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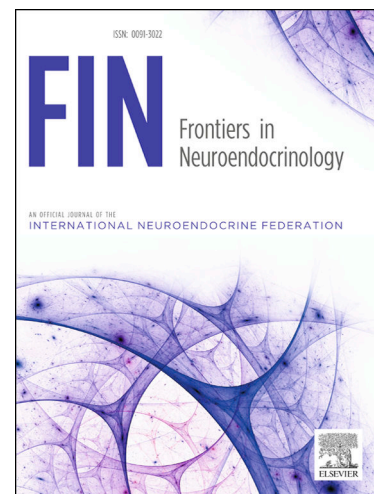
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How biological sex of the host shapes its gut microbiota

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Highlights

- Sex influences gut microbiota during human lifespan from the very beginning.
- Sex hormones are a potent driver of differences in the microbiome.
- Not only dynamic changes but also sustained hormonal differences are important.
- Diets, antibiotics and environment impact gut microbiota in a sex-dependent manner.
- Mechanisms underlying sex-dependent differences are mostly enigmatic.

Abstract

The gut microbiota is a complex system, consisting of a dynamic population of microorganisms, involved in the regulation of the host's homeostasis. A vast number of factors are driving the gut microbiota composition including diet, antibiotics, environment, and lifestyle. However, in the past decade, a growing number of studies also focused on the role of sex in relationship to changes in the gut microbiota composition in animal experiments as well as in human beings. Despite the progress in investigation techniques, still little is known about the mechanism behind the observed sex-related differences. In this review, we summarized current knowledge on the sex-dependent differences of the intestinal commensals and discuss the probable direct impact of sex hormones and more indirect effects such as dietary habits or antibiotics. While we have to conclude limited data on specific developmental stages, a clear role for sexual hormones and most probably for testosterone emerges.

Keywords: Sex difference, Gut microbiota, Microbiome, Sex hormone, Human lifespan, Diet, Antibiotic, Lifestyle, Environment.

1. Introduction

The “gut microbiota” is a term referring to the assembly of bacteria, archaea, viruses, and fungi that reside in the gastrointestinal (GI) tract of the host and to which a mutual, mostly beneficial relationship is formed (Bäckhed et al., 2005; Neish, 2009; Qin et al., 2010; Thursby and Juge, 2017). It is involved in several physiological functions, including preservation of the homeostasis of the host, regulation of immunity and metabolism and in the protection against pathogens (Baümler and Sperandio, 2016; Gensollen et al., 2016). However, dysbiosis, a detrimental alteration of the microbial composition, is associated with several diseases affecting the GI tract but also with disorders of other organ systems such as metabolic diseases or allergies (Fukui et al., 2018; Kim et al., 2020; Shanahan, 2013). Along lifespan, the GI tract is subjected to different stimuli e.g. mother’s health, mode of delivery, diet, antibiotics, and even psychological influences - all affecting the gut microbiota composition (Osadchiy et al., 2019). Beside these factors, studies carried out on human beings and animals discussed the importance of sex as a major contributor to the gut microbiota variability (Sinha et al., 2019; Yurkovetskiy et al., 2013). Recently, sex has been considered to be more than a simple statistical variable and several studies were initiated considering sex as an independent research question in regard to the microbiota. Despite this effort, still, little is known about the impact of sex on gut microbiota of human beings or of experimental animals and even less about the underlying mechanisms. We assumed that specific periods, when sex hormones undergo dynamic changes, might be the driving force for the observed differences between males and females and that the steady state hormonal levels may play an underlying role in such differences. To prove this, we summarized in this review current knowledge on human and rodent sex-dependent differences of gut microbiota throughout the life span. We discuss the impact of developmental stages and external factors such as diet, age, and geographic location in relation to sex and especially try to evaluate the role of sexual hormones.

2. The gut microbiota composition is affected by sex of the host during the entire lifespan

It has to be considered that important factors shaping the microbiota such as environment or hormones are depending on distinct life periods and might present differently herein in males and females. However, while the majority of studies concerning variation of the gut microbiota is conducted in children under 3 years of age and in adults (18-65 years and >65 years), relatively little is known about the age category of 3-12 years

(childhood) and 12-18 years (puberty/ adolescence). Both stages reveal especially highly dynamic changes regarding e.g. development or hormonal status. This lack of data might be based on restrictions by ethical or practical considerations (Derrien et al., 2019; Joseph et al., 2015).

2.1 Sex-related differences in gut microbiome observed in human postnatal studies (from birth to 12 months)

The development of the gut microbiota of human newborns is highly complex and orchestrated by several factors including the mode of delivery, the different type of nutrition as well as environmental factors (Cong et al., 2016; Jakobsson et al., 2014). The assembly of the gut microbial community during the first 2 years of age follows a pattern succession in which metabolic and host developmental pathways are critically affected (Robertson et al., 2019). During the first 6 months, the infants gut is rapidly colonized by facultative anaerobes, followed by obligate anaerobes such as *Bifidobacterium*, *Bacteroides*, and *Clostridium* (Koenig et al., 2011; Robertson et al., 2019; Yassour et al., 2016). Infancy is characterized by a domination of bacterial species involved in human milk oligosaccharide metabolism (it is estimated that 25–30% of the infant bacterial microbiota originates from breast milk) and with reduced difference in gut microbiota diversity (Pannaraj et al., 2017). In particular, this age period is characterized by colonization with several species belonging to *Bifidobacterium* (phylum Actinobacteria) and *Streptococcus* (phylum Firmicutes) such as *Bifidobacterium longum* and *Streptococcus thermophilus* (Robertson et al., 2019). The age period between 6 months and 2 years is then characterized by the introduction of solid foods into the infants' diet and by a concomitant rapid increase in the structural and functional diversity of the gut microbiota. This is a crucial period for the growth of the infant, in which the gut microbiota starts to approach adult-like features (Prendergast and Humphrey, 2014; Robertson et al., 2019).

Mainly, external environmental factors have been suggested to influence the gut microbial composition without considering contribution of sex at this early age. However, studies in rodent pups demonstrated a functional role of sex in gastrointestinal transit time, visceral sensitivity, and hormone-dependent effects on gut physiology (Gomez et al., 2014; Kilpatrick et al., 2010). This allows speculating also on a probable

influence of sex and sex-related hormones on human gut microbiota composition already at this very early stage of life.

Several recent studies conducted in human babies addressed potential sex-dependent differences in gut microbiota communities at different time points during their first year of life. Analysis of neonates' stool samples during the first 30 days revealed for example that male infants had a lower alpha-diversity as compared to females, which showed also higher abundance of Clostridiales (phylum Firmicutes), and lower abundance of Enterobacteriales (phylum Proteobacteria) (Cong et al., 2016). Among the earliest and most abundant bacterial colonizers of the newborns gut, *Bifidobacteria* (phylum Actinobacteria) are known to provide health benefits to the host intestine (Penders et al., 2006). Changes in *Bifidobacteria* abundance were observed in male and female vaginally-born infants from one day after birth to six months of age. In particular, a study conducted by Nagpal *et al.* (Nagpal et al., 2017) showed that boys had higher *Bifidobacterium* level at the first day of life as compared to girls. In the following, we shortly describe some examples in which sex induces differences in human babies' microbiota associated with a second parameter such as the mode of feeding (summary of these and additional findings from literature are illustrated in Figure 1 (upper part)).

a) Mode of feeding and sex-related gut microbiota differences

It has been shown that the gut microbiota composition is also affected by mode of feeding, relating to if the infants were fed with the mothers' own breastmilk (MBM) or not (non-MBM, formula-feeding) (Thompson et al., 2015): e.g. higher abundance of Clostridiales and Lactobacillales was observed in MBM-fed compared to non-MBM-fed infants. Nevertheless, also regarding feeding as one important determinant of the microbiota composition, Kozyrskyj *et al.* (Kozyrskyj et al., 2016) observed a lower abundance of *Bacteroides* species (phylum Bacteroidetes) in Caucasian male infants as compared to females at 3 months of age, pointing at a sex-dependent difference. Analyses carried out by Hoen *et al.* (Hoen et al., 2018) on stool samples from 204 US infants (118 males and 81 females) aged 6 weeks, showed a higher susceptibility of gut microbiota in formula-fed male infants as compared to the female counterpart in regard to arsenic.


Lactobacillus is one of the most dominant genera in the vaginal microbiota known to be regulated by female estrogens and to be associated with health benefit to the host (Shahani and Ayebo, 1980; Wang et al., 2017). *Lactobacilli* and *Bifidobacteria* both originate from the mother nipple milk ducts in the breast and are transmitted to newborns through human breast milk. They exert beneficial effects on neuro-behavior and cognitive development as well as reduced rates of infections (Asquith and Harrod, 1979; De Silva et al., 2004; Horwood et al., 2001; Vohr et al., 2006; Wall et al., 2009; WEST et al., 1979). The high presence of *Lactobacilli* in the infants' gut may be responsible for carbohydrate metabolism and for the high amount of lactic and acetic acids produced. In this process, several pathways are involved such as the Emden–Meyerhoff pathway, the phosphoketolase pathway, and the pentose phosphate pathway that release high amount of energy in term of ATP molecules, essential for the infant's growth (Gänzle, 2015). Similar to *Lactobacilli*, the *Bifidobacterial* species residing in the infant's gut are responsible for fermentation of oligosaccharides within the breast milk (Sela et al., 2008; Turrone et al., 2012) and for the ultimate production of short chain fatty acids (SCFAs) and other organic compounds which are beneficial for the babies' growth (Pokusaeva et al., 2011). In detail, the high caloric content of SCFAs is used by hepatocytes and colonocytes to produce energy in the form of ATP (Pokusaeva et al., 2011).

b) Asthma of the mother and sex-related differences of the gut microbiota of the offspring


Asthma is one of the most common chronic disorder occurring during pregnancy (between 8% and 13% of pregnant women are affected) and is associated to low birthweight of the infant (Koleva et al., 2017; Rejnö et al., 2014; Sawicki et al., 2011; V.E. and M., 2014). Koleva and colleagues analyzed the impact of maternal asthma on gut microbes such as *Lactobacilli* (phylum Firmicutes) in infants in relationship to their sex in a Canadian longitudinal study (Koleva et al., 2017). In particular, male infants delivered from women, which had pre-natal asthma, showed decreased *Lactobacilli* levels at 3-4 months of age as compared to infants with non-asthmatic mothers. Among infants delivered from asthmatic mothers, male babies also showed reduced *Lactobacilli* as compared to females, while females showed higher Bacteroidaceae levels, suggesting a possible dimorphic effect of prenatal asthma on infant gut microbiota.

c) Temperamental traits and sex-dependent gut microbiota differences in infants


The child's temperament has been reported to influence the gut microbiota composition in a sex-dependent manner. A population-based study conducted on 2.5-month-old infants (n=301, 159 boys and 142 girls) in southwest Finland showed association of gut microbiota composition with such temperament traits (Aatsinki et al., 2019). In detail, for boys a positive association of surgency with OTUs of *Bifidobacterium* (phylum Actinobacteria) and Clostridiaceae (phylum Firmicutes) and a negative one with *Veillonella* (phylum Firmicutes) was reported. Girls showed a negative association of fear reactivity and *Veillonella parvula* and *V. dispar*.



Age	Male	Female	Order / Genus / Species	Phylum	Methods	References
Day 1		↑	<i>Lactobacillus ruminis</i>	Firmicutes	qPCR, RT-qPCR	Martin et al., 2016
	↑		<i>Bifidobacterium</i>	Bacteroidetes	RT-qPCR	Nagpal et al., 2017
Day 30		↑	Clostridiales	Firmicutes	16S rRNA-seq V4 region, PCR	Cong et al., 2016
	↑		Enterobacteriales	Proteobacteria	16S rRNA-seq V4 region, PCR	Cong et al., 2016
2.5 months	↑		<i>Bifidobacterium</i>	Actinobacteria	16S rRNA-seq V4 region	Aatsinki et al., 2019
		↑	<i>Veillonella</i>	Firmicutes	16S rRNA-seq V4 region	Aatsinki et al., 2019
3 months		↑	<i>Bacteroides</i> spp.	Bacteroidetes	High-throughput gene seq	Kozyskiy et al., 2015
		↑	<i>Lactobacillus gasseri</i>	Firmicutes	qPCR, RT-qPCR	Martin et al., 2016
		↑	<i>Lactobacillus reuteri</i>	Firmicutes	qPCR, RT-qPCR	Martin et al., 2016
3-4 months		↑	<i>Lactobacillus</i>	Firmicutes	16S rRNA-seq	Koleva et al., 2017
		↑	Bacteroidaceae	Bacteroidetes	16S rRNA-seq	Koleva et al., 2017
6 months	↑		<i>Bifidobacterium longum</i>	Bacteroidetes	RT-qPCR	Nagpal et al., 2017
1.5 - 11 months	↑		<i>Lactobacillus reuteri</i>	Firmicutes	Quantitative analysis CFU/g	Ogunshie, 2016
	↑		<i>Lactobacillus bifidus</i>	Firmicutes	Quantitative analysis CFU/g	Ogunshie, 2016
	↑		<i>Lactobacillus acidophilus</i>	Firmicutes	Quantitative analysis CFU/g	Ogunshie, 2016
	↑		<i>Lactobacillus casei</i>	Firmicutes	Quantitative analysis CFU/g	Ogunshie, 2016
	↑		<i>Lactobacillus brevis</i>	Firmicutes	Quantitative analysis CFU/g	Ogunshie, 2016



Age	Male	Female	Order / Genus / Species	Phylum	Methods	References
1 - 5 years	↑		<i>Bifidobacterium</i>	Actinobacteria	RT-qPCR, 16S rRNA-seq	Solano-Aguilar et al., 2013
1.5 - 2.25 years	↑		<i>Dialister</i>	Firmicutes	bTEFAP, PCR	Christian et al., 2015
	↑		Ruminococcaceae	Firmicutes	bTEFAP, PCR	Christian et al., 2015
	↑		Rikenellaceae	Bacteroidetes	bTEFAP, PCR	Christian et al., 2015
	↑		<i>Parabacteroides</i>	Bacteroidetes	bTEFAP, PCR	Christian et al., 2015
		↑	Rikenellaceae	Bacteroidetes	bTEFAP, PCR	Christian et al., 2015
2 years	ns	ns	<i>Bifidobacterium</i>	Actinobacteria	16S rRNA-seq V4 region	Huda et al., 2019
7 - 12 years	ns	ns			16S rRNA-seq	Hollister et al. (2015)
		↑		Bacteroidetes	RT-qPCR	Mousavi et al., 2018



Age	Male	Female	Order / Genus / Species	Phylum	Methods	References
> 18 years	↑		Clostridiales	Firmicutes	16S rRNA-seq V3/4 region	Mahnich and Rupnik, 2018
		↑	<i>Akkermansia</i>	Verrucomicrobia	16S rRNA-seq	Mueller et al., 2006
		↑	Ruminococcaceae	Firmicutes	16S rRNA-seq	Mueller et al., 2006
20 - 50 years	↑			Bacteroides	16S rRNA-seq	Mueller et al., 2006
	↑			Prevotella	16S rRNA-seq	Mueller et al., 2006
20 - 89 years	↑		Prevotellaceae	Bacteroidetes	16S rRNA-seq V1/2 region	Oki et al., 2016
		↑	Ruminococcaceae	Firmicutes	16S rRNA-seq V1/2 region	Oki et al., 2016
	↑		<i>Prevotella</i>	Bacteroidetes	16S rRNA-seq V3/4 region	Takagi et al., 2018
	↑		<i>Megamonas</i>	Firmicutes	16S rRNA-seq V3/4 region	Takagi et al., 2018
	↑		<i>Megasphaera</i>	Firmicutes	16S rRNA-seq V3/4 region	Takagi et al., 2018
	↑		<i>Fusobacterium</i>	Fusobacteria	16S rRNA-seq V3/4 region	Takagi et al., 2018
		↑	<i>Ruminococcus</i>	Firmicutes	16S rRNA-seq V3/4 region	Takagi et al., 2018
		↑	<i>Bifidobacterium</i>	Actinobacteria	16S rRNA-seq V3/4 region	Takagi et al., 2018
		↑	<i>Akkermansia</i>	Verrucomicrobia	16S rRNA-seq V3/4 region	Takagi et al., 2018
75 years	↑			Bacteroides	16S rRNA-seq	Mueller et al., 2006
	↑			Prevotella	16S rRNA-seq	Mueller et al., 2006
> 100 years	ns	ns		Total microbiota	16S rRNA-seq V4 region	Wang et al., 2015

Fig. 1: Gut microbiota composition differences between male and female across human lifespan. The arrow pointing upwards represents increased levels of bacterial species or phyla in comparison to the other gender, while ns represents comparable amounts between male and female gender.

It may be speculated that changes in gut microbiome communities due to sex are related to the interaction of sex hormones with the immune system (Gomez et al., 2014). While multitude of data exist on adult hormone levels in humans, only limited data on sex-dependent differences in hormone levels for the newborn age stage are available. While no differences of cord blood supply with maternal hormones were observed in boys and girls at birth (Alawad and Al-Omary, 2019), early transient increase in testosterone levels in boys (Main et al., 2000) may suggest a possible role of testosterone in the gut microbiota differences in relation to sex in the newborn human being. In most of the investigated species, this so-called testosterone surge is rather transient; however, in some species as e.g. humans, the elevated testosterone level may persist for even weeks after birth (Corbier et al., 1992; De Zegher et al., 1992). Interestingly, this period coincides with the start of the colonization by microbiota.

2.2 Gut microbiota composition differences in relationship to sex during childhood (from 1 to 12 years)

In the first 12 months of life, the infant's intestine undergoes changes due to solid food introduction in the diet with a consequent variation of the microflora (Stark and Lee, 1982). In particular, the initial colonization of anaerobic bacterial populations in the infant's large bowel starts to resemble those of adults in number and composition after the introduction of solid food. Several studies suggested that the gut microbiota is more stable and resilient during adulthood as compared to childhood in the absence of external stressors such as dietary changes or antibiotic treatment (Derrien et al., 2019; Faith et al., 2013; Rajilić-Stojanović et al., 2013). This indicates that although a partial convergence occurs, there remain differences that make it valuable to analyze this stage of life time in relationship to sex differences. This is of especial interest as this age period is also accompanied by one of the most intense changes of hormonal status with onset of puberty. The evaluation of 277 children (154 girls and 123 boys) between 1 and 5 years of age from two different locations in Colombia (studying the presence of clinical diarrhea) showed that the fecal microbiota was affected by several host factors including age, health status, location but also by sex (Solano-Aguilar et al., 2013). Interestingly, a significantly stronger positive correlation between *Bifidobacterium* (phylum Actinobacteria) and *Lactobacillus* (phylum Firmicutes) species in healthy boys as compared to girls was assessed while no significant correlation was observed on children affected by diarrhea.

Below, some examples of how biological sex affects the gut microbiome in early childhood together with additional parameters are presented (for an overview see Figure 1).

a) Sex-dependent effect of early vitamin A supplementation

During infancy, vitamin A (VA) plays a crucial role in establishing the intestinal microbiome (Sherwin et al., 2012). Especially, VA is involved in intestinal immunity as well as in epithelial integrity and repair and its deficiency has been associated with impairment of the intestinal barrier at least in rodents (Reifen et al., 1998; Zile et al., 1977). VA supplementation affected, among others, the amount of *Bifidobacterium* and Proteobacteria (a phylum containing enteric pathogens) in a study including 306 Bangladeshi infants (Huda et al., 2019). *Bifidobacterium* abundance in early infancy (6 – 15 weeks old), but not in an older group (two years old), was lower in boys than in girls. However, boys receiving VA (50,000 IU within 48 hours) had higher levels than boys receiving placebo, while girls did not show these differences. In this regard, it is of interest that testosterone and VA signaling directly interact to regulate steroidogenic cell function as demonstrated in mice (Jauregui et al., 2018). This is not only depending on VA as a precursor for nucleic acid receptor ligand but also on retinoylation of proteins functional in steroidogenesis (Tucci et al., 2008). While testosterone itself does not increase in human plasma before the age of six, an increase of dehydroepiandrosterone (DHEA) has been observed at a bone age of already 5 years in boys (Sizonenko and Paunier, 1975) and may be associated with the observed phenomena. *Bifidobacterium*, which was increased by VA administration selectively in boys, itself may contribute to the beneficial effects of VA on the gut's immune system: mucosal CD103(+) dendritic cells within the lamina propria were increased by feeding *B. infantis* to mice (Konieczna et al., 2013).

b) Personality traits and the child's microbiome

Parental care as well as temperament in early childhood contribute to shape personality and behavior during childhood, adolescence, and adulthood (Rothbart and Posner, 2015). In line with this, Christian *et al.* reported a specific relationship between gut microbiota and temperament traits, such as surgency and fear reactivity, in a study conducted on 77 children aged between 18–27 months (Christian et al., 2015). However, they did not observe sex differences in the gut microbiome in general. In accordance with this, a study conducted by

Hollister *et al.* on pre-adolescent children aged between 7 and 12 years reported that sex did not contribute to variation of gut microbiota structure or function, while for example human ethnicity had a small effect (Hollister *et al.*, 2015).

c) Obesity and sex-dependent differences in gut microbiome

Another important aspect at this age might be growth and weight gain. A cross-sectional study carried out on 188 elementary school children (aged 7 - 12 years) investigated the role of obesity in relationship to gut microbiota considering also sex as a variable (Mousavi *et al.*, 2018). Children were segregated in three groups: obese, lean, and normal. Data revealed that Bacteroidetes levels increased in normal-weighted girls as compared to obese girls, resulting in a higher Bacteroidetes/Firmicutes ratio, while none of these differences was detected among boys. For girls, pre-pubertal obesity has been found to be accompanied by enhanced maturation of adrenal gland function and impaired gonadal secretion of estradiol (Genazzani *et al.*, 1978). In obese boys, increased DHEA was reported (Pintor *et al.*, 1984).

In summary, vitamin A supplementation and obesity seemed to influence the gut microbiome of young infants. A possible explanation for the observed differences may involve the interplay of sex hormones with the described factors.

2.3 Gut microbiota composition differences in relation to sex during adolescence (from 12 to 17 years)

Adolescence is a critical period of development, characterized by new experiences and explorative behaviors that build the basis for neurobiological, social, and emotional processing towards adulthood. Or rather, it is considered the second most important environmental shift in development due to simultaneous changes across multiple domains of behavior and neurobiology (Dahl *et al.*, 2018).

Sex hormones, such as estrogen and testosterone, seem highly plausible to have an important role in shaping the gut microbiota at this very age. A human study addressing the composition of the gut microbiota during adolescence showed higher fecal microbiota dissimilarity (greater UniFrac distances between the fecal bacterial communities of twins with different gender) in teenager (13–17 years) twin pairs with opposite sex as compared to twins with the same sex (Yatsunenکو *et al.*, 2012). A more recent study concluded that no difference in alpha- as well as beta- diversity occurs in non-pubertal as compared to pubertal subjects (aged

5-15 years, (Yuan et al., 2020)). With onset of puberty, levels of Clostridiales were lowered, while abundance of Betaproteobacteria increased. In pubertal subjects *Adlercreutzia*, *Ruminococcus*, *Dorea*, *Clostridium*, and *Parabacteroides* were found to be associated with testosterone levels. Unfortunately, no subgroups of males and females were analyzed – probably due to restricted participant numbers (42 for non-pubertal and 47 for pubertal).

Considering the limited data available on this phase of life in humans - studies from rodent models have to be used as a proxy. Experiments conducted in mice showed that sex differences in the gut microbiota appear at the onset of puberty in relation to sex hormone levels (Markle et al., 2013; Yurkovetskiy et al., 2013). In particular, differences in alpha-diversity were detected only in non-obese diabetic (NOD) post-pubescent mice aged between 10 to 13-week-old with significant higher levels in females as compared to males. Moreover, male mice showed higher abundance of Porphyromonadaceae (phylum Bacteroidetes), Veillonellaceae, Peptococcaceae, Lactobacillaceae (phylum Firmicutes), and Enterobacteriaceae (phylum Proteobacteria) as compared to females. These differences disappeared when male mice were subjected to castration (Yurkovetskiy et al., 2013). This was confirmed using the same mouse model for autoimmunity (NOD) by another study, comparing microbiota by 16SrRNA sequencing: weanling NOD males and females were indistinguishable, while sex-dependent differences were apparent in 6 week old pubescent mice (Markle et al., 2013). These differences were even aggravated in early adults (14 weeks). All observations were made before onset of the type 1 diabetes in this mouse model and thereby might be suitable for generalization. Interestingly, transfer of adult males' gut microbiome into immature females resulted in elevated testosterone levels in the latter animals. This might hint at a mutual influence of sex hormones and microbial sex-typical commensals.

2.4 Gut microbiota composition differences in relationship to sex during adulthood

The gut microbiome is for the most part instable in the first years of life, but it becomes more stable in adulthood before undergoing possible changes in bacterial richness and composition in conjunction with the onset of specific diseases, such as cancer or with increased frailty (Aleman and Valenzano, 2019). Adulthood is characterized by an increased stability and uniformity of the gut microbiome due to continuous adaptation

to stress, infection, diet, and antibiotic exposition (Spor et al., 2011). The concomitant development of the gut microbiota with the maturation of the immune and nervous system throughout the lifespan is sexually dimorphic, leading to different microbial communities as well as immune and neuro-inflammatory pathways in adult males and females (Jašarević et al., 2016; McCarthy et al., 2017). Multiple factors, including age, environment (diet and physical activity), and geographic location, seem to play a role in these sex-dependent gut microbiota differences (Shin et al., 2016). Including 82 subjects from the control group of a colon cancer study, Dominianni and colleagues (Dominianni et al., 2015) concluded sex being significantly associated with gut microbiome composition overall. However, this study included a comparably low number of participants with a rather wide range of age (30 to 83 years), which might have confounded the outcome of the study. A cross-sectional study on intestinal microbiota composition performed on 230 healthy subjects at four European locations (France, Germany, Italy, and Sweden) showed gut microbiota differences in relationship to sex and country in two groups of age. The first group contains adults aged between 20 to 50 years (age average of 35 years; n=85), while the second group includes adults aged > 60 years (age average of 75 years; n=145) (Mueller et al., 2006). Sex effects were observed for the *Bacteroides-Prevotella* group in the totality of the studied population, with higher levels in males as compared to females. Mahnic and Rupnik reported about an increased general bacterial diversity in relation to age in the Slovenian female population compared to the male population in a large study including 186 healthy volunteers aged > 18 years (Mahnic and Rupnik, 2018). A significant increase in the abundance of several operational taxonomic units (OTUs) from the order Clostridiales (phylum Firmicutes) was detected in males, while females displayed higher abundance of *Akkermansia* (phylum Verrucomicrobia) in addition to multiple OTUs corresponding to the family Ruminococcaceae (phylum Firmicutes). Moreover, fecal samples collected from 516 healthy Japanese adults (325 females, 191 males; age 21-88 years) showed higher abundance of Prevotellaceae (phylum Bacteroidetes) in men and higher abundance of Ruminococcaceae (phylum Firmicutes) in women (Oki et al., 2016). In particular, among the above mentioned OTUs, *Clostridium* is the only one showing differences levels in both, female infants (Cong et al., 2016) and adult women (Oki et al., 2016) in comparison with their

corresponding male counterparts. This may be explained by the fluctuation of estrogen's concentration across the entire lifespan; however, this hypothesis has not been addressed so far in detail.

Relationship between age, sex and gut microbiota alpha-diversity within three large cohorts of adults aged between 20 and 69 years were assessed in four geographical regions including United States, United Kingdom and two cohorts from Colombia and China (de la Cuesta-Zuluaga et al., 2019). Interestingly, young adult women (aged 20–45 years) showed higher alpha-diversity in comparison to men of the same age-range. Moreover, these differences were observed in all the three cohorts except for the Chinese one, in which no link between alpha-diversity and age or sex was detected. In agreement, association between sex and alpha-diversity was more pronounced in younger adults than in middle-aged adults, while no differences in alpha-diversity were observed between women and men when the average age of the participants was 60 years (Haro et al., 2016). This could be explained by the fact that women in the menopausal phase show a decline in estrogens levels (Scavello et al., 2019). In addition, a strong association between estrogen levels and Clostridia taxa in fecal microbiota has been demonstrated (Flores et al., 2012) suggesting that women enter in the adult age with a more diverse gut microbiota as compared to men. However, this difference flattens out over time, disappearing by the age of 40 years.

Significant differences in the microbial structure were shown in 277 male and female healthy Japanese with an age of between 20-89 years, although no differences were detected in the alpha-diversity. A higher number of the genera *Prevotella* (phylum Bacteroidetes), *Megamonas*, *Megasphaera* (phylum Firmicutes), and *Fusobacterium* (phylum Fusobacteria) was detected in men, while *Ruminococcus* (phylum Firmicutes) *Bifidobacterium* (phylum Actinobacteria), and *Akkermansia* (phylum Verrucomicrobia) were detected at higher levels in women (Takagi et al., 2019).

Increasing attention is being paid to studies about centenarians, people aged >100 years, which have been found to exhibit marked delays in age-related lethal diseases such as heart disease, cancer, and stroke (Evert et al., 2003). Only few studies investigated the gut microbiota composition in centenarians in comparison to normal elderly (aged <100 years). In particular, Wang *et al.* (Wang et al., 2015) reported a significantly increased number of *Roseburia* (phylum Firmicutes) and *Escherichia* (phylum Proteobacteria) genera in

centenarians (aged 100-108 years) compared to non-centenarian (aged 85-99 years), whereas *Lactobacillus*, *Faecalibacterium*, *Coprococcus*, *Megamonas*, *Mitsuokella* (Phylum Firmicutes), *Parabacteroides*, *Butyricimonas* (Phylum Bacteroidetes), *Sutterella* (Phylum Protobacteria), and *Akkermansia* (Phylum Verrucomicrobia) were less abundant in the centenarians. However, whether gender differences occurred between centenarians and non-centenarian has not been reported in this investigation. This may be due to the small total number of participants (n=24) enrolled in this study because of the limited recruiting success regarding extremely aged people.

Additional factors that may explain the observed sex-dependent microbiota differences in adults may be fat distribution and obesity in men and women. In particular, fat distribution has been correlated to *Holdemanella* and *Gemmiger* (phylum Firmicutes) in both, adult men and women, but in opposite direction (android fat ratio negatively correlated with *Holdemanella* and positively with *Gemmiger* in women, while positively with *Holdemanella* and negatively with *Gemmiger* in men) (Min et al., 2019). *Holdemanella* is able to produce 3-hydroxyoctadecaenoic acid from prebiotic fructooligosaccharides and thereby would promote the anti-inflammatory properties of this food ingredient (Pujo et al., 2020). This might be of relevance as a male-specific circuit in which androgens promote inflammation in fat tissue was uncovered in mice recently and might be also existing in humans (Vasanthakumar et al., 2020).

In summary, population studies conducted in men and women from different countries highlighted the presence of sex-related difference in gut microbiota composition during adulthood (for a summary see the lower part of Fig. 1). This means, that not only dynamic changes in sex hormones influences the gut commensals but also the persistent difference between men and women in adulthood is able to sustain obtained differences.

3. Probable role of sex hormones on gut microbiota composition

Sex steroids hormones, such as estrogen, progesterone, and testosterone are major determinants of the differences between male and female mammals (Lauretta et al., 2018). Males and females show the same type of hormones but with different production sites, blood concentrations, and interactions with the target organs (Svechnikov and Söder, 2008). Serum estradiol concentration variates from 20 to 80 pg/mL during the

early to mid-follicular phase of the female menstrual cycle, reaching the peak at 200 to 500 pg/mL in the pre-ovulatory phase, while in males the level remains constant and is less than 40 pg/mL. Serum testosterone level ranges from 300 to 1000 ng/dL in adult men, while it ranges from 20 to 50 ng/dL in adult women (Carmina et al., 2019). In summary, males mainly produce constantly testosterone within the testes in a daily pattern according to the circadian rhythm, while females mainly produce estrogens and progesterone from the ovaries following the estrus cycle in cyclical pattern (Nelson and Bulun, 2001; Simpson, 2003).

A matter of discussion concerns whether sex steroids participate in the regulation of the composition or function of gut microbiota, or if vice versa gut microbes may regulate sex steroid balance. The potential interaction between microbiome and estrogens has been theorized by (Chen and Madak-Erdogan, 2016), in a way that endogenous estrogens or estrogens ingested with the diet may be metabolized by gut microbes producing estrogenic metabolites that in turn affect the host metabolism. Plottel and Blaser coined the term the “estrobolome” that defined the totality of enteric bacterial genes whose products are capable of metabolizing estrogens (Plottel and Blaser, 2011).

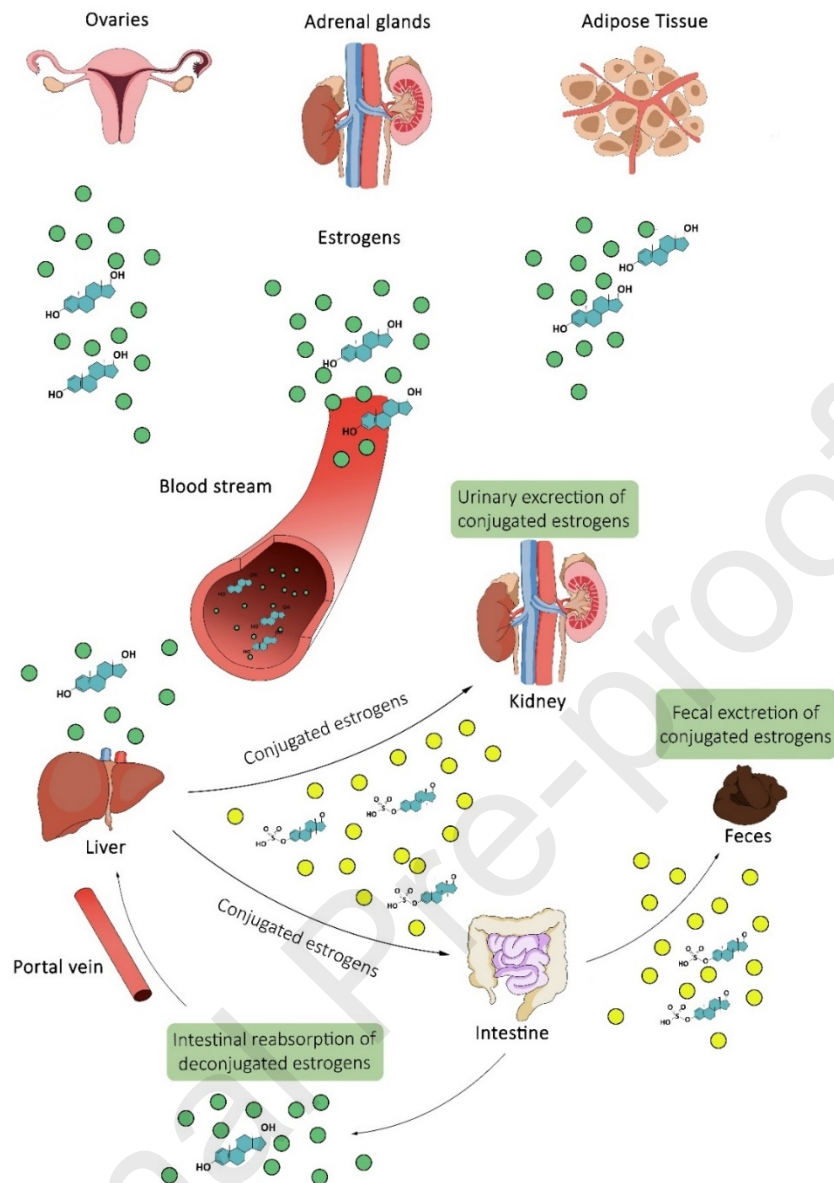


Fig. 2: Estrogen circulation pathways. Estrogens are locally produced in several tissues (mainly ovaries but also adrenal glands and adipose tissue). They circulate in the bloodstream, reaching several target organs such as liver and kidney. Once reaching the liver, they undergo conjugation by glucuronidation or sulfonation reactions. Moreover, conjugated estrogens are converted into water-soluble molecules and can subsequently be excreted in bile, urine, and feces (Kwa et al., 2016). Some fractions of conjugated estrogens can be subjected to deconjugation by gut microbes in the intestine and be reabsorbed through the portal vein into the liver (Kwa et al., 2016; Rose, 1993).

Gonadal steroid hormones are not the only hormones that cause sex differences in human beings but also non-steroid hormones, such as insulin, catecholamine, growth hormones (GH) and cortisol are involved (Bemben et al., 1992; Bonen, 1992; Bunt, 1990; Matute and Kalkhoff, 1973; McKerns et al., 1958; Reinke et al., 1972). For example, women showed increased daily variation of GH levels as compared to men (Johansson, 1999). Moreover, ovarian hormones also interact in indirect manner with non-ovarian hormones

as shown by estrogen supplementation in rats which increases sensitivity to catecholamine and hormone-sensitive lipase (HSL) activity (Benoit et al., 1982). On one hand, the gastrointestinal tract controls the enterohepatic circulation of non-ovarian estrogens in men and post-menopausal women, affecting local and systemic sex hormone levels (Flores et al., 2012). On the other hand, sex hormones modulate gastrointestinal (GI) health in women (Chang and Heitkemper, 2002; Drossman et al., 1993).

Colon tissue of adult rats was shown recently to express molecules involved in steroidogenesis and in the subsequent synthesis and metabolism of steroid hormones such as testosterone (Diviccaro et al., 2020). Levels of active metabolites of testosterone were even higher in colon than in plasma, pinpointing at a rather direct interference of locally produced sex hormones, enteric nervous system, and microbiome.

3.1 Impact of sex hormones on gut microbiota: animal studies

The developmental stage at which sex hormones start to shape the gut microbiota is still a matter of discussion. Studies conducted on mice reported that sex hormones alter the gut microbiota composition only after puberty: quantification of 17 β -estradiol and testosterone in non-obese diabetic (NOD) male and female mice at three different developmental stages revealed differences in microbiome composition just during puberty (6-week-old) and adulthood (14-week-old) but not at the time of weaning (3-weeks-old). Same levels of 17 β -estradiol were found in germ-free (GF) and specific pathogen-free (SPF) male mice, while higher levels of testosterone in GF females than in SPF females and lower levels in the GF males compared to SPF males were observed. This suggested an impact of gut microbiota on testosterone level in mice (Markle et al., 2013). In agreement, no significant sex differences were detected in the alpha-diversity of 4-week-old (prepubescent) NOD mice while significant differences were shown in 13-week-old (post-pubescent) mice (Yurkovetskiy et al., 2013). Among the microbial families involved, the Porphyromonadaceae (phylum Bacteroidetes), Veillonellaceae (phylum Firmicutes), Kineosporiaceae (phylum Actinobacteria), Peptococcaceae (phylum Firmicutes), Enterobacteriaceae phylum (Proteobacteria) and Lactobacillaceae (phylum Firmicutes) showed higher abundance in adult males as compared to adult females or castrated males. Moreover, male castrated mice presented comparable gut microbiota composition to that of female

mice suggesting an involvement of male sex hormones in shaping the gut microbiota. This is supported by the fact that administration of testosterone in three different mouse strains subjected to gonadectomy (GDX) prevented the significant changes in gut microbiota composition that were observed in untreated GDX males (Org et al., 2016). Kaliannan *et al.* reported a decrease in the abundance of Proteobacteria phylum, decreased Firmicutes/Bacteroides ratio, higher *Bifidobacterium*/Enterobacteriaceae ratio and increased *Akkermansia* (phylum Verrucomicrobia) abundance in control females as compared to control male and ovariectomized (OVX) female mice (Kaliannan et al., 2018). 17 β -estradiol treatment (4000 ng/ml water, for a six-week treatment) significantly reduced Proteobacteria and in male and OVX female mice while elevated *Akkermansia* species levels in males and increased the *Bifidobacterium*/Enterobacteriaceae ratio in OVX females. Interestingly, treatment of female mice with high levels of 17 β -estradiol during pregnancy also induced changes in the composition and diversity of gut microbiota including an enrichment of Lactobacillaceae (phylum Firmicutes), Coriobacteriaceae (phylum Actinobacteria), and an unidentified family from the Bacillales order (phylum Firmicutes) (Benedek et al., 2017). Other studies demonstrated sex-specific effects of maternal dihydrotestosterone (DHT) on the gut microbiome of offspring derived from dams with polycystic ovary syndrome (PCSO), such as a reduction in bacterial richness and diversity in males and an increase in females. The only bacterial genera showing the same reduction in male and female offspring mice were Lachnospiraceae *NK4A136* (phylum Firmicutes) and *Bifidobacterium* (phylum Actinobacteria). Moreover, samples from dams showed increased levels of *Akkermansia* family (phylum Verrucomicrobia) (Lindheim et al., 2018) as also described elsewhere in rodent studies (Jašarević et al., 2017; Org et al., 2016; W. et al., 2015). Moreover, microbiome analysis from fecal samples of female rats subjected to neonatal androgenization or to adult ovariectomy, as a model of menopause, demonstrated changes in gut microbiota composition (Moreno-Indias et al., 2016). In particular, lower microbial diversity (with a high Firmicutes to Bacteroidetes ratio) observed in androgenized rats suggested the existence of enduring alteration in gut microbiota composition even in adulthood.

3.2 Sex hormones are associated to gut microbiota sex-dependent differences in humans

A study conducted on 57 adult Korean men (n=31) and women (n=26) from 25 to 67 years of age demonstrated a fundamental correlation between sex hormones such as testosterone and estradiol and gut microbial composition (Shin et al., 2019). The involved bacterial genera that significantly correlated with high testosterone levels were *Acinetobacter* (phylum Proteobacteria), *Dorea* (phylum Firmicutes), *Ruminococcus* (phylum Firmicutes), and *Megamonas* (phylum Firmicutes) in men, while *Slackia* (phylum Actinobacteria) and *Butyricimonas* (phylum Bacteroidetes) correlated with high estradiol levels in women. On the other hand, high estradiol levels were associated with more Bacteroidetes and less Firmicutes phyla in women. Additionally, it was demonstrated that gut microbiota composition was profoundly altered in pregnant women due to changed estrogen levels during the first three months of gestation (Koren et al., 2012). A study conducted by Flores and colleagues on men (n=25), premenopausal (n=19) and postmenopausal (n=7) women showed a strong association between the non-ovarian urine estrogens with fecal Clostridia taxa, including non-Clostridiales and three genera in the Ruminococcaceae family (phylum Firmicutes) (Flores et al., 2012). Another European (Spain) study including 46 adult volunteers (15 patients with PCOS, 16 non-hyperandrogenic women, and 15 healthy men) reported a correlation of gut microbiota with testosterone and estradiol hormones (Insenser et al., 2018). In particular, *Paraprevotella* (phylum Bacteroidetes) exhibited positive correlation with testosterone and negative with estradiol. In addition, an abnormal increase in the abundance of the *Catenibacterium* and *Kandleria* genera (phylum Firmicutes) was shown in women with PCOS compared with both, female and male controls.

The use of anti-androgen oral contraceptives, normally used to prevent pregnancy and androgen-dependent conditions in women such as acne or excessive facial/body hair growth (e.g. (van der Spuy et al., 2003)), was positively associated with *Bacteroides caccae* (phylum Bacteroidetes) and *Coprobacillus* unclassified species. In contrast, oral contraceptives correlated with an increase in *Rothia mucilaginosa* (phylum Actinobacteria) species in a large Netherland population-based study (Sinha et al., 2019). In addition, data from a population based-cohort indicated that women who underwent bilateral ovariectomy show increased abundance of the species *Clostridium bolteae* (phylum Firmicutes) as comparably described in mouse studies (Cox-York et al., 2015; Org et al., 2016).

3.3 Interactions of sex with environmental factors affecting the microbiome

Still, the distinct pathways leading to interference of sex hormones with gut microbiota, also in relation to different ages, have not been characterized yet, neither in animals nor in humans. A study based on nearly 700 mice from 89 different inbred strains crucially contributed to the needed insight (Org et al., 2016): mice within a controlled environment clearly displayed a sexual dimorphic microbiome with differences most apparently e.g. in C57BL/6J mice. However, when the respective strains were not considered separately and analyzed together using Bray-Curtis dissimilarity, no clear distinguishing patterns were observed anymore. Genetic differences may therefore obscure sex-dependent effects on the microbiome, which is even more relevant when considering studies on human beings. Additionally, sex-hormone-evoked effects on bile acid pools were reported to be amenable to diet (chow versus high fat/ high sugar diet). Bile acids, are known to affect the gut microbiota (Clarke et al., 2012; Li and Chiang, 2015) and, together with the observed sex-diet interaction, offer possible indirect ways to explain how sex can influence the microbiome composition. Probably, hormonal difference also influences daily habits that in turn affect the gut microbiota. In the following, we describe according data on dietary habits, antibiotics treatment, and exposition to environment as examples of such potential indirect parameters.

a) Dietary habits affect the gut microbiota in relation to sex differences

Diet represents one of the most influencing factors inducing differences in the gut microbiota composition (Makki et al., 2018). Several studies conducted in experimental animals as well as in humans showed that the type of diet influences the gut microbiome also in relation to sex.

Olive oil, the main ingredient of the Mediterranean diet contained in several aliments as the main source of fat, is recently been associated with an effect on gut microbiota. Ingestion of olive pomace-enriched biscuits for 8 weeks in adults (aged between 30 and 65 years) showed significant sex-dependent differences in relative abundances of fecal bacteria (Conterno et al., 2019). In particular, women showed higher levels of *Akkermansia* (phylum Verrucomicrobia), *Bifidobacterium* (phylum Actinobacteria), *Bacteroides*, Rikenellaceae, Barnesiellaceae (phylum Bacteroidetes), and Enterobacteriaceae (phylum Proteobacteria) as compared to men. Contrarily, men showed higher level of *Prevotella* (phylum Bacteroidetes). A second

example is given by tuna oil and algae oil mixture treatment, both, having been associated with anti-aging effects. Male mice showed higher abundance of *Lactobacillus* (phylum Firmicutes) bacteria and of several probiotic-like butyric acid producers than female mice, whereas lower levels of *Clostridium XIVa* (phylum Firmicutes), an inflammation-related genus, were selectively detected in male mice after ingesting the oils (Zhang et al., 2018).

The Western pattern diet (WD), a modern food style generally characterized by high intakes of red meat, butter, sweets, and fried foods (Halton et al., 2006), has also been investigated. Chronic administration of WD (21% fat, 34% sucrose and 0.2% cholesterol for 3 weeks) after weaning increased the Firmicutes to Bacteroidetes ratio in male farnesoid X receptor (FXR) knockout mice as compared to females (Sheng et al., 2017). In addition, WD administration exerted effects also in WT mice in a sex-dependent way, increasing levels of S24-7 family (Phylum Bacteroidetes) in male mice as compared to females. On the other hand, treatment of C57BL/6 pups with WD (45% fat, 35% sugar, 197 mg cholesterol/kg) for 6 weeks revealed no differences in gut microbiota composition between males and females (Steegenga et al., 2017). Male CD-1 mice treated with WD supplemented with 30% dairy fat showed increased abundance of Bacteroidetes at 13.5 compared to 10.5 months of age while opposite effect of diet was observed in female mice at the same age points (Unger et al., 2019). Feeding with WD supplemented with 30% echium oil resulted in decreased abundance of Bacteroidetes in male mice at 13.5 as compared to 10.5 months of age, while no effect of diet was detected in relation to age in female mice.

Excessive high-fat diet (HFD) consumption contributes to gut microbiota alteration which might lead to subsequent development of diabetes and chronic diseases in humans (Murphy et al., 2015). Female pups from murine dams treated with HFD showed decreased alpha-diversity, decreased Bacteroidetes, and increased Firmicutes levels as compared to control female pups, while male mice showed a reverse trend in relation to control males (Guo et al., 2018). In line with the previous report, microbiome analysis of cecal content in offspring mice subjected to maternal HFD showed greater alpha- and beta-diversity in male as compared to female mice. Among Firmicutes families involved, Lachnospiraceae and Clostridiaceae showed higher abundance in postnatal HFD treated male offspring than in females (Wankhade et al., 2018).

Moreover, exposure of dams to HFD has been associated to behavioral deficits in male offspring mice in the open field and auditory fear conditioning tests (Bruce-Keller et al., 2017) which might be at least partly related to altered microbiota. Combination of HFD and arachidonic acid (AA) (45% fat with 10 g/kg of arachidonic acid) resulted in a significant decrease in microbial richness of Proteobacteria and Verrucomicrobia phyla in female mice but not in males (Zhuang et al., 2017). In particular, for male mice alteration of 78 OTUs was reported (55 decreased and 23 increased within 17 families) while 76 OTUs (50 decreased and 26 increased within 16 families) were affected in females. HFD supplemented with honokiol (HON, an extract from Chinese herbal medicine) induced a significant increase of *Akkermansia* (phylum Verrucomicrobia) selectively in male mice (Ding et al., 2019).

Gut microbiota sex-differences in relation to HFD were additionally described for rats. Lee *et al.* observed that the amount of *Akkermansia muciniphila* (phylum Verrucomicrobia) and *Desulfovibrio spp.* (phylum Proteobacteria) increased in response to HFD in young rats independently from sex and in aged female rats, but not in aged male rats (Lee et al., 2018). Another experiment conducted in rats reported that oligofructose (OF) supplementation diet induces a reduction in the total fecal community only in males and that it increased the fecal content of Bacteroidetes only in females (Shastri et al., 2015).

Inadequate eating habits are characterized by altered circulating lipids and lipoproteins increasing the risks for dyslipidemia (DLP) diseases (Udenigwe and Rouvinen-Watt, 2015). A recent study conducted in Wistar rats showed that DLP disease was associated with alteration in the gut microbiota composition and different fat weight levels in male and female rats (Pinheiro et al., 2019). In detail, male offspring from DLP dams showed higher visceral fat weight and lower *Lactobacillus spp.* as compared to male controls (from non-dyslipidemic dams) while higher liver fat levels were observed in female offspring from not optimally nourished dams as compared to female controls. In accordance, increased anxiety and depressive-like behaviors, assessed with greater distance travelled in the open field test, have also been detected selectively in male mice receiving a diet enriched in long-chain omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA). This also correlated with increased level of *Allobaculum* and *Ruminococcus* (phylum Firmicutes) (Davis et al., 2017).

Besides these relatively abundant reports on the effect of fat content and quality, only few reports are accessible about single food additives or specific medications. One study, for example, showed that carboxymethylcellulose (CMC) and polysorbate 80 (P80), two emulsifiers commonly used as additives of human food, also affected gut microbial communities of mice in relation to sex. On one hand, CMC treatment induced the elimination of the existing microbial differences between male and female mice concerning the genera *Bacteroides*, *Clostridium*, *Lactobacillus*, and *Coprococcus* (Holder et al., 2019). On the other hand, CMC treatment increased the abundance of the genera *Staphylococcus* and *Ruminococcus* (phylum Firmicutes) in males, while females displayed more bacteria within the phylum Deferribacteres. P80 treatment induced an increase of the genus *Pseudomonas* (phylum Proteobacteria) only in males. In addition, these treatments altered anxiety-like behavior such as increased distance travelled in the elevated plus maze and decreased time spent in the center of the arena in the open field test in males and reduced social behavior only in females.

Daikenchuto (TU-100), an herbal medication comprised of ginger, ginseng, and Japanese pepper, increased the genera *Bacteroides*, Rikenellaceae, *Turicibacter*, Clostridiaceae (phylum Bacteroidetes) and decreased one genus of the order Clostridiales and one genus of the family Desulfovibrionaceae in female mice after chronic administration for 24 weeks (Miyoshi et al., 2018). In addition, male mice showed increased levels of Coriobacteriaceae (phylum Actinobacteria), *Allobaculum* (phylum Firmicutes), and decreased *Turicibacter* and *Ruminococcus* (phylum Firmicutes). Chondroitin sulfate, a family of sulfated glycosamino-glycans (GAGs), actively involved in the pathological process of osteoarthritis, has been studied in relation to their effects on the gut microbiota. Administration of diet supplemented with CS induced increased abundance of Bacteroidetes and decreased the amount of Firmicutes in male mice as compared to females. Among the other differences observed, the bacterial genus *Odoribacter* (phylum Bacteroidetes) decreased in male mice and increased in females after treatment with both, CS and chondroitin sulfate oligosaccharide (CSO). Dietary treatment with just CSO increased abundance of *Lactobacillus* only in female mice, while both, CS and CSO treatment, decreased the abundance of *Lactobacillus* in male mice (Shang et al., 2016).

Inoculation of fecal bacteria from a single, male human individual with short-term vegetarian and inulin-supplemented diet into male and female germ-free C57BL/6J mice identified 46 operational taxonomic units (OTUs) to be differing between both sexes (Wang et al., 2016). In detail, 13 OTUs including *Parabacteroides distasonis* (phylum Bacteroidetes) and *Blautia faecis* (phylum Firmicutes) were elevated in males while 33 OTUs belonging to *Clostridium* groups and *Escherichia fergusonii/Shigella sonnei* predominated in females.

Another study addressed whether omeprazole (OM) treatment, an over-the-counter proton pump inhibitor, has an effect on the structure of and composition of gut microbiota in relationship to diet and sex differences. Female mice that received standard chow (STD, 6% fat) showed higher OTU richness compared to female on STD or high-energy chow (HiE) with omeprazole supplementation. Moreover, male mice on HiE diet with omeprazole supplementation showed reduced OTUs number as compared with males on STD diet (M. et al., 2017).

The effect of corn/soybean meal (CS) or wheat/barley/by-products (WBP) based diet were investigated in pigs in relation to sex. Especially, pigs fed with WBP diet showed differences in feed efficiency associated with 17 OTU in males and 7 OTU in females. Moreover, 3 OTU relevant for discriminating between low and high feed efficiency were in common for both sexes (Verschuren et al., 2018).

Acesulfame-potassium (Ace-K), an FDA-approved artificial sweetener, has been investigated to induce different effects on gut microbiota composition in relation to sex in mice (Bian et al., 2017). Especially, male CD-1 mice (~ 8 weeks old) chronically treated with Ace-K (37.5 mg/kg body weight/day) for 4 weeks showed increased levels of *Bacteroides* as compared to female. Female mice, however, showed significantly reduced abundance of multiple genera, including *Lactobacillus*, *Clostridium*, an unassigned genus of Ruminococcaceae and an unassigned genus of Oxalobacteraceae, and an increased abundance of *Mucispirillum* as compared to males.

In summary, all presented studies show how different types of diet can affect the gut microbiota in relation to sex differences in diverse animal models such as mice, rats and pigs but also in humans. How the same diets can differently alter the richness or composition of male and female gut microbiota is still an open question. Different fat distribution between men and women (Shimokata et al., 1989) may be considered as

a potential factor involved in the previously reported sex-dependent gut microbiota differences caused by diet. In agreement with this hypothesis, Min *et al.* (Min et al., 2019) associated different fat distribution between male and female with differences in the gut microbiota composition. Interestingly, Erysipelotrichaceae family and *Holdemanella* genus (phylum Firmicutes) showed significant negative association with fat ratio in female participants and negative with male participants. Moreover, body mass index (BMI) has been associated to different gut microbiota composition between men and women. A study conducted on 551 Chinese adult participants categorized as underweight, normal, overweight, or obese, showed higher alpha-diversity in women compared to men regardless to BMI (Gao et al., 2018). When BMI was taken in consideration, no differences in Principal coordinates analysis (PCoA) were observed anymore. However, increased levels of Fusobacteria phylum were detected in obese men as compared to obese women, while obese women showed increased relative abundance of Actinobacteria phylum suggesting that BMI-associated differences in the gut microbiota may be driven by gender. In addition, lower abundance of the *Bacteroides* genus was observed in men as compared to women in relation to low BMI (<33 kg/m²). Moreover, decreased abundance of *Bacteroides* genus went along with the increase of BMI (>33 kg/m²) in men, while it remained unchanged in women. In accordance with this, Dominianni and colleagues reported a significant association between lower abundance of Bacteroidetes with BMI only in women but not in men (Dominianni et al., 2015).

b) Antibiotic treatment induces sex-dependent gut microbiota composition differences

Interestingly, chronic antibiotic treatment elicited sex-dependent gut microbiota differences in human as well as animal studies. Analysis of 1135 gut metagenomes from the Netherlands (participants aged between 18 – 81 years) revealed that bacteria derived from women had higher numbers of antibiotic resistance genes belonging to *lincosamide nucleotidyltransferase* (LNU) family as compared to the microbiota of men (Sinha et al., 2019). In addition, higher Shannon diversity and higher levels of *Akkermansia muciniphila* (phylum Verrucomicrobia), a bacterial species that has been already associated with healthier glucose metabolism and leanness in mice and humans (Dao et al., 2016; Shin et al., 2014), was observed in females compared to

males. Furthermore, sex-dependent differences in *Akkermansia muciniphila* may suggest a more protective phenotype in regard to diabetes and insulin resistance in females (Sinha et al., 2019).

Moreover, gut microbiota alpha-diversity and richness significantly decreased in female mice exposed for 14 days to vancomycin (Vanc) or ciprofloxacin-metronidazole (CiMe), while male mice showed reduction in taxon-richness and alpha-diversity only after Vanc treatment (Gao et al., 2019). In detail, at phylum level, decreased Firmicutes abundance was observed in female mice exposed to both antibiotics while a clear reduction of Firmicutes abundance was observed only in male mice exposed to Vanc antibiotic. On the other hand, Vanc treatment increased the abundance of Proteobacteria phylum in both, male and female mice, while CiMe treatment increased the abundance of *Proteus* (phylum Proteobacteria) only in female mice. It seems that Vanc treatment led to lower Firmicutes and Proteobacteria levels in males compared to females and that CiMe treatment induced lower Firmicutes and higher Proteobacteria levels in female compared to males. However, no statistical analysis whether the antibiotic treatments induce differences between male and female mice at phylum level were provided. Harrison *et al.* showed that CiMe reduced the Porphyromonadaceae family (phylum Bacteroidetes) abundance in both, male and female mice, while reduction of Lachnospiraceae (phylum Firmicutes) after treatment was observed only in male but not in female animals (Harrison et al., 2019). Moreover, increased level of Lactobacillaceae (phylum Firmicutes) was reported in both sexes although, not in sex-specific manner. In addition, Enterococcaceae (phylum Firmicutes) remained absent in male mice treated with antibiotics while it rapidly expanded in female within the first two weeks of antibiotic administration and it remained at higher level throughout the entire duration of the treatment (2 weeks). SNF1 mice, used as a model for studying spontaneous lupus disease, treated with a broad-spectrum of antibiotics (ampicillin, vancomycin, neomycin and metronidazole) after weaning showed same levels of bacterial depletion between male and female mice (Johnson et al., 2020). Interestingly, the depletion of microbiota was associated with a 40% reduced possibility of developing severe nephritis in female mice while no association was detected in male mice.

An experiment conducted in piglets reported that a single parental antibiotic injection of ceftiofur (5.0 mg/kg body weight) affected gut microbiota in relation to sex (Ruczizka et al., 2020). In particular, antibiotic-treated

female piglets showed 2.1-fold more of Tenericutes phylum than antibiotic-treated males 28 days after the administration. 97 days after the administration, female pigs treated with antibiotics showed a 1.8-fold increase of Proteobacteria phylum as compared to males treated with antibiotics. Moreover, it was assumed that the changes in bacterial abundance might be associated with the body weight loss observed in female pigs at day 97.

c) Exposition to different environmental elements induces sex-dependent differences in the gut microbiota. An experiment performed on mice assessed that chronic low-dose cadmium exposition (100 nM, LDC) in early life induced alteration of the gut microbiota composition at 8 weeks of age leading to a decreased level of *Bifidobacterium* (phylum Actinobacteria) and *Prevotella* (phylum Bacteroidetes) only in male mice (Ba et al., 2017). In addition, early LDC exposition led to a fat accumulation only in adult male mice, suggesting again a correlation between sex-specific body fat and microbiota. Arsenic is one of the main agents affecting human health with more than 100 million people being exposed worldwide by the drinking water (Hughes et al., 2011). Arsenic has been shown to induce changes in the gut microbiota in both, male and female mice after 4 weeks of treatment (10 ppm arsenic in drinking water) (Chi et al., 2016). Interestingly, female mice showed significantly elevated beta-diversity as compared to males and among the bacteria, *Dorea* (phylum Firmicutes) abundance decreased in female mice, while it significantly increased in treated males. In addition, *Akkermansia* (phylum Verrucomicrobia) levels were higher in treated females in comparison to treated males.

Organophosphate insecticides, known to be applied in agricultural environments, are one of the most common causes of declining avian populations worldwide (Golden and Rattner, 2003). Changes in the cecal microbiota composition due to organophosphate insecticides have been investigated in Japanese quail (*Coturnix japonica*) in relation to sex (Crisol-Martínez et al., 2016). Female quail treated with trichlorfon showed significant reduction in the genus *Lactobacillus* (phylum Firmicutes) and a significant increase of Proteobacteria genera in comparison to untreated females, while no differences were observed between treated and untreated males. Moreover, treated females showed decreased beta-diversity in caecum

microbiota in comparison to untreated females, while again no differences were detected between treated and untreated males.

Nicotine, the active substance in tobacco, is known to increase Proteobacteria and Bacteroidetes phyla and to decrease Actinobacteria and Firmicutes phyla (Biedermann et al., 2013; Savin et al., 2018). An experiment carried out in C57BL/6J mice showed sex-dependent differences in the gut community composition in relation to nicotine exposure (Chi et al., 2017). In particular, female mice treated with nicotine (60 mg/L for 13 weeks via drinking water) showed reduced Christensenellaceae (phylum Firmicutes), Anaeroplasmataceae (phylum Tenericutes), F16, and unassigned families in the orders Bacillales and of the Enterococcaceae bacterium RF39 as compared to untreated female. Treated males, however, showed increased levels of F16, Turicibacteraceae and Peptococcaceae, and decreased level of Dehalobacteriaceae in comparison to untreated males. Although statistical analysis between treated male and female mice was not provided, treated male mice seemed to show higher levels of F16, an unassigned family in the order RF39, Turicibacteraceae and Peptococcaceae as compared to treated female animals. In addition, it has been reported that, six months of chronic cigarette smoke exposition changed the cecal content of microbial community in both, male and female mice (Tam et al., 2020). In particular, a significant reduction of *Alistipes* and uncultured *Bacteroidales* bacterium (phylum Bacteroidetes), higher level of *Prevotellaceae* NK3B31 group and *Bacteroides* were observed in the cecal content of male and ovariectomized female mice after chronic smoke exposition in comparison to control-treated mice.

d) Stress and early life adversity affect the microbiome according to sex

Stress, another important environmental factor for today's society, is known to induce perturbation of homeostasis in human ordinary life and in animals (Chovatiya and Medzhitov, 2014). Especially, continuous exposition to different types of stress is assumed to alter the gut microbiota composition. The impact of stress in relation to sex-dependent gut microbiota differences was for example observed in offspring mice from dams that received stress during days 1–7 of gestation ((early prenatal stress, EPS; (Jašarević et al., 2016)). In detail, EPS (60 min of fox odor exposure) disrupted the abundance of the early colonizers *Lactobacillus* and *Streptococcus* (phylum Firmicutes) regardless of sex. Afterwards, at the age of weaning, sex

differences appeared, showing enrichment of *Mucispirillum* (phylum Deferribacteres), *Odoribacter* (phylum Bacteroidetes), and *Desulfovibrio* (phylum Proteobacteria) in female mice, while *Dehalobacterium* (phylum Firmicutes) and *Flexispira* (phylum Proteobacteria) were enriched in males (Jašarević et al., 2016). In addition, chronic limited nesting stress (LNS) exposure from post-natal days 2-10 induced a lower diversity in fecal microbiota with a distinct composition characterized by increased abundance of Gram-positive cocci in both, male and female Wistar rats at weaning (Moussaoui et al., 2017). Despite the fact that differences were observed mainly between LNS exposed- and control-rats, two unclassified OTUs were significantly associated with sex. Furthermore, Rincel *et al.* addressed the importance of multifactorial early-life adversity on behavior and gut microbiota composition in relation to sex differences (Rincel et al., 2019). C3H/HeN mice, a strain more susceptible to maternal immune activation and maternal separation (MS) than C57BL/6 was used for this purpose. Animals were exposed to a combination of maternal immune activation (lipopolysaccharide injection on embryonic day 17, 120 µg/kg, i.p.), maternal separation (3 h per day from postnatal day 2 to 14) and to a maternal unpredictable chronic mild stress. Interestingly, male mice subjected to early adversity events showed increased abundance of taxa belonging to *Bacteroides*, *Lactobacillus*, *Alloprevotella*, *Porphyromonas* and unclassified Firmicutes and decreased abundance of unclassified Lachnospiraceae (phylum Firmicutes), and Porphyromonadaceae families (phylum Bacteroidetes), while females showed a decreased level of *Lactobacillus* and *Mucispirillum* genera.

e) Other daily habits affecting the microbiome in a sex-dependent manner

Different daily habits also may play an important role in shaping the gut microbiota in a sex-dependent manner: a study conducted in a community of human hunter-gatherers, the Hadza of Tanzania, observed different abundance of bacterial species in relation to sexual division of labor (Schnorr et al., 2014). Increased levels of *Treponema* (phylum Spirochaetes) were detected in women, which mainly forage for tubers and plant foods and spend more time with the children in the central camp. Increased levels of *Eubacterium* and *Blautia* (phylum Firmicutes) were observed in men, which are highly mobile and normally move from the central camp to hunt down animals and to collect honey.

The influence of sex and spatial tactics (i.e. floater vs. territorial), was also analyzed in free-ranging Namibian cheetahs (*Acinonyx jubatus*) (Wasimuddin et al., 2017). Interestingly, male cheetahs, which are bigger than females and differ in their diet composition from females (Caro, 1994; Voigt et al., 2014), showed higher mean abundance of the genera *Blautia* and *Eubacterium* - similar to the previously reported data on human hunter-gathers (Schnorr et al., 2014). However, the influence of spatial tactics, as a potential vehicle for the host exposition to environmental bacteria, did not affect the alpha-diversity and UniFrac distances in male cheetah (Wasimuddin et al., 2017), suggesting that other factors (e.g. diet and genetics) may have a more relevant function here.

Details of the cross talk between the here described environmental factors and sex are still to be unraveled. A factor that also should be considered when discussing the impact of sex hormones on the gut microbiota is age in general – not only referring to the prominent stages of puberty or onset of menopause. The gut-blood-barrier and also other barriers, such as the blood-brain-barrier, loose integrity with higher age (e.g. (Man et al., 2014; Marques et al., 2013)) and the cells belonging to the respective organ system also alter their functionality, entering into senescence or displaying reduced proliferation capacity (Moorefield et al., 2017; Rawji et al., 2016)). This, on one hand, intensifies the entry/escape of blood-transported factors such as sex hormones or bacterial metabolites but, on the other hand, increases the vulnerability of the respective organ to pathogens or other harmful substances. In addition, the age-dependent change in the cytochrome P450 system has to be taken into account when considering ingested pharmaceuticals or chemicals. Here, age affects the total amount of the xenobiotic-degrading enzymes but also their composition (George et al., 1995). Interestingly, increased CYP27B1 expression has been specifically found in duodenum of mice and humans during puberty (Gawlik et al., 2015) suggesting also its involvement on the microbial gut commensals composition.

5. Conclusions

In the current review, we provide summary of current knowledge on the impact of sex in relation to the gut microbiota composition in both, human and animal studies. From the very first day of life, the gut microbiota starts to shape in relation to sex, showing differences in composition and alpha-diversity. Boys and girls

showed for example significant differences in Actinobacteria, Firmicutes, and Bacteroidetes phyla amount with higher Bacteroidetes/Firmicutes ratio in boys compared to girls. We hypothesized that dynamic changes in sexual hormones such as the testosterone peak at early postnatal stage in boys or within puberty might be the leading cause of observed differences. However, studies carried out in adult population showed that men and women maintain different amount of gut microbiota phyla (Firmicutes, Verrucomicrobia, Bacteroidetes, Prevotella, Fusobacteria and Actinobacteria). In line with this, steady-state levels of sexual hormones may have an important role in shaping the gut microbiome. It may be too simplistic to conclude that sex hormones are the only players in generating gut microbiota differences between men and women. However, experiments conducted on castrated male mice showed similarity in the gut microbiota composition to that of female mice, suggesting an active role of male sex hormones in structuring the gut microbiota (Org et al., 2016). For humans, also educational aspects concerning sex or sex-dependent habitual differences might play an additional role, which are not abundant in laboratory animal models. This clearly hampers deducing impact of sex in humans from outcome of studies conducted exclusively in animal experiments. Moreover, longitudinal studies would be needed to unravel the impact of sexual hormones on the long run: higher androgen levels in newborn boys, for example, predicted greater negative affectivity (Alexander and Saenz, 2011). Such personality traits contribute to general development of sex-linked personality characteristics (Crockenberg et al., 2008) and by this might influence later life periods converging with other parameters such as the social organization (Archie and Tung, 2015), particularly important for shaping the individuals microbiota. Investigations in post-menopausal women are also still limited but they might help to evaluate the role of sex hormones. A metagenome-wide association study revealed a clear difference between pre- and post-menopausal women with, for example, a depletion of Firmicutes and *Roseburia spp.* (Zhao et al., 2019). Moreover, a small study indicated that post-menopausal women tended to be more similar to age-matched men as to pre-menopausal women regarding e.g. Firmicutes to Bacteroidetes ratio (Santos-Marcos et al., 2018). In addition, a recent study of Mayneris-Perxachs and colleagues reported that an androgenization of the microbiome occurs as a result of the menopause of women and that testosterone plays a major role in shaping the microbiota (data from a one-year follow-up

study on about 100 human subjects) (Mayneris-Perxachs et al., 2020). More such studies – together with data obtained from pubescents – should enlighten the distinct role of sexual hormones as direct or indirect choirmasters of our gut microbiota in future.

Authors' roles

FV collected the information, designed the pictures and drafted the manuscript. KE critically revised the manuscript. All authors have seen and approved the final version.

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