotoxic T-lymphocytes, release of cytokines



Picture 7 Differentiation of Th0 cells*. Conversion of naïve T cells is based on APC present antigen. Cytokines regulate the formation of a concrete subset of Th cells. Specific immune response and cytokine production depend on every Th subset. Misregulation can lead to an outbreak of severe diseases. (Th17 – autoimmunity; Th1 – autoimmunity, and inflammation; Th2 – asthma, and allergy). Source:* (Angel, 2013)



Picture 8 Detailed immune functions of Th1 and Th2. *Th1 cells are usually stimulated against intracellular bacteria or viruses. Th2 cells are mostly triggered by extracellular bacteria and parasites and use direct antibody defense.* ***GM-CSF*** *– granulocyte-macrophage colony stimulating factor;* ***IFN****-interferon;* ***TNF****-tumor necrosis factor;* ***IL****-interleukin. Source:* (Haynes, Soderberg, & Fauci, 2015)

2.2.6 How gut microbiota affects gut immunity

The process of colonization begins *in utero* and is changing throughout life. The biggest changes happen during the first few years of life. Colonization in infants is crucial for appropriate development of the IS (Houghteling & Walker, 2015; Marietta, Rishi, & Taneja, 2015). In addition, many studies with GF-animals revealed, the microbiota is necessary for shaping host innate and adaptive immunity (Belkaid & Hand, 2014; Min & Rhee, 2015) (Table 1).

GF mice show reduced development of GALT, structureless secondary lymphoid tissues (e.g. spleen, peripheral lymph nodes) and altered mucosal barrier function. Lack of microbiota results in lower expression and localization of PRR (TLRs, NODs), deficiency of antimicrobial peptides, and hypoplastic PPs. Effective units of adaptive immunity are disturbed due to the decreased number of IgA producing plasma cells, $CD4^+$ T-cells and related subsets. Inversion of these immunologic deficiencies can be achieved by administration of healthy commensal microbiota in GF mice (Belkaid & Hand, 2014; Lin & Zhang, 2017; Round & Mazmanian, 2009).

Intestinal organ development	Site	Phenotype in Germfree mice
Small Intestine	Peyers Patches	fewer, less cellular
	Lamina propria	thinner, less cellular
	Germinal centers	fewer plasma cells
	Isolated lymphoid follicles	smaller, less cellular
Mesenteric Lymph nodes	Germinal centers	smaller, less cellular
		fewer plasma cells

Cellular Defects	Cell Type	Phenotype in Germfree mice
Intestinal epithelial lymphocytes	CD8+ T cells	fewer, reduced cytotoxicity
Lamina propria lymphocytes	CD4+ T cells	proportional decrease in number
		decreased Th17 cells (Small intestine)
		increased Th17 cells (Colon)
Mesenteric lymph nodes	CD4+CD25+ T cells	reduced expression of Foxp3
		reduced suppressive capacity

Molecular immune deficiencies	Molecule	Phenotype in Germfree mice
Paneth Cells	Angiogenin-4	reduced expression
	RegIIIγ	reduced expression
B cells	Secretory IgA	reduced production
Intestine	ATP	reduced
Intestinal epithelial cells	MHC class II	reduced expression
	TLR 9	reduced expression
	IL-25	elevated

Table 1 Intestinal immunologic defects in germ-free mice. Isolated lymphoid follicles (ILFs) *are lymphoid structures contributing to homeostasis and production of S-IgA.* ***Mesenteric lymph nodes (MLNs)*** *are located at the base of the mesentery collecting lymph from the intestinal mucosa.* ***Germinal centers*** *are found in secondary lymphoid organs*

and serve as a site for proliferating, differentiating and maturation of B cells. ***Paneth cells*** *are located at the bases of the Lieberkühn crypt in small intestine secreting lysozyme.* ***RegIIIγ*** *is antibacterial peptide preventing microbial-epithelium interaction.* ***MHC class II (major histocompatibility complex) molecules*** *are located on APCs presenting processed antigens.* ***IL-25*** *is pro-inflammatory cytokine favoring Th2-type of immune response and belongs to IL-17 cytokine family. Source:* (Round & Mazmanian, 2009)

2.2.7 Gender differences in immunology and microbiota

Every individual is considered to have a specific composition of the microbiota. There are many factors that can have an effect on the relative abundance and composition (e.g. diet, BMI, age, genetic background, and sex hormones). Multiple studies in mice and human proved sex differences in microbiota composition (Elderman, de Vos, & Faas, 2018; Org et al., 2016). Sex-specific microbiota can contribute to a well-known fact of sex-specific immune responses, where females have stronger innate and adaptive responses than males. The enhanced active IS in females leads to higher incidence and severity of autoimmune diseases (e.g. systemic lupus erythematosus, Grave´s disease, Hashimoto´s thyroiditis,…). On the other hand, men have a higher frequency of infectious diseases. These differences can be attributed to diverse sex hormones and chromosomes, which also have an effect on the composition of the microbiota and vice versa (Elderman et al., 2018; Rubtsova, Marrack, & Rubtsov, 2015). Whether there are other factors contributing to sexually different responses and what is their specific role is not completely understood and further investigation needs to be undertaken.

3 OBJECTIVE AND HYPOTHESIS

It is a thought, that there are gender-specific differences in the IS and composition of the microbiota. These differences in the opposite sex can contribute to diverse reactions to treatment, vaccination, development of autoimmune diseases, nutritional interventions, etc. The aim of this study is to establish possible sexual dimorphism in GM and intestinal immunity in adult rats.

4 MATERIAL AND METHODS

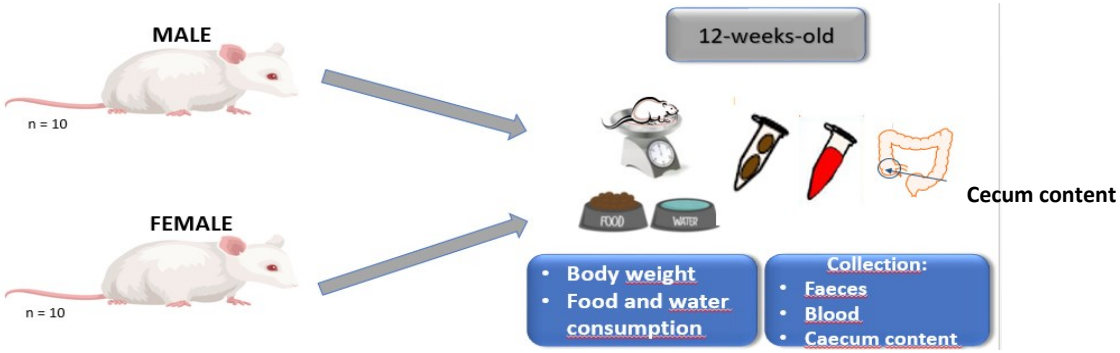
4.1 ANIMALS

Twenty Wistar rats were obtained from Janvier Labs (Saint-Berthevin, France) and maintained at the animal facility of the Faculty of Pharmacy and Food Science, University of Barcelona. The animals (9-weeks-old) were separated into 2 groups of 10 females and 10 males receiving standard diet AIN-93M (Harlan, Barcelona, Spain) for 3 weeks. Gender equality was a condition build on a recommendation of the Ethical Committee for Animal Experimentation of the University of Barcelona. Approval link of this procedure is 351/17.

Animals were individually housed in plastic, stainless steel cages with controlled humidity (55 %) and temperature (21 ± 2°C) and a 12/12-h light/dark cycle. Conditions were applied based on the Unit of Animal Experimentation at the Faculty of Pharmacy and Food Science of the University of Barcelona. The access of animals to food and water during the experimental period was *ad libitum*.

4.2 EXPERIMENTAL DESIGN

The focus of this study is concentrated on sexual differences in GM – specifically on the intestinal secretion of IgA, IgA-coated bacteria (IgA-CB), and GM composition.
In the age of 12 weeks animals were anesthetized by intramuscular injection of ketamine (90 mg/kg) (Merial Laboratorios, S.A, Barcelona, Spain) and xylazine (10 mg/kg) (Bayer, A.G, Leverkusen, Germany) and samples of faeces, blood and caecum content were collected for further determinations. Additionally, parameters of body weight, food, and water consumption were recorded for each rat (Picture 9).



Picture 9 Experimental design – *12-week-old animals (10 males and 10 females) were anesthetized at the age of 12 weeks old. At the endpoint body weight and food and water, consumption was measured. Faeces, blood and caecum content were collected for further testing.*

4.3 SAMPLE COLLECTION AND PROCESSING

Fresh faeces were collected for analysis of faecal parameters measurements (faecal weight, humidity, and pH), IgA concentration and percentage of IgA-CB. Faecal samples were diluted in sterile PBS 7.2 (1:20 w/v) and homogenized using Pellet Pestels Cordless Motor (Sigma.Aldrich, Madrid, Spain). For IgA-CB determination, the homogenates were centrifuged at low speed (300 *g* for 1 min). On the contrary, faecal homogenates to quantify IgA concentration by ELISA technique were centrifuged at high speed (538 *g* for 10 min). Then, both supernatants were frozen at -20°C to be preserved.

At the same time point, caecal content was obtained and put into sterile and RNase free eppendorfs and kept at -20°C for microbial DNA sequencing and to determine its pH. Moreover, the small intestine was also excised and divided into two halves. The distal part was carefully rinsed with cold sterile 0.9% sodium chloride (NaCl) solution in order to remove faecal content. Then it was opened lengthwise and was used to obtain the gut wash for IgA quantification by ELISA assay.

Blood was also collected, and serum was separated after centrifugation and kept at - 20°C until IgA concentration quantification by ELISA technique.

4.4 MICROBIAL GENOME SEQUENCING

The analysis of microbiota composition in rats was conducted by 16S rRNA sequencing as previously described in the laboratory (Azagra-boronat et al., n.d.). In brief, the genomic DNA was isolated from caecal samples using Qiamp Stool Mini Kit (Qiagen). Another purification and concentration of extracted DNA were achieved by Qiamp Micro Kit (Qigen). Amplification (50 ng)

and sequencing was performed by following the 16S Metagenomic Sequencing Library Illumina 15044223 B protocol (Illumina Inc, San Diego, California, USA). The DNA-sequencing provided a microbial characterization of phyla, families, genera, and species. To simplify a massive data amount only dominant groups (those whose presence was higher than 0.5 %) were studied and non-dominant groups (present in <0.5%) in each taxonomical level were classified as ''others". Final data were stated for each group in relative proportion abundance. The richness and diversity of microbiota composition were defined by Shannon and CHAO1 tests, respectively, which were performed at a species level. The diversity was also displayed by Venn diagrams (Bioinformatics & Evolutionary Genomics Venn calculator).

4.5 QUANTIFICATION OF S-IgA IN SERUM, FAECES, AND GUT WASH BY ELISA

ELISA is an immunological experiment that can quantitatively evaluate antibodies from an obtained sample. For our experiment, a sandwich ELISA was carried out. This method works on the principle of binding specific capture antibody with the target antigen and after adding a detection antibody creating antigen-antibody complex. The detection antibody is conjugated with an enzyme. To see the results, the substrate is converted to colored substance and is read by the photometer (Veterinary & Laboratories, 2011).

Samples of serum, faecal homogenates, and gut wash were quantified by ELISA assay following the manufacturer´s instructions for the determination of total secretory-IgA (S-IgA) (Bethyl Laboratories Inc, Montgomery, USA).

A plate of 96 wells NUNC Maxisorb (Labclinics) was coated with capture antibody (Goat anti-rat IgA, 1mg/mL A110-102A) diluted in coating buffer (Carbonat-bicarbonat, pH 9.6, dil 1:500). 100 μL of diluted antibody was added to each well and the plate was incubated overnight at 4°C in a humid chamber. Next day the plate was emptied and washed 5x with 200 μL of TBS-0.05% Tween 20. After blocking with 200 μL TBS+BSA 1% in each well, the plate was incubated for 1 h at room temperature in a humid chamber. The plate was emptied and washed (5x) again and 100 μL of appropriately diluted samples and standards (Rat reference serum 0.2 mg/mL RS10-100) in increasing scale of concentration were added. TBS+0.05% Tween 20+BSA 1% was used as a solution for dilution of the standards. After incubation for 2 h at room temperature in a humid chamber, the plate was wash again. 100 μL of the detection antibody (Goat anti-rat IgA HRP conjugated A110-102P-28) (dil 1/50 000) was added in each well and incubated for 2 h (the incubation period for samples of faeces and gut wash was 3 h) and afterward washed.

Lastly, the extemporaneous solution for enzymatic development was prepared. 1 tablet of OPD (0.4 mg/mL) in 50 mL phosphate-citrate (pH 5), containing 0.04% of hydrogen peroxide solution 30% (Sigma-Aldrich, Madrid, Spain), was dissolved. After dissolution, 200 µL was put in each well and plate was agitated for 10 – 30 min. The enzyme-substrate reaction was stopped by 50 µL/well of 3M H_2SO_4. Absorbance was measured by microplate photometer (LabSystem Multiskan) at 492 nm and data were analysed by ASCENT version 2.6 software (Thermo Fisher Scientific) into standard curves and expressed as ng/g of serum, ng/g of faeces, and ng/g of tissue in the gut wash.

4.6 ANALYSIS OF IgA-COATED BACTERIA BY FLOW CYTOMETRY

Flow cytometry is a technique to determinate each individual cell characteristics. In addition, categorization of specifically marked cells can be provided by specialized flow cytometer called fluorescence activated cell sorter (FACS).

A volume of 10 µL of faecal homogenate was diluted in 500 µL PBS-FBS-1% and after a high-speed centrifugation (8000 *g* for 5 min at 4°C), the resulting pellet was resuspended in 50 µL of diluted fluorescein isothiocyanate (FITC)-anti-rat Ig antibodies (Abcam; Cambridge, UK, g/ml) (1:40) in PBS-FBS-1 %. All samples were incubated for 30 min at 4°C in the dark and then washed twice (8000 *g*, 5 min) using sterile PBS (pH 7.2). The resultant pellet was resuspended in 200 µL of 4% PFA and kept in the dark at 4°C. To define all bacteria, samples were mixed with 4 µL of propidium iodide (PI, Sigma Aldrich, Madrid, Spain) and then analysed by flow cytometry (FCM) using FACSAria SORP sorter (BD Biosciences). Negative samples were established to control the output: 1 negative control (PI- IgA-), 1 PI + control (PI + IgA-), 1 IgA+ control (PI- IgA+) and the examined sample (PI+ IgA+).

Bacterial morphology was selected according to their FSC/SSC signal[4] (FACsAria SORP sorter (BD, San José, CA, USA). IgA-CB quantification was settled as positive FITC counts within PI+ bacteria in the above FSC/SSC morphology gate. FlowJo version 7.6.5 software (Tree Star, Inc.), the propriety of Scientific and Technological Centres of University Barcelona (CCIT-UB) was used to evaluate the analysis.

[4] FSC/SSC signal stands for forward-scattered light (FSC) which is proportional to cell-surface area or size and side-scattered light (SSC) which is proportional to cell granularity or internal complexity

4.7 ANALYSIS OF FAECAL AND CAECAL PARAMETERS AND BODY WEIGHT

Analysis of pH of faecal and caecal content was conducted by a surface electrode (Crison Instruments, S.A., Barcelona, Spain).

At the end of the study, each rat and its faeces were weighed. Afterward, the faeces were dried along at 60°C for 24 h in the oven. Faecal humidity was later calculated based on the weight difference of each sample after the drying process.

4.8 STATISTICAL ANALYSIS

All statistical analysis was accomplished by software package IBM SPSS Statistics 22.0 (SPSS Inc., Chicago, IL, USA). Levene´s and Shapiro-Wilk test were performed to assess equality of variance and normal distribution, respectively. When the variance equality and normal distribution existed, conventional one-way ANOVA was carried out. Otherwise, the non-parametric Kruskal-Wallis test was used. Significant differences were established when $p<0.05$ (* $p<0.05$, ** $p<0.01$). The graphics were completed using SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA, USA) and GraphPad Prism® (Graphpad Software Inc.).

5 RESULTS

5.1 BIOLOGICAL, FAECAL, AND CAECAL PARAMETERS

At the end of the study body weight, food intake, and water consumption were measured. Additionally, feed efficiency was calculated by dividing food consumption by gained body weight of each rat. Data are shown in Figure 1. In these biological features, male rats showed higher food consumption and body weight. Otherwise, female rats had a significantly bigger quotient of feed efficiency.

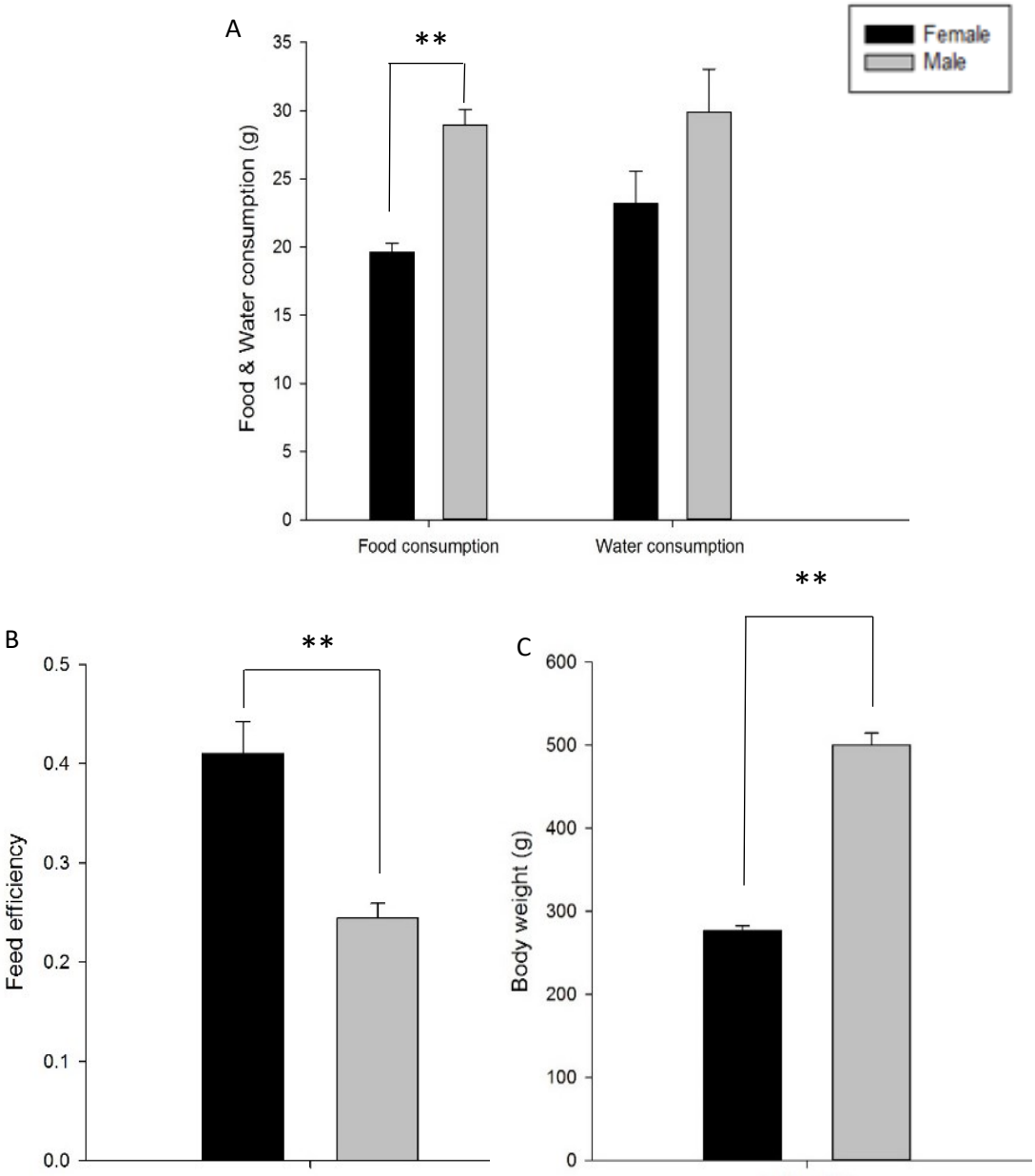


Figure 1 *(A) Food and water consumption [g] (B) Calculated feed efficiency (C) Body weight [g] measured at the end of the experiment. Values are expressed as mean ± SEM. A significant difference ($p < 0.01$) in food intake, feed efficiency and body weight between males and females.*

Caecal and faecal parameters summarized together caecal pH, faecal humidity, faecal wet weight, and faecal pH. Graphs with these data are shown in Figure 2. Caecal pH and humidity of faeces did not show any significant differences between male and female rats. The difference in male faecal wet weight corresponded to the previous figure concerning food consumption. Surprisingly, female rats displayed higher faecal pH compare to the males.

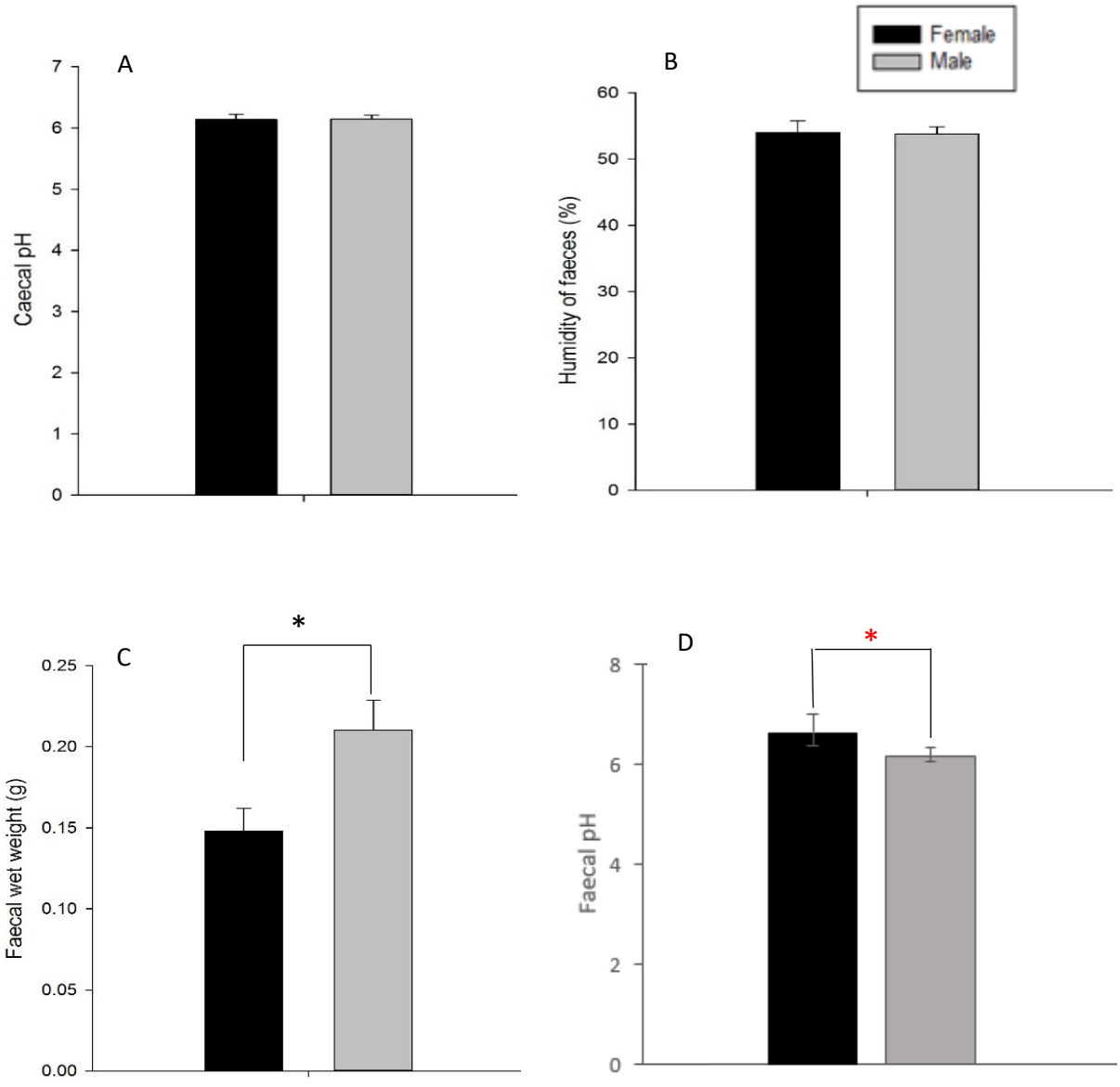


Figure 2 *(A) Caecal pH and (B) humidity of faeces have no significant differences. (C) Faecal wet weight showed significant difference ($p < 0.05$) and (D) faecal pH was significant in the Kruskal-Wallis test. Values in A, B, and C are expressed as a mean ± SEM. In part D values are expressed as median ± percentile of 75% and 25%.*

5.2 IMMUNOLOGY

Immunological parameters included the quantification of IgA and IgA-CB, the graphs are shown in Figure 3. Levels of IgA were detected by ELISA test from three different types of samples – gut wash, faeces, serum. Analysis of all data showed no sexual bias.

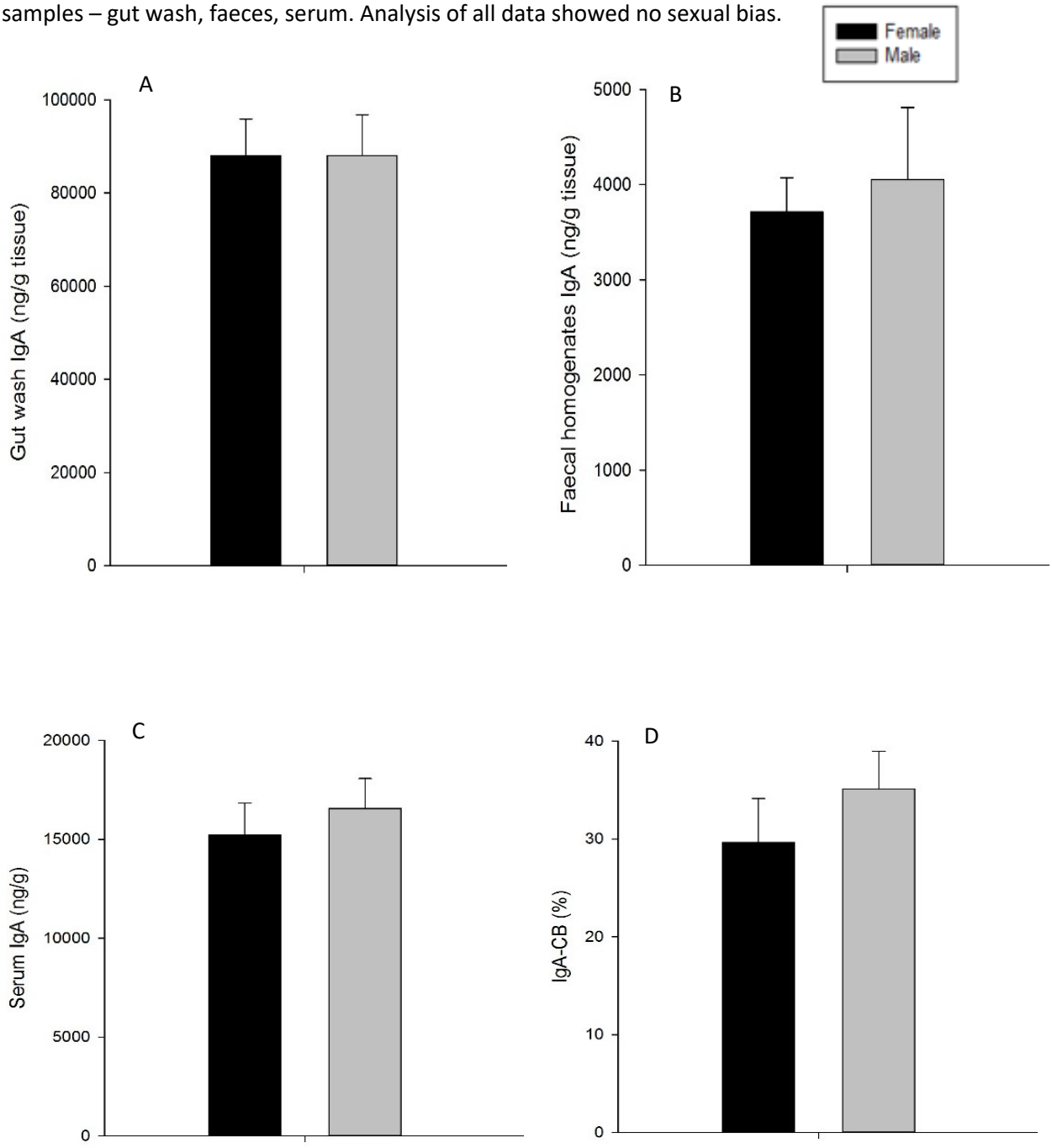


Figure 3 *Immunological parameters: (A) Gut wash IgA (ng/g), (B) Faecal homogenate IgA (ng/g), (C) Serum IgA (ng/g), (D) IgA-Coated Bacteria (%). Values are expressed as mean ± SEM. No significant differences were detected between sexes.*

5.3 MICROBIOTA

The composition of the microbiota was detected by DNA sequencing and divided into 4 levels – phylum, family, genus, and species. In *Figure 4* is shown the relative proportion of the most predominant phyla. Male animals displayed a higher proportion of *Firmicutes*. Females had a higher percentage of *Bacteroidetes* and *Verrucomicrobia*.

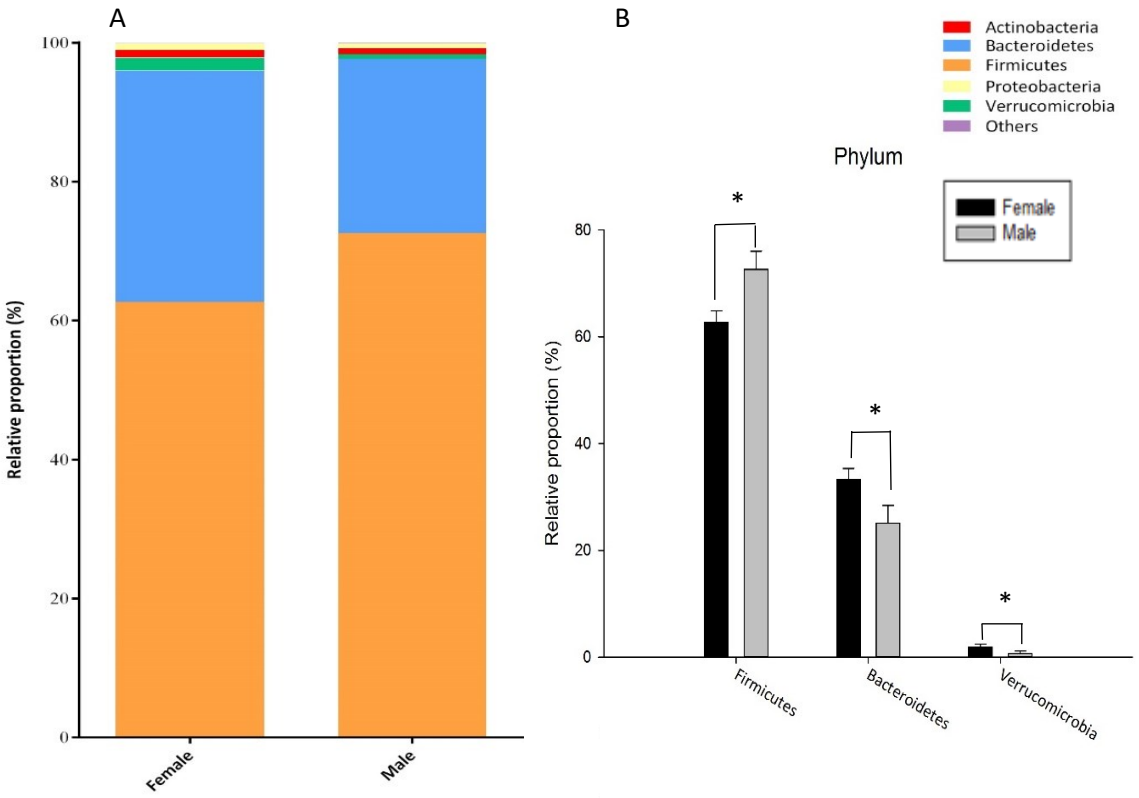


Figure 4 *(A) General overview of the relative proportion of the most predominant phyla in female and male rats. (B) Phyla with a significant difference between females and males. Values are expressed as mean in percentage ± SEM.*

The relative proportion of the most predominant families presented in female and male rats are shown in *Figure 5 (A)*. Overall, 6 families shown in *Figure 5 (B)* exhibited a significant difference between sexes. As can be observed, 5 families (*Porphyromonadaceae, Prevotellaceae, Ruminococcaceae, Akkermansiaceae, Oscillospiraceae*) had more abundance in the female sex, whereas *Lactobacillaceae* was highly predominant in male rats.

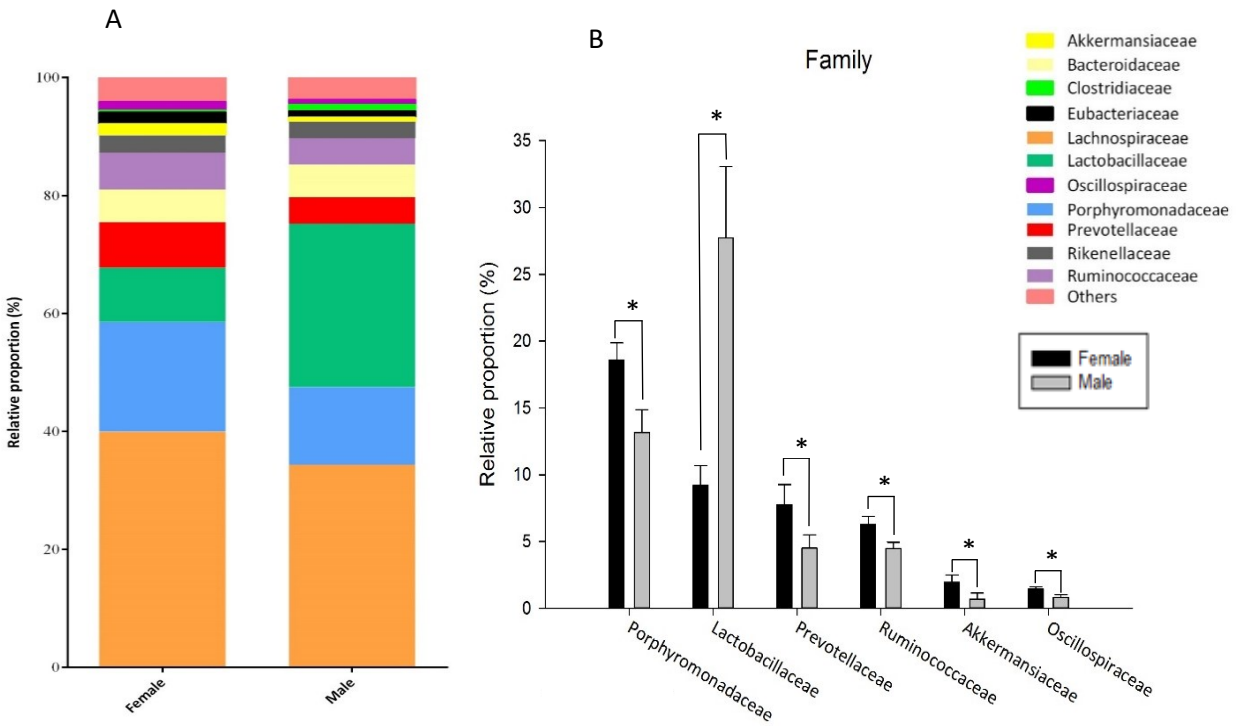


Figure 5 *(A) Relative proportion at the level of family. The most predominant families are displayed. (B) Only families with significant difference between female and male rats. Values are expressed as mean in percentage ± SEM.*

Results of analysis at the genus level are shown in *Figure 6*. Figure 6A presents a full overview of the most predominant genera detected and Figure 6B highlights the 4 genera with a significant difference when comparing the genders. As in the previous level, *Lactobacillus* prevailed in male sex and whereas other genera such as *Muribaculum, Prevotella, and Akkermansia* were present in a higher proportion in female animals.

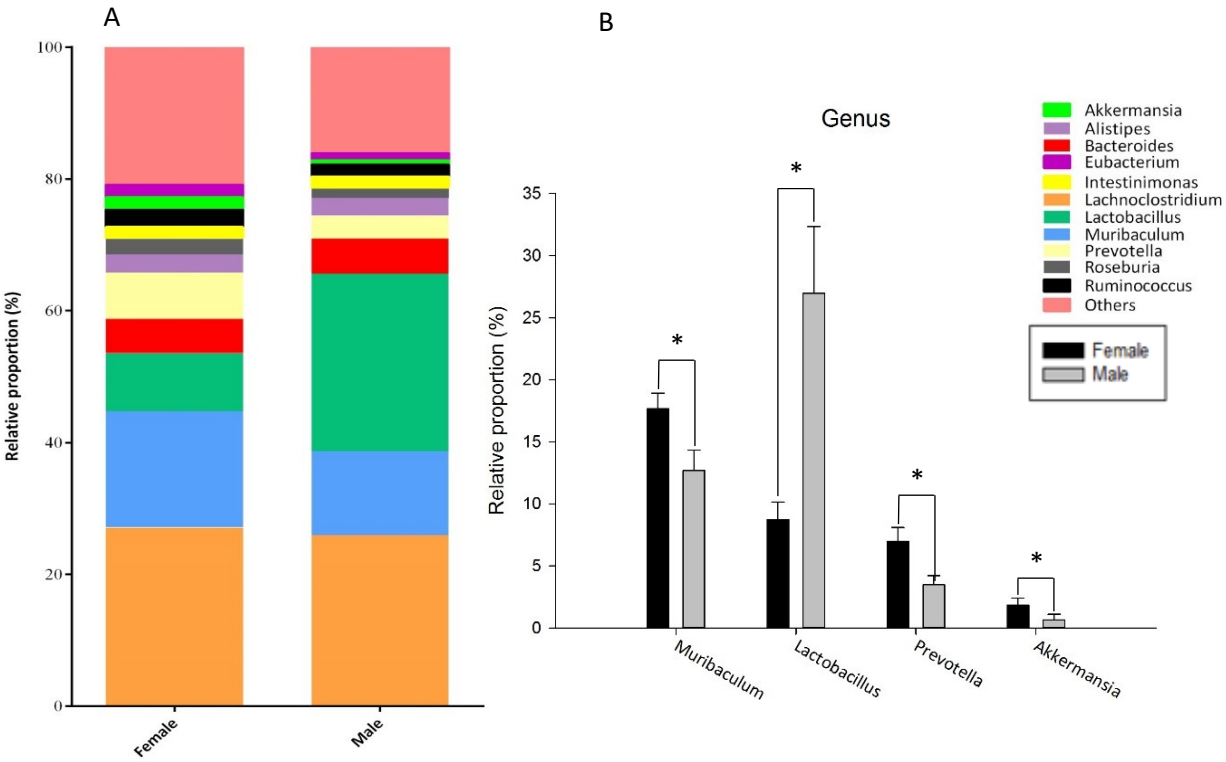


Figure 6 *(A) Relative proportion of the most predominant genera found in rats. (B) Genera displaying significant difference between genders. Values are expressed as mean in percentage ± SEM.*

The most detailed level displays concrete species of the microbiota. Those species are shown in *Figure 7.* For clearer imagination, *Figure 8* (A, B, C) is categorized by specific species with the same generic name and significant difference between female and male rats are marked out. This analysis revealed 5 species significantly different between genders and 2 species with a p-value near to the significance. *Lactobacillus animalis* showed a higher incidence in male animals than female. *L. reuteri,* higher in male animals with p = 0.053, is also worth mentioning. Two species belonging to the *Prevotella* genus - *Prevotella shahii, Prevotella stercorea* - resulted higher in female rats than male. Similarly, *Roseburia faecis* and *Akkermansia mucuniphila* were also found in a higher proportion of female rats. *Muribaculum intestinale,* higher in female rats with p = 0.059, could be responsible for the elevated incidence of genus *Maribaculum* and family *Porphyromonadaceae*. Other results in species graphs also correspond with the previous superior level of genus.

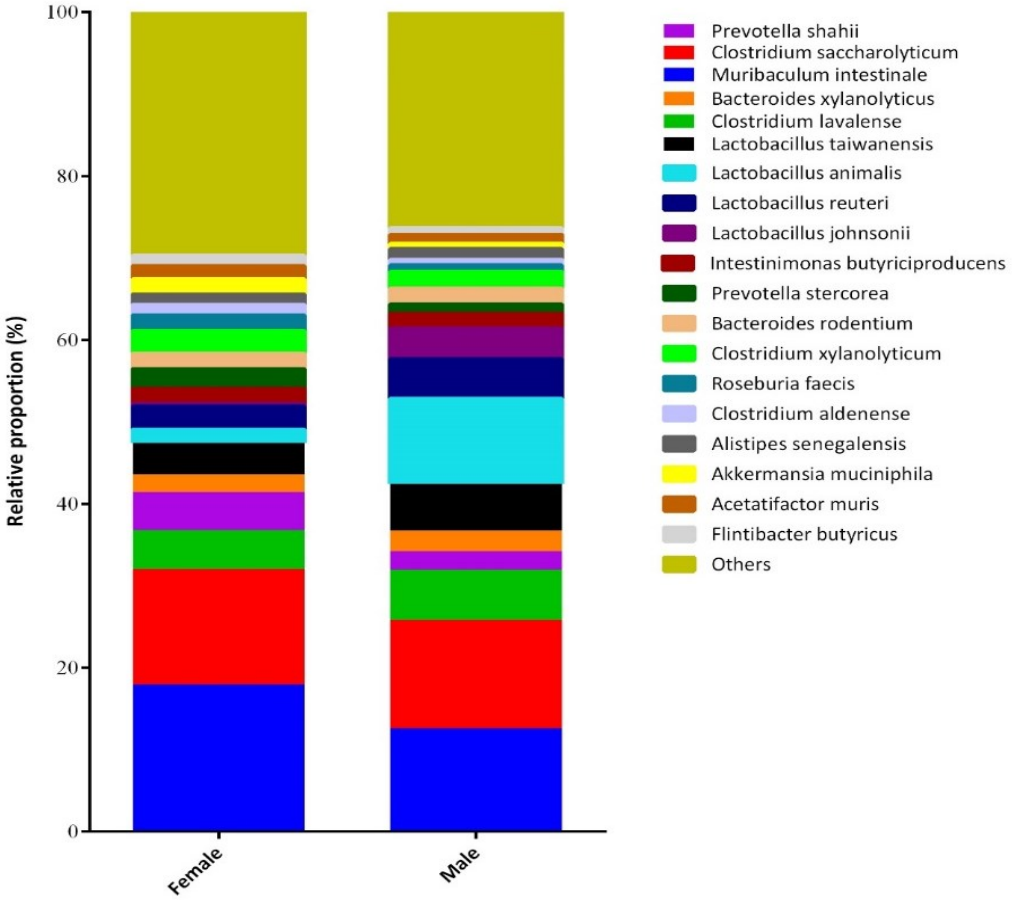
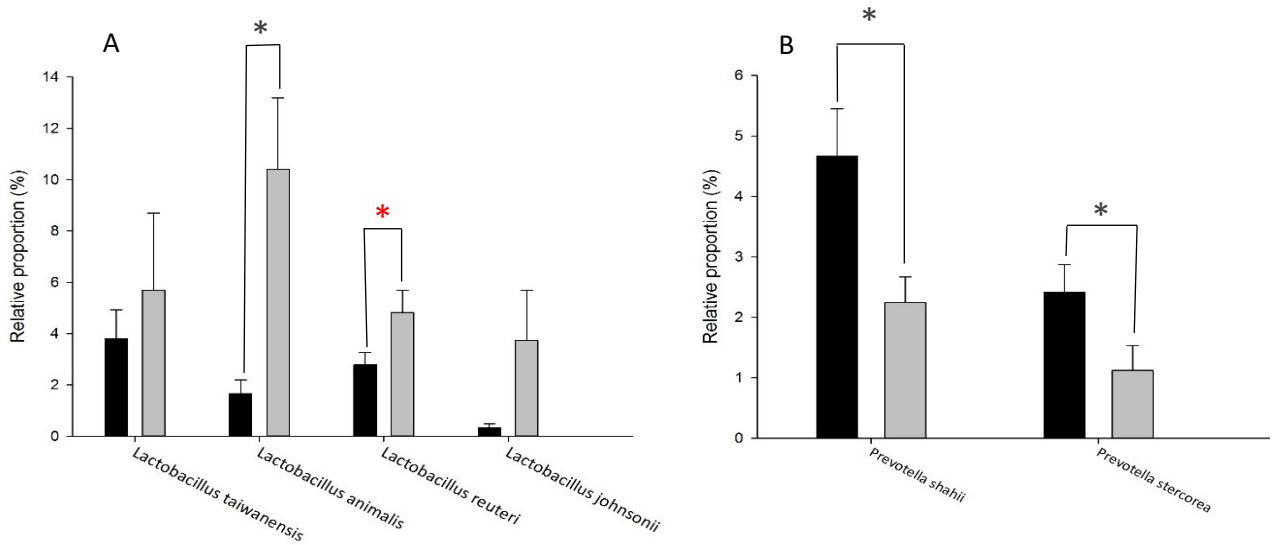


Figure 7 *Graph with relative proportion of the most predominant species detected in female and male rats. Values are expressed as mean in percentage ± SEM.*



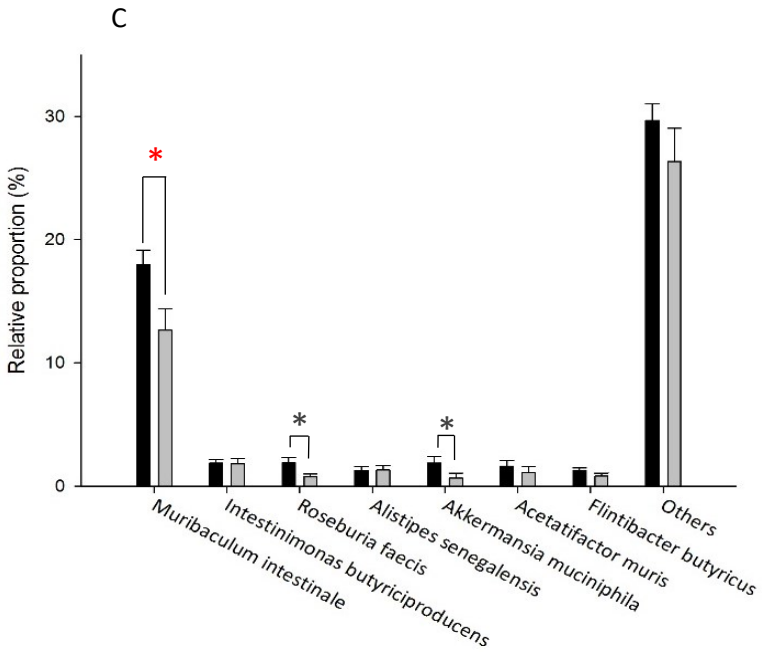


Figure 8 *(A) Analysis of Lactobacillus with 2 species with significant difference higher in males. *Lactobacillus reuteri - p = 0,053 (B) Analysis of Prevotella, where both species are higher in females. (C) Other species with single representative with 3 species significantly higher in females. *Muribaculum intestinale - p = 0,059. Values are expressed as mean in percentage ± SEM.*

To summarize the results of microbiota composition, Table 2***Table 2*** compiles all microbiota in phylum, family, genus, and species level with significant differences between sexes (plus M. intestinale and L. reuteri).

	PHYLUM	FAMILY	GENUS	SPECIES
Females	Verrucomicrobia	Akkermansiaceae	Akkermansia	Akkermansia muciniphila
	Bacteroidetes	Prevotellaceae	Prevotella	Prevotela shahii, P. stercorea
	Bacteroidetes	Porphyromonadaceae	Muribaculum	Muribaculum intestinale*
				Roseburia faecis
		Ruminococcaceae		
		Oscillospiraceae		
Males	Firmicutes	Lactobacillaceae	Lactobacillus	Lactobacillus animalis, L. reuteri*

Table 2 *Compendious table displaying taxonomic ranks with significantly higher proportion (p – 0.05) in relevant sex. *Species that do not cross the value of significance but are worth mentioning: Lactobacillus reuteri - p = 0,053; Muribaculum intestinale - p = 0,059.*

5.4 DIVERSITY AND RICHNESS - SHANNON and CHAO 1 index

In species level values for SHANNON and CHAO 1 index were carried out (*Figure 9*). SHANNON represents diversity and CHAO 1 richness of microbiota composition. SHANNON index indicates a higher diversity of microbiota colonization. This result was verified by Venn diagrams (*Figure 10*) for each taxonomic rank. Female rats had 1 phylum, 4 families, 13 genera, and 13 species that were not present in males. Only 1 male-specific microbe was observed at the species level.

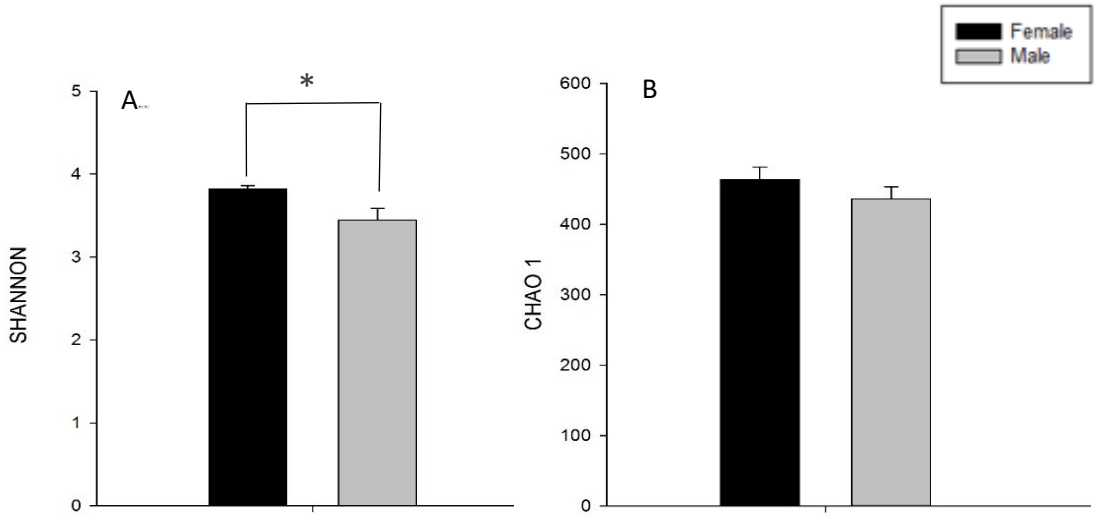


Figure 9 *(A) Only SHANNON index shows significant difference (p > 0.05). Values are expressed as mean in percentage ± SEM.*

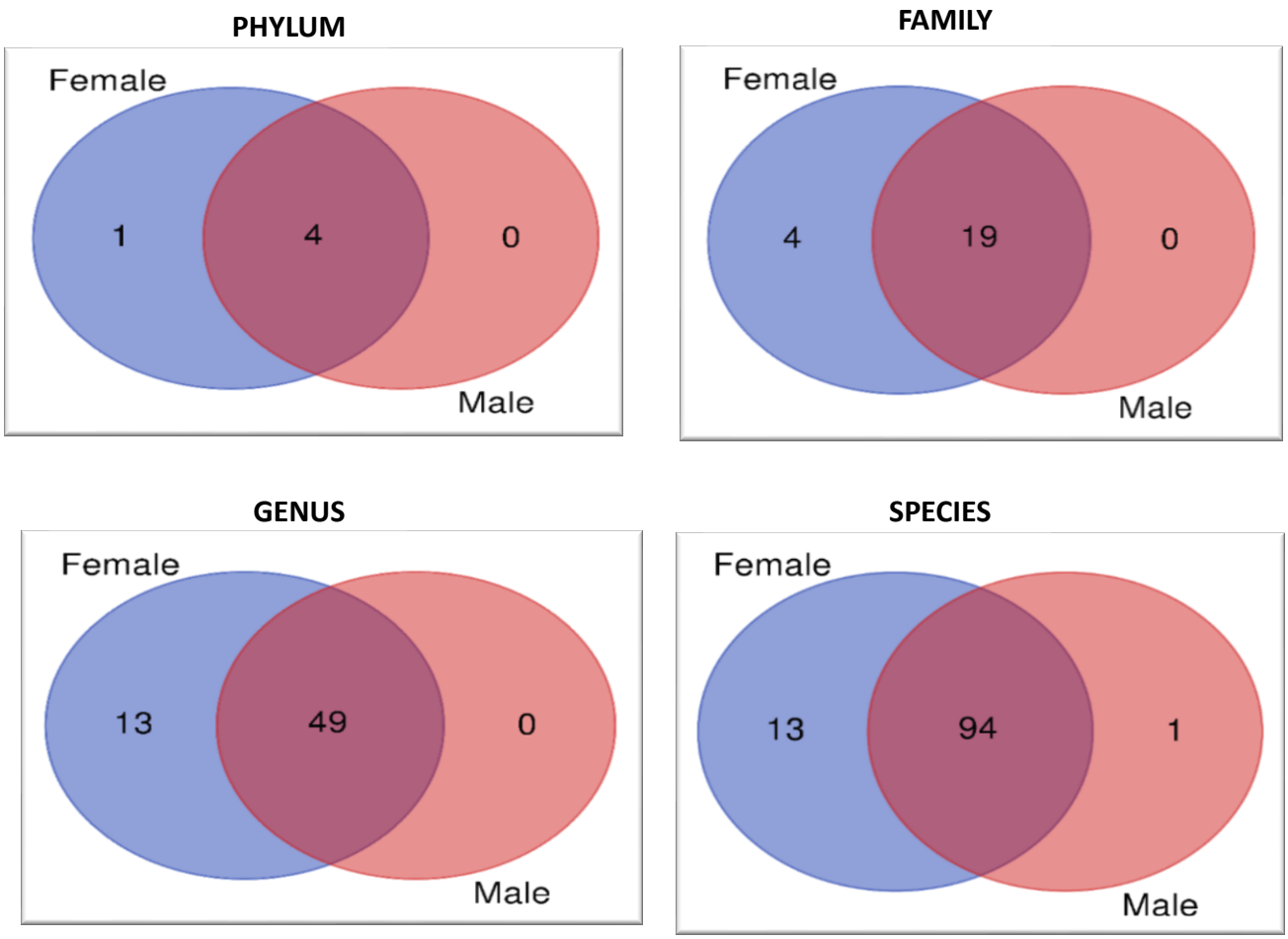


Figure 10 *Venn diagrams display Phylum, Family, Species, and Genus. There is higher number of female-specific microbiota in every taxonomic rank than in males.*
Female specific phylum: *Verrucomicrobia*
Female specific families*: Defluviitaleaceae, Campylobacteraceae, Akkermansiaceae, Peptococcaceae*
Female specific genera*: Ihubacter, Dorea, Peptococcus, Rodentibacter, Akkermansia, Hydrogenoanaerobacterium, Herbinix, Enterorhabdus, Anaerosporobacter, Natranaerovirga, Syntrophococcus, Fusicatenibacter, Campylobacter*
Female specific species*: Romboutsia timonensis, Eubacterium rectale, Hydrogenoanaerobacterium saccharovorans, Ethanoligenens harbinense, Coprococcus catus, Ruminococcus gauvreauii Ruminococcus faecis, Campylobacter jejuni, Akkermansia muciniphila, Anaerosporobacter mobilis, Rodentibacter pneumotropicus, Peptococcus niger, Herbinix hemicellulosilytica*
Male specific species: *Clostridium symbiosum*

6 DISCUSSION

The main objective of this study was to observe possible sexual dimorphism of GM composition in rats. We also observed potential alterations of immunological values and biological and physiological parameters with sexual bias.

Biological parameters include measurement of body weight, which was marked down at the end of the study. Our results showing that male rats have significantly higher values of body weight correspond with official tables of The Animal Resources Centre ("Rat and Mice Weights," 2019) and other studies containing this information in their research (van Hest, van Haaren, & van de Poll, 1988). Concerning considerable difference in food intake, studies confirm that steroid hormones affect feeding behavior in rodents. Testosterone in males is partly responsible for a bigger size of the body as demonstrated at the previous point about body weight. On the other hand oestrogens in females can reduce feeding (Fukushima et al.,2015; Lalitha et al., 2014). The outcome of these effects echoes with our results, where male rats have higher food intake than females.

Sex differences in faecal wet weight are comparable with differences in body weight and food consumption. Male rats, due to larger body weight and food intake, showed higher values of collected faecal wet weight.

The biochemical parameter of pH resulted differently in the caecal and faecal analysis. Caecal pH showed no sexual difference, while analysis of faecal samples revealed higher pH for female rats. The standard value of pH was conducted by a study of McConnell and his colleagues, where intestinal pH in rats was concluded to a value of 6.6 (L McConnell, W Basit, & Murdan, 2008). GM composition and fermentation patterns are affected by the pH. Studies with human samples proved that changes in pH can alter SCFAs production, or vice versa and alongside also include changes in GM (Bradshaw & Marsh, 1998; Walker, Duncan, McWilliam Leitch, Child, & Flint, 2005). Our results of pH analysis and DNA-sequencing showed more alkalic pH and bigger occurrence of *Bacteroides* in female rats. These findings correlate with the studies which suggest that mildly acidic pH can cause reduction of *Bacteroides*. We can assume a similar outcome with *Lactobacillus* species. *Lactobacillus* is producing lactic acids and its numbers increase in an acidic environment. Therefore *Lactobacillus*, which was more present in the male sex, could also contribute to the fact, that male rats have slightly lower faecal pH than females. We need to

take in an account all differences in species, type of samples and methodology between those studies and our results. To prove these suggestions further testing needs to be done (Ilhan, Marcus, Kang, Rittmann, & Krajmalnik-Brown, 2017; Walker et al., 2005).

Next part of our study was focused on the analysis of immunological parameters and their differences between sexes. Diverse immune responses of females and males are broadly accepted but not all responsible mechanisms are known and the information at gut levels is not so clear. The contributing mechanisms are sex hormones, genes, GM, different environmental factors, and others (Klein & Flanagan, 2016). Generally, the female IS reacts stronger and it causes better endurance against infections, but also a higher occurrence of autoimmune diseases such as systemic lupus erythematosus or autoimmune thyroid disease. Evidence supporting these observations are based on a comparison of the actors of the peripheral IS including T-cells and B cells, cytokines, interleukins, lymphocytes and others (Ahmed et al., 1999; Elderman et al., 2018; Ngo, Steyn, & McCombe, 2014). In our research, we focused only on the intestinal IS and its most distinctive feature – S-IgA.

Our analysis of S-IgA in various samples and IgA-CB did not show any significant influence of sex on the concentration. As previously described S-IgA is a product of a cascade of actions involving DCs. Study of Netherlands researcher Elderman and his colleagues concentrate, among others, on the level of DCs in PPs and how it differs between male and female mice. Their results revealed no sexual interference on the percentage of DCs on the PPs, which can loosely correlate with our findings (Elderman et al., 2016). A study containing an analysis of serum IgA in conventional mice (Fransen et al., 2017) and another study with IgA obtained from caecal content (Shastri, McCarville, Kalmokoff, Brooks, & Green-Johnson, 2015) confirms our results where sex has no effect on the concentration of IgA. Nevertheless, we still need to keep in mind the species differences of rats and mice and the possible divergent process of collecting and analysing samples.

The DNA sequencing of rat faecal sample revealed big microbial diversity in the female intestine. Results of Venn diagrams show that specifically only female rat inhabits 1 phylum, 4 families, 13 genera and 13 species in their intestine and are not present in male rats. The analysis displaying quantitatively significant differences in each taxonomic rank also echoes with findings that female have a higher abundance of GM than males. Interestingly, on the other hand, male rats harbor in their intestine much higher percentage of *Lactobacillus* species. This single microbe modifies the male microbiota analysis up to the phylum rank, respectively.

The composition of GM is altered by many different internal and external factors. Those factors based on their severity can modify the GM permanently or temporarily. For example, internal factors include a method of delivery at birth, infant feeding, genetic, etc. External factors such as previously experienced infections, medications (mainly antibiotics) and diet, which is one of the most altering and diverse factors, can all change the bacterial community (Wen & Duffy, 2017). Changes in the GM can lead to disruption and reduction of diversity and richness. These potential harmful alterations cause dysbiosis and are associated with many severe diseases inside the intestine, e.g. IBD, IBS, and coeliac disease. Dysbiosis is also considered to be partly responsible for the pathogenesis of outside-intestine disorders, including metabolic syndrome, type 2 diabetes, obesity, cardiovascular disease, etc. (Carding, Verbeke, Vipond, Corfe, & Owen, 2015; Liang, Leung, Guan, & Au, 2018). These findings are applicable to human beings and can change the GM in intra-individual level. We also need to take into account interspecific differences between human, mice, and rats. Mice are number one experimental animal in the research of the intestine and microbiota. They have comparable gut physiology and anatomy to humans and allow various interventions and gene modifications. Rats share some of these similarities and are possibly more representative of the human GM than mice. Despite some similarities, different species cannot fully substitute human systems and consequences of external ("real life") factors. Moreover, individual conditions set during experiments are also relevant to the final results of research (Nguyen, Vieira-Silva, Liston, & Raes, 2015). Due to multiple reasons mentioned above, our results have confirming and disapproving studies. Most of those studies use mice as experimental animals for their benefits and therefore there is a lack of studies with rats.

A recent review of Elderman and his colleagues confirm our finding of a higher diversity of microbiota composition in females and admit sexual dimorphism in intestinal microbiota in mice and humans minding the influencing factors (Elderman et al., 2018). The composition of microbiota in phylum level in our research showed a higher abundance of *Firmicutes* in males which is presumably connected with higher levels of *Lactobacillaceae* and subordinate genus of *Lactobacillus*. These results are in line with studies on GF NOD (germ-free non-obese diabetic) mice (Yurkovetskiy et al., 2013) and rats (Shastri et al., 2015). *Lactobacillus* is a member of the lactic acid bacteria group, which produce lactic acid in the intestine (Walter, 2008). Major functions of *Lactobacillus* in the intestine are a production of vitamins and enzymes, maintenance of gut homeostasis by excluding pathogens from adhesion to gut mucosa and production of antibacterial substances, reduction of cholesterol level, and stimulation of the IS (Denev, 2006).

Increased level of phylum *Bacteroidetes* in female rats can be caused by a higher abundance of low proportion families of *Porphyromonadaceae* and *Prevotellaceae* and their belonging genera of *Maribaculum* and *Prevotella*. Our findings do not correlate with Dominiani et al. who demonstrate on 82 humans lower abundance of *Bacteroidetes* in women (Dominianni et al., 2015) and another study on mice (Yurkovetskiy et al., 2013). The differences could be explained by different animal species and experimental conditions.

Akkermansia has been found decreased in various states of pathogenicity (IBD, appendicitis or obesity) and therefore can be categorized as a marker of a healthy intestine. Specifically, the presence of *Akkermansia muciniphila* leads to activation of immune responses and enlargement of the gut barrier (La Rosa et al., 2019). *Akkermansia* species, linked to the family of *Akkermansiaceae* and *Verrumicrobia* phylum, resulted higher in female rats. This finding is in line with mice study of Xie and his colleagues. Unfortunately, their study states only the level of *Verrumicrobia* phylum and does not specify family or genus (Xie et al., 2017).

Species *Roseburia faecis* and family *Ruminococcaceae* is taxonomically subordinate to phylum *Firmicutes*, nevertheless, our results show its level increased in female rats. *Roseburia spp.* is considered to play an important role in the gut inflammatory processes, maturation of the IS and production of butyrate as the main energy source for the intestinal cells (La Rosa et al., 2019). Study of Org et al. tested 89 strains of mice and presented two different results with a sexual bias regarding *Roseburia* in two different strains of mice. We might consider a genetic influence on the occurrence of *Roseburia.* The same study correlates with our findings concerning the increase of family *Ruminococcaceae* in female rats. (Org et al., 2016). The study, using gonadectomy and hormone replacement, indicates that sex differences in microbiota might be also a consequence of the sex-specific hormones (Yurkovetskiy et al., 2013).

Studies focused on the research of GM and its influence on the host struggle with interspecific differences and multiple internal and external factors modifying the results. Despite these obstacles, it is important to further investigate mechanisms of actions and effects of GM on the host to help reveal unclear circumstances of the development of some diseases and adjust the treatment accordingly.

7 CONCLUSION

The main aim of this study was to investigate possible sexual bias in GM composition and intestinal immunity in adult rats. Analysis of biological, caecal and faecal parameters was conducted in this study. In line with standard nature laws male resulted significantly higher in body weight and faecal wet weight. Other biological parameters showed that female rats had higher feed efficiency and more alkaline faecal pH than males.

Our results confirm that female and male intestine harbour different types and number of the microbiota. Female rats provided higher diversity in the microbial species which was verified by 1 female-specific phylum, 4 families, 13 genera and 13 species displayed in Venn diagrams. Sexual dimorphism in GM composition was also confirmed by an analysis comparing the mean values of occurring microbiota between female and male rat intestine showing that female intestine had a higher occurrence of *Bacteroidetes* phylum associated with P*orphyromonadaceae,* family *Prevotellaceae* and *Prevotella shahii, P. stercorea* and another phylum *Verrucomicrobia,* family *Akkermansiaceae,* and *Akkermansia muciniphila.* The female intestine showed also higher abundance in species *Roseburia faecis* and families *Ruminococcaceae* and *Oscillospiraceae*. The DNA-sequencing revealed only one significant difference where the occurrence of the microbiota was higher in males. The species is *Lactobacillus animalis* belonging into family *Lactobacillaceae* and phylum *Firmicutes*, respectively.

Analysis by ELISA was carried out to determine the level of IgA and IgA-CB on multiple samples (faeces, caecal content, and serum). The outcomes showed no significant difference between female and male rats. This result does not support the hypothesis of sexual dimorphism in intestinal immunity. However, we need to take into account that IgA is only one part of the whole IS and further testing needs to be performed to confirm or disprove our results.

To conclude, we can claim that sexual dimorphism does occur in GM composition and its diversity in rats. This fact should be considered not only in the treatment of diseases associated with a disturbance in the intestine and GM but also in designing preclinical studies which somehow inspect any influence on the microbiota.

ABBREVIATION

AcMo – Monoclonal Antibody

APC - Antigen-presenting cell

DC – Dendritic cell

ELISA – Enzyme-Linked Immunosorbent Assay

FACS - Fluorescence-activated cell sorter

FCM – Flow cytometry

FSC/SSC - Forward-scattered light / size and side-scattered light

GF animals – Germ-free animals

GM – Gut microbiota

IBD – Inflammatory bowel disease

IBS – Irritable bowel syndrome

IFN – Interferon

IgA – Immunoglobulin A

IgA-CB – Immunoglobulin A coated bacteria

IL – Interleukin

IS – Immune system

PAMPs - Pathogen-associated molecular patterns

pIgR - Polymeric Ig receptor

PPs – Peyer´s patches

PRRs - Pattern recognition receptors

SC - Secretory component

SCFAs – Short-chain fatty acids

S-IgA – Secretory IgA

TGF – Transforming growth factor

TLRs – Toll-like receptors

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Figure 10 Venn diagrams display Phylum, Family, Species, and Genus. There is higher number of female-specific microbiota in every taxonomic rank than in males. **Female specific phylum:** Verrucomicrobia **Female specific families**: Defluviitaleaceae, Campylobacteraceae, Akkermansiaceae, Peptococcaceae **Female specific genera**: Ihubacter, Dorea, Peptococcus, Rodentibacter, Akkermansia, Hydrogenoanaerobacterium, Herbinix, Enterorhabdus, Anaerosporobacter, Natranaerovirga, Syntrophococcus, Fusicatenibacter, Campylobacter **Female specific species**: Romboutsia timonensis, Eubacterium rectale, Hydrogenoanaerobacterium saccharovorans, Ethanoligenens harbinense, Coprococcus catus, Ruminococcus gauvreauii Ruminococcus faecis, Campylobacter jejuni, Akkermansia muciniphila, Anaerosporobacter mobilis, Rodentibacter pneumotropicus, Peptococcus niger, Herbinix hemicellulosilytica **Male specific species:** Clostridium symbiosum 36

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