Toxicological studies of di-n-butyl phthalate (DBP)

Impact on the reproductive system and gut microbiota

Radwa Almamoun
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Abstract
The potential health impact of exposure to anthropogenic chemicals has raised major concerns worldwide. Phthalates are mainly used in the plastic industry and have been associated with adverse effects in humans. Di-n-butyl phthalate (DBP) is one of the dominant phthalates with a ubiquitous presence in the environment. While many studies have examined the endocrine disrupting properties of DBP, with a focus on developmental and reproductive dysfunctions, studies of its effects on the adult reproductive system and gut microbiota are limited. This thesis aimed to determine persistent effects of DBP on the adult male reproductive system, provide a high-throughput screening tool for identifying reproductive toxicants, and characterize the effects of DBP on the gut microbiota.

Paper I investigated if adult DBP exposure can induce persistent effects on the mature reproductive system. Adult male mice were orally exposed to 10 or 100 mg/kg/day for five weeks and testes were collected one week after the last dose. The results demonstrated a significant decrease in testosterone levels in the DBP-exposed groups. Mechanistically, the levels of steroidogenic enzymes, cell-specific markers and oxidative stress were increased. In paper II, elements of the in vivo testicular microenvironment, including functional testosterone production, were modeled using a three-dimensional (3D) heterogenous testicular cell co-culture derived from neonatal mice. Automated high-content imaging of cell-specific markers confirmed the presence of germ cells (DAZL+), Leydig cells (CYP11A1+), and Sertoli cells (SOX9+). DBP exposure decreased testosterone production, as well as levels of the steroidogenic enzyme CYP11A1, and the steroidogenic regulator StAR. Overall, this in vitro 3D model recapitulates the testicular pathways involved in DBP toxicity, making it a relevant tool for assessing reprotoxic effects of chemicals.

Paper III investigated the impact of oral DBP exposure on the gut microbiota and the potential interplay with immune and testicular toxicity using 16S rRNA sequencing. DBP-treated mice showed a distinct microbial composition and numerous differentially abundant amplicon sequence variants. Interestingly, the microbial alterations correlated with an increase in non-classical monocytes observed in DBP-exposed mice. In paper IV, a shotgun metagenomic analysis was conducted to achieve a more comprehensive characterization of the DBP-induced effects on gut microbiota composition and function. The DBP-exposed mice had a higher abundance of Adlercreutzia mucosicola, a bacterium linked with intestinal inflammation. In contrast, the beneficial Akkermansia muciniphila was less abundant in DBP-exposed mice. Functional analysis demonstrated that DBP exposure increased the abundance of genes involved in environmental sensing and antimicrobial resistance.

In conclusion, this doctoral thesis demonstrates the antiandrogenic effects of DBP as well as potential underlying mechanisms of testicular dysfunction in adult mice. In addition, we established a powerful in vitro tool for screening reprotoxic effects. The gut microbiota was also impaired by DBP exposure, which may play a potential role in initiating or exacerbating the DBP-induced toxicity. Overall, this work highlights the potential health impact of the interplay between the two exposome components, chemical exposure and gut microbiota.

Keywords: Dibutyl phthalate, Antiandrogen, Gut microbiota, Reproductive toxicity, Endocrine disrupting chemicals, Environmental contaminants, Phthalates, Plastics, 3D cell culture model, Exposome, Toxicology.
TOXICOLOGICAL STUDIES
OF DI-N-BUTYL PHTHALATE (DBP)

Radwa Almamoun
Toxicological studies of di-n-butyl phthalate (DBP)
Impact on the reproductive system and gut microbiota

Radwa Almamoun
To Roya
“All disease begins in the gut"
Hippocrates
>2000 years ago
List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals. Shared first authorship is indicated with an asterisk.


II. Almamoun, R., Pierozan, P., and Karlsson, O. Mechanistic screening of reproductive toxicity in a 3D testicular co-culture shows significant impairments following exposure to low dibutyl phthalate concentrations. *Submitted*


Publications not included in this thesis


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### Abbreviations

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<th>Full Form</th>
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<tbody>
<tr>
<td>16S rRNA</td>
<td>16S subunit ribosomal RNA</td>
</tr>
<tr>
<td>5-αR</td>
<td>5-alpha-reductase</td>
</tr>
<tr>
<td>ASVs</td>
<td>Amplicon sequence variants</td>
</tr>
<tr>
<td>BBP</td>
<td>Benzyl butyl phthalate</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic-adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DAZL</td>
<td>Deleted in azoospermia-like</td>
</tr>
<tr>
<td>DBP</td>
<td>Di-n-butyl phthalate</td>
</tr>
<tr>
<td>DEHP</td>
<td>Di(2-ethylhexyl) phthalate</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECHA</td>
<td>European Chemicals Agency</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine disrupting chemical</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>FSHR</td>
<td>Follicle stimulating hormone receptor</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GTDB</td>
<td>Genome Taxonomy Database</td>
</tr>
<tr>
<td>HPLC-MS</td>
<td>High pressure liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>HPT</td>
<td>Hypothalamus-pituitary-testis</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSD</td>
<td>Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INFγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LHR</td>
<td>Luteinizing hormone receptor</td>
</tr>
<tr>
<td>LOAEL</td>
<td>Lowest Observed Adverse Effect Level</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAGs</td>
<td>Metagenome-assembled genomes</td>
</tr>
<tr>
<td>MBP</td>
<td>Mono-butyl phthalate</td>
</tr>
<tr>
<td>MBP-G</td>
<td>Mono-butyl phthalate glucuronide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NOAEL</td>
<td>No Observed Adverse Effect Level</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCoA</td>
<td>Principle coordinate analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PERMANOVA</td>
<td>Permutational multivariate analysis of variance</td>
</tr>
<tr>
<td>PMN-MDSC</td>
<td>Polymorphonuclear myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>REACH</td>
<td>Registration, evaluation, authorisation, and restriction of chemicals</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short-chain fatty acids</td>
</tr>
<tr>
<td>SOX9</td>
<td>Transcription Factor 9</td>
</tr>
<tr>
<td>STAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>SULT1E1</td>
<td>Sulfotransferase Family 1E Member 1</td>
</tr>
<tr>
<td>TDI</td>
<td>Tolerable daily intake</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens protein 1</td>
</tr>
</tbody>
</table>
1. Introduction

Today, there are no plastic-free areas in our environment. Over the past decades, the global production of plastics has increased to approximately 400 million tons per year [1]. Plastic waste is considered one of the main contributors to anthropogenic pollution [2]. In the environment, plastics can take thousands of years to decompose [1, 2]. Considering this ubiquitous contamination of the environment, the potential impact of plastics on human health and diverse biota is significant. In parallel, there is a severe decline in birth rates worldwide, particularly in the industrialized world [3, 4]. This reproductive health decline has been linked not only to behavioral and economic factors but also to biological factors [3]. Exposure to environmental contaminants, particularly phthalates, may contribute to amplifying reproductive dysfunction by altering testosterone levels and semen parameters [3, 5, 6]. Recent research has also highlighted the critical role of the gut microbiome, and its interactions with environmental exposures, in influencing human health and disease [7].

Phthalates are high-volume industrial compounds widely used as plasticizers to improve the elasticity and durability of polyvinyl chloride (PVC) plastics since the 1930s. The worldwide production of plasticizers reached about 10.4 million tons in 2021, and is expected to exceed 13.9 million tons by 2027 [8]. Once manufactured, a plastic product may contain up to 10-60% of phthalates [9, 10]. Di-n-butyl phthalate (DBP) is among the top hazardous chemicals and considered as an endocrine disrupting chemical (EDC). The use of some phthalates, including DBP has been restricted in the EU [11]. This thesis aims to expand the current understanding of DBP-induced male reproductive toxicity and develop a high-throughput in vitro model for studying relevant cell signaling pathways involved in the testicular toxicity. Since DBP exposure occurs mainly via ingestion, the thesis also aims to explore the gut microbiota as a possible target for DBP toxicity and if the potential microbial alterations may crosstalk with other DBP toxicological effects.

1.1. Di-n-butyl phthalate

DBP is an odourless, faint yellow oily liquid that belongs to a family of manmade chemicals called phthalates. Phthalates are synthetic compounds
consisting of different alkyl esters of phthalic acid, which are extensively used as plasticizers (> 90%) in the production of PVC plastics to make them flexible and durable. DBP is a di-ester of phthalic acid obtained by the formal condensation of carboxyl groups of phthalic acid with two molecules of butyl alcohol. DBP was first produced in the 1930s, and initially intended to be used as an insect repellent and acaricide [12, 13]. Subsequently it gained popularity as an excellent plasticizer in PVC production. DBP is of great commercial and economic interest with diverse applications in plastic-based consumer products, such as packaging material, building material, including wall covering and flooring, electric cables, food packaging and synthetic leather. DBP is also used as a solvent and an additive in adhesives, paints, insects repellent, pharmaceutical coating and cosmetics, such as nail polish and perfume [14-16]. Phthalates are not covalently bound to the plastic matrix and have been shown to migrate from materials to the environment. Therefore, they have become ubiquitous environmental contaminants.

1.1.1. Exposure to DBP

DBP can leach out to the environment, water, and food, making it a pervasive contaminant [14, 15, 17, 18]. Various plasticizers involved in food packaging, including DBP, have been shown to migrate to foods [10, 19]. The migration is particularly considerable in fatty food and is enhanced with increased temperature [20]. An early study revealed the presence of DBP, di(2-ethylhexyl) phthalate (DEHP) and benzyl butyl phthalate (BBP) in retail samples of margarine and the butter, particularly on the contacting surfaces of the food material, due to migration from the coating material [21]. A more recent study found that the migration of DBP and DEHP from polypropylene food containers is increased under strong acidity condition and prolonged heating. The study further showed that DBP had the highest migration level, exceeding regulatory specific migration limits [20]. DBP has also been detected in different environments, such as soil, household dust, indoor air, wastewater and sewage [15, 22-25]. The general population is, therefore, continuously exposed to DBP [15].

The exposure to phthalates occurs through multiple routes, including ingestion, inhalation and dermal absorption. Among these, the consumption of phthalate-contaminated food and water is considered to be the most frequent, accounting for over 67% of exposure [15, 17, 18, 26]. Moreover, DBP and its metabolites can cross the placenta, and has been detected in amniotic fluid as well as breast milk, making developing offspring susceptible to exposure both before and after birth [27-30]. Exposure via dermal and inhalational routes
may be particularly important in high-exposure occupational settings, including plastic industry workers and beauty bar workers, specifically manicurists [31-34].

Human studies have reported a wide range of DBP exposure levels. For exposure assessment, the urinary levels of the primary metabolite of DBP, monobutyl phthalate (MBP), has been used as a biomarker of DBP exposure [35]. One large retrospective human biomonitoring study assessed the intake of various phthalates based on urinary metabolites in 634 subjects in Germany and estimated a median daily DBP intake of 4.1 µg/kg/day, with a peak value of 116 µg/kg/day [36]. A study in south Delhi in India that estimated DBP intake based on analysis of food, water and air samples reported 7.1 µg/kg/day as a lower estimate and 38.4 µg/kg/day as a higher estimate [37]. Moreover, a retrospective study in USA that included 289 adults, estimated a DBP intake of 1.5 µg/kg/day [38, 39]. In addition, studies have investigated the exposure to DBP through the coating of medications. For example, one study analyzed DBP levels in phthalate-containing medications and found that the amounts of DBP in the defined daily dose of bisacodyl, budesonide, and lithium exceeded 10 µg/kg/day [16]. Another study determined high exposure levels of DBP, up to 233 µg/kg/day, in patients taking mesalamine medication (Asacol) that contains DBP in the enteric coating [40].

1.1.2. Toxicokinetics

Studies in rats have shown that orally administered DBP undergoes rapid hydrolysis in the gastrointestinal tract to its respective monoester metabolite, MBP, by intestinal esterases and lipases before absorption. In the liver, MBP can be further conjugated to MBP-glucuronide (MBP-G) [41, 42]. Rodent studies have also demonstrated that unconjugated MBP is the main metabolite in plasma (>77%), with no significant tissue bioaccumulation, followed by MBP-G (19%). However, MBP-G was found to be the primary metabolite in urine (53-69%) [43]. Other less abundant metabolites including mono-butanolic phthalic acid, mono-hydroxybutylphthalate, and its glucuronide, were also detected in plasma and urine [44]. In rodents, the elimination halftime of DBP is 2-3 hours, with the majority excreted within 24 hours in urine (>90% as MBP-G) [44].

Data from human studies have shown similar DBP metabolism and elimination rates obtained from rat studies (>90% excreted within 24 hours) [45]. In a comparative study, DBP conversion by gastrointestinal enzymatic activity has been shown to occur at similar rates in rodents and in humans [46]. However, in humans, MBP-G is the principal DBP metabolite detected in both plasma and urine [47, 48].
1.1.3. Toxicological effects of DBP

Over the last two decades accumulating evidence has indicated that DBP, among other phthalates, can interfere with hormone signaling pathways and functions, and are considered as endocrine disrupting chemicals. The most studied adverse effect of DBP exposure is testicular toxicity, particularly when the exposure occurs during fetal development. However, animal studies have also reported effects on other organs, such as the liver, immune and endocrine systems [49-52]. For example, in vivo studies in rodents exposed to 50-700 mg/kg/day DBP reported reduced serum cholesterol and/or triglycerides, suggesting impaired lipid metabolism and hepatic toxicity [49, 53]. Rats exposed to DBP at 350 mg/kg/day or more showed induction of the hepatic peroxisomal enzyme activity [49]. In vitro exposure to 50 µM DBP weakly activated the three subtypes of the peroxisome proliferator-activated receptor (PPAR) in transiently transfected HepG2 cells. The PPAR is involved in various metabolic processes, such as lipid homeostasis and may play a significant role in the DBP-induced hepatic toxicity [49, 52].

Studies have also demonstrated that DBP can affect immune cells and alter cytokine profiles. For instance, in vitro DBP exposure reduced the production of proinflammatory cytokines tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), and altered the expression of granulocyte markers in response to lipopolysaccharide (LPS) inflammatory stimuli in human whole blood assay [54]. Our group, has previously shown that oral exposure to DBP (10 and 100 mg/kg/day) for five weeks resulted in leukopenia, decreased populations of T helper (Th) cells and classical monocytes, as well as altered levels of several cytokines in the blood of exposed mice. These changes persisted at least one week after the final DBP dose and indicate that the compound can cause an immune impairment [51]. In addition, exposure to dust containing different phthalates, including DBP, has been associated with airway diseases such as asthma [55, 56]. In the meantime, there is a mounting evidence that the gut microbiota is implicated in numerous disorders, in particular metabolic and immune-mediated diseases. Given the fact that exposure to DBP occurs mainly through ingestion, we hypothesized that DBP might perturb the gut microbiota composition and function, which could be further interlinked with DBP-induced immune and metabolic toxicity.

1.1.4. Developmental and reproductive toxicity of DBP

Reproductive and developmental toxicity of DBP has been extensively studied in male rodents. In terms of in utero DBP exposure, studies have shown that the compound interferes with the development of testis and markedly reduces the fetal testosterone levels essential for male sexual differentiation, resulting in subsequent abnormalities in the male reproductive system [57-60]. One of
the major adverse effects of phthalates toxicity, including DBP, is the so-called “phthalate syndrome”, which is hypothesized to cause a syndrome similar to the testicular dysgenesis syndrome [61, 62]. The phthalate syndrome originates in fetal life following gestational exposure and is characterized by malformed seminiferous tubules, cryptorchidism (undescended testes), hypospadias, reduced anogenital distance, and low sperm count or decreased sperm quality. Multiple in vivo studies have found that gestational exposure to ≥ 500 mg/kg/day DBP resulted in testicular lesions of Leydig cells and multinucleated germ cells, as well as low fetal testosterone levels in rats [62-64]. Studies have also shown that DBP exposure can result in impairment of Sertoli cells function leading to degeneration of the seminiferous tubules and impaired sperm production [65-67]. There is a growing evidence that oxidative stress may play an important part in mediating DBP toxicity [68, 69]. Although several animal studies have investigated the potential mechanism by which DBP exerts toxic effects on the male reproductive system, especially during fetal development using high dosages, a fully detailed mechanism still needs to be elucidated and include all stages of life. The mechanisms behind DBP-induced germ cell toxicity also remains to be revealed.

Most of the experimental models used to study the developmental and reproductive effects of DBP are focused on gestational and perinatal exposure. Studies specifically focusing on neonatal exposure to phthalates are scarce. There is also an unmet demand for relevant alternative testing methods for developmental and reproductive toxicity that are less labor-intensive, fast, relevant, cost-effective, and use a relatively small number of animals compared to in vivo studies. Such in vitro models are particularly important due to the sensitivity of the early postnatal genital developmental window that can modify reproductive function and fertility later in life [70].

Epidemiological studies have suggested links between phthalate exposure, including DBP and several health issues, primarily reproductive dysfunction, in humans [55, 56, 71-73]. For instance, a study in 134 young boys that measured the urinary levels of four phthalate metabolites in mothers, including the DBP metabolite MBP, found that gestational exposure to phthalates was associated with decreased anogenital distance index and incomplete testicular descent [74]. The anogenital distance defines the distance between the anus and genitals, which tends to be longer in newborn males compared to females [75]. Studies in humans and rodents have shown that shorter male anogenital distance is linked with defects in fetal testosterone synthesis and may influence sperm count and testicular function in adults [72, 76]. Another study found an association between altered semen parameters and MBP urinary level in 463 men attending an infertility clinic [77]. Furthermore, in a study evaluating the impact of occupational exposure to DBP and DEHP from 74 male workers at
a PVC plastic factory, elevated urinary levels of MBP were associated with lower serum testosterone levels [34].

1.1.5. Current risk assessment of DBP

DBP is classified as toxic for reproduction (category 1B) and toxic to aquatic life according to the harmonised classification and labelling approved by the EU. Therefore, it is included in the Authorisation List of substances in Annex XIV of the European Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). This means that DBP cannot be used for any application within the EU without permission from 2015 [11, 78]. Nevertheless, DBP is still ubiquitous in the environment and in materials that are still in use, and remains prevalent in the global market [15, 79]. The safe reference dose (tolerable daily intake; TDI) for human exposure to DBP is set to 7 µg/kg/day, according to the European Chemical Agency (ECHA). Other regulatory bodies, such as the European Food Safety Authority (EFSA) define a similar TDI of 10 µg/kg/day. This TDI was estimated based on the Lowest Observed Adverse Effect Level (LOAEL) of 2 mg/kg/day in a rat study that showed effects, such as decreased numbers of spermatocytes and nipple retention after developmental exposure to DBP in feed [80]. The US Environmental Protection Agency (EPA) safe dose is 100 µg/kg/day. Notably, the long-term effects of adult exposure to DBP have not been evaluated.

1.2. The gut microbiome

The microbiota is made up of a diverse range of bacteria, viruses, fungi, and protozoa that reside various sites of multicellular organisms, both internally and externally [81]. These microbes can be found in the gastrointestinal tract, skin, oral mucosa, saliva, vaginal tract, seminal fluid, conjunctiva, and lung. Most of these microbial communities are either commensal or mutualistic, in which one or both organisms benefit from the interaction [81]. The microbiome has been referred as the "superorganism" due to its large genome collection of all microbial genes, which encodes over 3 million genes compared to the human genome of 23,000 genes [82, 83]. The human gastrointestinal tract harbors more than 100 trillion microorganisms, and the number of microbial cells in the human body is estimated to be equal to the total number of human cells [83, 84]. The gut microbiota plays significant roles in maintaining human health, such as digesting indigestible carbohydrates into the beneficial short-chain fatty acids (SCFA), protecting against pathogens by competing for space, inhibiting their growth, and producing antimicrobial agents, such as bacteriocins and hydrogen peroxide. The gut bacteria also plays a role in the
synthesis and modification of vitamins, bile acids and amino acids, modulating gut endocrine and neurological signaling, and regulating the development and function of the immune system [81].

The composition of the microbiota varies between sites. For example, the skin microbiota is dominated by gram-positive bacteria. *Staphylococcus* and *Corynebacterium* are the most common genera found in skin [85]. Fungus and virus species, such as *Malassezia* spp. and *Candida* spp. are also considered skin microbiota [86, 87]. The vaginal microbiota is characterized by the dominance of *Lactobacillus* spp., which plays protecting roles in the vaginal ecosystem by producing antimicrobial compounds, like lactic acid, bacteriocins, and hydrogen peroxide [88]. The stomach was previously believed to be a sterile environment because of its low pH levels. However, after the discovery of *Helicobacter pylori*, a bacterium that can survive in the acidic environment in the stomach, it was found that other species, such as *Lactobacilli* and *Streptococci*, also can inhabit the stomach [89].

The highest concentration of bacteria is found in the colon, estimated to be $10^{11}$ to $10^{12}$ bacterial cells/mL. The colon microbiota consists of six phyla, including *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* [82]. *Firmicutes* and *Bacteroidetes* are the dominant representatives, making up 90% of the gut microbiota. The anaerobic species belonging to the *Bacteroidetes* and *Actinobacteria* and comprises the majority of the intestinal microbiota [90]. Several factors can influence the gut microbiota composition, including delivery mode at birth, infant feeding, dietary habits, use of medications, life style and other environmental factors as well as host genetics [7, 91-96]. In addition to these well-known factors, gender has been suggested as a potential variable influencing the gut microbial population. However, the results from available studies are inconsistent. For example, gender effects were observed in a cross-sectional study, where males had a higher abundance of *Bacteroides-Prevotella* group, than females [97]. In a large cohort, gender was associated with gut microbiota composition and metabolic pathways. Females had higher presence of *Akkermansia* and an increased abundance of antibiotic resistance genes compared to males [98]. Similarly, another study showed that gender explained about 2% of the variations in the gut microbiota [99]. In contrast to these studies, a study from five European countries found no significant grouping of the gut microbiota according to the gender [100].
1.2.1. Gut dysbiosis and health effects

Gut dysbiosis is the disruption of the gut microbiota homeostasis and describes alterations in microbial composition and functions [101, 102]. Perturbation of the gut microbiota under defined environmental context may lead to an impaired intestinal barrier and abnormal metabolic, immune, hormonal, and neural pathways, contributing to disease development [101, 102]. Dysbiosis has been associated with numerous gastrointestinal tract diseases, as well as extra-intestinal disorders. These include inflammatory bowel disease (IBD), obesity, diabetes, allergy and asthma, liver disease, atherosclerosis and colorectal cancer [103] (Figure 1). For example, accumulating evidence indicates that a higher Firmicutes to Bacteroides ratio in the gut microbiota is associated with obesity in mice and humans [104]. Studies in mice showed that microbial short-chain fatty acids, specifically butyrate and propionate, have anti-obesogenic effects and can help prevent diet-induced obesity by stimulating anorexigenic hormones and leptin synthesis [105, 106]. Moreover, mice treated with the butyrate prodrug tributyrin were protected from diet-induced obesity and associated insulin resistance [107]. Studies have also shown significant associations between altered gut bacteria and metabolic diseases. For instance, the decreased abundance of Firmicutes and the class Clostridia in type 2 diabetes mellitus compared to the non-diabetic group [108].

Extensive research has been conducted to study the interplay between gut microbes and the immune system. Abnormal interaction between microbiota and the immune system has been found to play a central role in the development of complex immune-mediated diseases, as in the context of IBD, including ulcerative colitis and Crohn’s disease (CD) [109-111]. In IBD patients, certain environmental factors, such as antibiotic drug use coupled with host genetic susceptibility, can lead to decreased microbial diversity, marked shifts in specific taxa abundance, and altered metabolite profiles. Such changes can result in a defective mucosal barrier and translocation of bacteria into the mucosa, leading to aberrant host immune responses [109, 112]. Microbiota from IBD patients have been shown to display microbial patterns that are characterized by reduced richness, with less abundant Firmicutes, Bacteroides and more abundant Gammaproteobacteria such as Enterobacteriaceae [113, 114]. The reported aberrant immune responses include augmented Th17, Th1, and Th2 responses, attenuated T regulatory cells, and impaired antibody-mediated immune response [112]. Although these changes are believed to significantly contribute to IBD pathogenesis, a causal relationship is still debated and remains to be proven [109, 111].

Moreover, the crosstalk between the microbiota and brain has led to the emergence of a distinct microbiota-gut-brain axis [115]. Growing evidence suggest
that gut dysbiosis is implicated in a number of neural disorders, including autism, anxiety, Parkinson’s disease, schizophrenia, and Alzheimer’s disease. Gut microbiota can interact with the brain via various pathways, including the immune system, the neurohormonal system, the vagus nerve and the enteric nervous system, involving microbial metabolites such as short-chain fatty acids, branched chain amino acids, and peptidoglycans [115]. Given the above mentioned implications of the gut microbiota in numerous diseases, it is crucial to study the impact of DBP exposure on the gut microbiota, and the potential interplay with DBP-induced toxicity. There may also be a potential interplay between changes in gut microbiota and effects on the male reproductive system. These effects are hypothesized to be indirect via gut dysbiosis-mediated alterations in systemic inflammation, metabolites, endotoxins and reactive oxygen species levels [116, 117]. However, overall, the exact pathways for this interaction is understudied.

![Figure 1. Gut microbial dysbiosis and its associations with various diseases. Figure created with BioRender.com](image)

1.2.2. Tools for studying the gut microbiota

Previous knowledge of gut microorganisms was shaped by culture-dependent methods that include cultivation of microbial cells. However, recent advances in high-throughput sequencing technologies, together with advances in bioinformatics have revolutionized our understanding of the entire gut microbial communities and their functional potentials [103, 118]. In particular, various omics-based approaches are employed to verify the presence of microorganisms ecosystem in dynamic and interactive microbial communities within a
habitat or environment. These approaches include DNA sequencing technologies: 16S rRNA amplicon sequencing and shotgun metagenomics, metatranscriptomics and metaproteomics. Furthermore, the gut bacteria can produce bioactive metabolites including antimicrobial compounds, toxins, signaling molecules and host energy substrates, which in turn can enter the host bloodstream through absorption into the enterohepatic circulation. Certain gut microbiota metabolites may be linked to specific disease phenotypes and can be identified using mass spectrometry-based metabolomics of urine or faeces [101]. Currently, there is therefore a growing interest in conducting joint analysis of the microbiome, metabolome and host phenotypes to uncover potential mechanistic links [118].

1.2.3. The exposome concept and toxicology

The exposome concept describes the cumulative environmental exposures and their associated biological responses throughout the lifetime of the individual [119]. This includes all non-genetic exposure features that can influence health and disease, for example environmental chemicals, diet, microbiota, radiation, drugs, air pollutants, climate and psychosocial stress, as well as internal chemicals produced by infection, oxidative stress and microbial metabolites and products [119]. Omics tools, such as exposomics, metabolomics, and metagenomics can be used to characterize such multiple exposures. Applying the exposome concept in toxicology can advance our understanding of the interplay among complex environmental exposures, including chemical mixtures, and the corresponding toxicity outcomes [120-122]. For instance, diet is a key modulator of the gut microbiota. In parallel, exposure to environmental chemicals have been shown to affect the gut microbiota. The interaction between these three exposome components and its potential role in the chemical toxicity outcome needs to further investigated [121].
2. Aims

The overall aim of this thesis is to increase the understanding of the toxicological effects of DBP exposure in males by using an in vivo mouse model and in vitro studies. The main focus was persistent effects of adult DBP exposure on the male reproductive system and the gut microbiota. This included, to our knowledge, the first characterization of the composition and potential function in the gut microbiota after oral exposure to DBP using two DNA sequencing approaches. This thesis also addresses the need for developing methodologies for mechanistic high-throughput screening of developmental and reproductive toxicants. A three-dimensional (3D) in vitro testicular co-culture model was developed to study the specific cell signaling pathways involved in neonatal reproductive development in a high-throughput format.

The specific aims of the studies are:

**Paper I:** Study the persistent effects of oral exposure to DBP on the male reproductive system in adult mice and the underlying mechanisms of potential changes. This work focused on the testosterone regulation and biosynthesis pathway, testicular cell markers and relevant mechanistic pathways.

**Paper II:** Establish and develop a 3D heterogenous testicular co-culture system that allows mechanistic screening of reproductive toxicants during the postnatal developmental in a high-throughput format. DBP was used as a model compound to elucidate potential reprotoxic effects of DBP on the developing testicular cells, and demonstrate the applicability of the in vitro model.

**Paper III:** Investigate the impact of oral DBP exposure on the gut microbiota composition and examine possible relationships with DBP-induced immune system impairment and reproductive toxicity.

**Paper IV:** Further investigate the effects of oral DBP exposure on the gut microbiota in adult mice by conducting a more comprehensive compositional and functional profiling of the whole genome of the gut microbiota using shotgun metagenomic sequencing.
3. Methodology

A summary of the experimental procedures used in this thesis is given in Table 1. The methodology sections provide a brief overview of the study models and methods. For more detailed descriptions, the reader is referred to the Methods and Materials section in each respective paper.

**Table 1. Overview of the experimental models and methods used in this thesis**

<table>
<thead>
<tr>
<th>Method</th>
<th>To investigate</th>
<th>Model, age and species</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunofluorescence staining</td>
<td>Effects on testicular cell markers and levels of key proteins</td>
<td>In vivo Adult DBP exposed C57BL/6N mice</td>
<td>I</td>
</tr>
<tr>
<td>Automated high-content analysis</td>
<td>Mechanistic screening of reproductive toxicity by analyzing cell-type specific markers and levels of key proteins</td>
<td>In vitro DBP exposed 3D testicular co-culture from neonatal mice</td>
<td>II</td>
</tr>
<tr>
<td>MTT assay</td>
<td>Cell viability</td>
<td>In vitro DBP exposed 3D testicular co-culture from neonatal mice</td>
<td>II</td>
</tr>
<tr>
<td>ELISA</td>
<td>Effects on testosterone levels</td>
<td>In vitro DBP exposed 3D testicular co-culture from neonatal mice</td>
<td>II</td>
</tr>
<tr>
<td>Western blot</td>
<td>Levels of testicular cell markers, steroidogenic enzymes and critical proteins</td>
<td>In vivo Adult DBP exposed C57BL/6N mice</td>
<td>I</td>
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<tr>
<td></td>
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<td>In vitro DBP exposed</td>
<td>II</td>
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</table>
3D testicular co-culture from neonatal mice  
HPLC-MS  Effects on steroid hormones  
DNA extraction  Isolate microbial DNA  
16S rRNA amplicon sequencing  Characterize the taxonomic composition of the gut microbiota  
Shotgun metagenomics sequencing  Characterize the taxonomic and functional profile of the gut microbiota  

<table>
<thead>
<tr>
<th>Method</th>
<th>Objective</th>
<th>In vivo</th>
<th>Study</th>
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<tbody>
<tr>
<td>HPLC-MS</td>
<td>Effects on steroid hormones</td>
<td>In vivo</td>
<td>Paper I, III and IV</td>
</tr>
<tr>
<td>DNA extraction</td>
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<td>Adult DBP exposed C57BL/6N mice</td>
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<td>Adult DBP exposed C57BL/6N mice</td>
</tr>
</tbody>
</table>

3.1. Ethics

All animal experiments conducted in this thesis were approved by the Regional Animal Ethical Committee and were carried out in accordance with the Swedish legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Union Directive on the Protection of Animals Used for Scientific Purposes (2010/63/EU).

3.2. *In vivo* DBP exposure model

C57BL/6N male mice at the age of eight-week were purchased from Charles River (Sulzfeld, Germany) and orally exposed to DBP to study the reproductive toxicity and the effects on the gut microbiota (Paper I, III and IV). This mouse strain is frequently used as an *in vivo* model and has well-documented genome sequence, phenotypic, and genetic information. The mice were housed in a controlled environment and provided with standard food pellets and water *ad libitum*. After one week of acclimation, the mice were orally exposed to DBP diluted in corn oil at dosage of 10 mg/kg/day or 100 mg/kg/day via gavage once per day for five weeks. The control group was administered corn oil only. Each group consisted of 12 mice. The mice were
euthanized via decapitation one week after the final dose to allow investigation of more persistent effects. The plasma and testicular tissues were collected directly after euthanasia for evaluation of testosterone levels and key testicular cell markers (Paper I). Fecal pellets were collected before euthanasia to conduct microbiome analysis (Paper III and IV). A schematic diagram of the in vivo model is illustrated in Figure 2.

![Figure 2. Schematic overview of the in vivo DBP exposure model (Paper I, III and IV). Figure created with BioRender.com](image)

### 3.3. Mouse 3D testicular co-culture

The purpose of modeling the testicular cells in 3D in vitro conditions is to develop a robust and relevant, high-throughput platform recapitulating the heterogenous cellular interactions in vivo. In paper II, Matrigel, a soft matrix that reconstitutes into a solid gel at 37°C, was used to support the testicular cells. Matrigel is a mixture of extracellular matrix (ECM) proteins and growth factors derived from Engelbreth-Holm-Swarm mouse sarcoma. The addition of Matrigel provides a 3D architecture that can mimic in vivo cell-to-cell and cell-ECM interactions and connectivity [123, 124]. Using this 3D testicular co-culture, we were able to recapitulate and study Leydig cell steroidogenesis function and regulation, demonstrated by the production of testosterone and the expression of key enzymes in the testosterone biosynthesis pathway, which is not possible with Leydig cell lines [125]. The productivity of the 3D testicular co-culture was improved by optimizing cell seeding in a 96-well plate instead of culture dishes used in the previously published protocol [126]. This allows high-throughput analysis to improve the time efficiency and include assessment of further parameters, such as the protein components of tight junctions between adjacent Sertoli cells. The relatively small number of
In brief, testicular tissues were harvested from postnatal day seven (PND 7) old male mice and used to obtain a single cell suspension through a two-step digestion protocol described by Wegner and colleagues with modifications [126]. In brief, the fresh samples were mechanically decapsulated and dissected into small fragments under dissecting microscope. For the first step of digestion, the tissues were digested in minimum essential medium (MEM) supplemented with collagenase, hyaluronidase and DNase I to dissociate the interstitial compartment and digest excessive cellular aggregation. In the second step, the remaining small tissues, mainly seminiferous tubules fragments were processed in culture medium supplemented with collagenase and DNase I. This was followed by washing steps and trypsin digestion. The isolated testicular cells were resuspended in complete medium containing a mixture of nutrients. The viability and the count of the isolated cells were determined and the cells were seeded in 96-well plate or 6-well plates.

To set up the 3D system, drops of ice-cold Matrigel were added to the center of each well and gently swirled with the cell suspension to facilitate an in vivo-like supportive matrix, thereby enhancing the cell viability and providing beneficial cell-to-cell interactions [124]. The 3D testicular co-culture was acclimated for 48 h before treatment with DBP. A wide range of DBP concentrations, including 1 nM, 10 nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM, 50 µM and 100 µM were used. The controls were treated with the solvent only (0.1% DMSO). The duration of exposure was seven days and the exposure medium was replaced every other day. The viability of the testicular cells was evaluated using the MTT colorimetric assay. This assay estimates the number of viable cells by measuring the cleavage of the tetrazolium ring of MTT dye by the dehydrogenase and oxidoreductase enzymes present in the active mitochondria of living cells. The absorbance of the generated purple formazan is proportional to the number of viable cells present. Mechanistic screening of reproductive toxicity was conducted by analyzing cell-specific markers and levels of key proteins with immunofluorescence staining and automated high-content analysis. A schematic workflow of the 3D testicular co-culture is illustrated in Figure 3.
3.4. ELISA

ELISA is an immunoassay for detecting and measuring antigens through highly specific antibody-antigen interactions. A testosterone ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) was used to measure testosterone in collected cell medium according to the manufacturer protocol (Paper II). In brief, this ELISA uses a monoclonal antibody that binds to testosterone in a competitive manner. The testosterone in the sample competes with an enzyme-labeled antigen for a limited number of antibody binding sites on a 96-well microtiter plate coated with Goat anti-Mouse IgG. After incubations, the excess reagents are washed away and the enzyme substrate added. The enzymatic reaction is ended by adding the stopping solution and the absorbance measured on a microplate reader (Molecular Devices, San Jose, CA, USA). The intensity of the yellow color is inversely proportional to the testosterone concentrations in the sample or standards. The measured optical density and the generated standard curve were used to calculate the concentration of testosterone in the samples.
3.5. Immunofluorescence

Immunofluorescence, also known as cell imaging, is a technique that uses a fluorophore-labeled antibodies to identify and localize a specific target antigen. The presence of Leydig cells, Sertoli and germ cells was confirmed by immunofluorescence staining of cell-specific markers, including CYP11A1 and SULT1E1 (Leydig cell), SOX9 and vimentin (Sertoli cell), and DAZL (germ cell marker) (Paper I and II).

In paper I, cryosections from frozen mouse testes were fixed with ice-cold methanol followed by washing steps. Non-specific antibody binding was prevented by blocking with serum, such as 1% bovine serum albumin (BSA) and 10% serum from the same species of the secondary antibody. The tissue slides were incubated with primary antibodies overnight at 4 °C, followed by washing steps. Next, the sections were incubated with secondary antibodies conjugated with different fluorophores to produce the signal. The nuclei were counterstained with DAPI and the sections were mounted using DPX-mounting media. Images were acquired using a fluorescent microscope (Olympus BX53F2, Tokyo, Japan) with constant acquisition settings for all the samples. The fluorescence intensity at the specific wavelengths was determined. In the 3D testicular co-culture model (Paper II), the testicular cells, seeded in 96-well plate, were fixed with 4% paraformaldehyde. After blocking, cells were permeabilized with phosphate buffered saline (PBS) containing 0.25% Triton X-100. After blocking, cells were stained with different primary and secondary antibodies. The nuclei were counterstained with DAPI. Image acquisition and analysis were conducted by using an ImageXpress Micro XLS Confocal High-Content Analysis System and the MetaXpress Software (Molecular devices, Sunnyvale, CA, USA). One advantage of this technique is the ability to detect multiple antigens simultaneously (multiplexing) by using multiple fluorophores excited at different wavelengths. The use of automated high-content imaging with multiplexed quantification of fluorescence intensities has significantly improved the analysis time efficiency.

3.6. Western blot

Western blot is a common technique for detecting and measuring proteins. This method was used to quantify the protein levels and complement the results of the immunofluorescence for testicular cell markers and steroidogenic proteins (Paper I and II).

Western blot was used to measure the levels of a series of steroidogenic oxidative enzymes, including the cytochrome P450 side chain cleavage enzyme (P450scc or CYP11A1), 3β-hydroxysteroid dehydrogenase type II (HSD3β2),...
CYP17A1, 17β-hydroxysteroid dehydrogenase type III (HSD17β3), and 5-α reductase (5-αR). In addition, quantification of cell markers, including DAZL, vimentin, SOX9 and SULT1E1 was performed. Western blot was also used to quantify the levels of mechanistic markers, including the steroidogenic acute regulatory protein (StAR) that facilitates the transport of cholesterol from the outer to the inner mitochondrial membrane, luteinizing hormone receptor (LHR), follicle stimulating hormone receptor (FSHR) and the oxidative stress marker nitrotyrosine.

In brief, to measure the specific proteins, equal amount of proteins was separated based on their molecular weight using gel electrophoresis. The proteins were then transferred to a nitrocellulose membrane where they can be detected using antibodies. To prevent non-specific antibody binding, the membrane was blocked by adding milk. Next, the membrane is incubated with a primary antibody specific to the target protein overnight, followed by a secondary antibody that is enzyme-labelled (horseradish peroxidase (HRP)-conjugated secondary antibody). The membrane was then incubated with a chemiluminescent substrate, which produces a light signal that can be detected using a digital imaging system (iBright CL750 Imaging System, ThermoFisher, Rockford, IL, USA). The amount of protein is directly proportional to the intensity of the signal. The protein content was normalized against the loading control β-tubulin or β-actin.

3.7. High-pressure liquid chromatography-mass spectrometry

To measure the levels of steroid hormones in the testis and adrenal glands, high-pressure liquid chromatography-mass spectrometry (HPLC-MS) was used (Paper I). The tissues were homogenized and then centrifuged. The hormones were extracted from the tissue sample, with liquid-liquid extraction using acetonitrile:ethyl acetate (9:1) with the addition of internal standards. The remaining proteins were then precipitated, followed by another centrifugation step. Calibration curves were prepared by adding internal standards at the same concentrations as in the samples. The chromatographic separation was done using an ultra-high pressure liquid chromatography system (Ultimate 3000, Thermo Scientific, CA, USA). Mass spectral data were acquired using a Q-Exactive HF-X Orbitrap (Thermo Fisher Scientific, Bremen, Germany).
3.8. Sequencing of the gut microbiota

Two DNA sequencing approaches, 16S rRNA sequencing and shotgun metagenomics, were used to characterize the gut microbiota in DBP-exposed mice and its relationship with health and disease (Paper III and IV). The 16S rRNA gene amplicon sequencing approach targets the small-subunit ribosomal RNA gene, which is well conserved in almost all bacteria. This gene is about 1,500 bp and consists of both regions that are identical across diverse bacteria, and regions that are variable across bacteria, which can be used to distinguish between different bacteria. These nine hypervariable regions (V1-V9) are located between several conserved regions. The variable regions can be amplified by polymerase chain reaction (PCR) and sequenced. The 16S analysis is a cost-effective method frequently used to identify taxonomic and phylogenetic profiling of the microbial community in a sample (Paper III).

In brief, DNA was extracted from fecal samples of DBP-treated and control mice. The library preparation was done using two-step PCR, in the first PCR reaction the variable region V3-V4 of the 16S rRNA gene was amplified. In the second PCR reaction, the indexes and Illumina adaptors were incorporated. The amplified DNA was denatured into single-stranded DNA, which then was sequenced. The process for analyzing the amplicon data involves several steps. First, quality control and trimming of any low-quality bases, primers, and adapters. Algorithm models for denoising and chimera removal were used to correct or discard amplicon sequencing errors. To identify the microbial community, amplicon sequence variants (ASVs) were generated. ASVs are variants that can differ by as little as one nucleotide with no fixed threshold for dissimilarity. Finally, the SILVA database was used for ASVs taxonomic annotations. The main caveats of using 16S sequencing are the low taxonomic resolution below the genus level, as well as the lack of functional genes compared to shotgun metagenomics [127]. Another limitation is the choice of the PCR primers, which may introduce biases, such as favoring the amplification of particular bacterial taxa and possible chimeric molecule formation.

In contrast to 16S sequencing, shotgun metagenomics (Paper IV) enables sequencing of the entire microbial genome in a sample. Shotgun metagenomics has the advantage of providing higher sequencing depth and identifying genes and pathways with functional potential in the microbial community. In brief, the DNA was fragmented, amplified and adapters were ligated. After quality control of reads, bioinformatic tools were applied to reconstruct the microbial genomes present in each sample using the assembly-based approach. Using binning tools, representative assembled sequences (contigs) were grouped into collections or bins that resemble the genome in the sample. The output from the binning step was evaluated to select a non-redundant set of high-quality
bins, which is called metagenome-assembled genome (MAGs). Taxonomic assignments were performed of assembled contigs (UniRef100 database) or MAGs using the Genome Taxonomy Database (GTDB). An overview of the 16S rRNA sequencing and shotgun metagenomics analysis workflow used in paper III and IV, respectively is illustrated in Figure 4.

**Figure 4.** Overview of 16S rRNA and shotgun sequencing pipelines applied in paper III and IV.
4. Results and discussion

4.1. Effects of DBP on the testosterone biosynthesis pathway and cell markers

4.1.1. DBP mouse model (Paper I)
The effects of 5-weeks adult oral DBP exposure (10 mg/kg/day and 100 mg/kg/day) on testosterone levels and specific testicular cell markers were investigated in adult mice, one week after the last DBP dose was administered. The DBP-exposed mice showed a significant decrease in the testosterone levels in the testis at both tested dosages. No significant changes were observed in the levels of the steroid hormones progesterone or corticosterone in the adrenal gland. The DBP-induced decrease in testosterone levels is consistent with other studies, although the previously reported effects were observed at higher DBP concentrations (≥ 50 mg/kg/day) [69, 128-130]. The mechanistic investigation revealed a significant increase in the levels of multiple steroidogenic enzymes, including CYP11A1, HSD3B2 and CYP17A1 (Figure 5). This upregulation of the enzymes levels could indicate an attempt to compensate for the decrease in testosterone. Testosterone is the primary androgen produced by Leydig cells in the interstitial compartment of the testis. The reproductive capacity and testosterone synthesis are regulated by the complex interaction between hypothalamus-pituitary-testis (HPT) axis [131]. The gonadotropin LH, acts on Leydig cells to stimulate testosterone secretion, while the Follicle-stimulating hormone (FSH) acts on Sertoli cells to provide structural and nutritional support for the developing germ cells [131, 132]. The serum levels of LH and FSH were not studied in the current work. However, the DBP exposure increased the LHR levels, which is expressed in Leydig cells. This increase is likely attributed to positive feedback in the HPT axis due to the reduced testosterone levels [133].

DBP exposure also induced oxidative stress in the testis, as demonstrated by the increase in nitrotyrosine levels, a marker for protein damage [134]. Increased oxidative stress can reduce testosterone production by either directly damaging Leydig cells or interfering with other endocrine structures such as the anterior pituitary [135]. One previous in vivo study reported that oral ex-
posure to DBP (≥ 200 mg/kg/day) reduced the availability of antioxidant enzymes in adult mice [69]. Altogether, the DBP-induced decline in testosterone levels is likely triggered by multilevel crosstalk between the Leydig cell steroidogenic apparatus and oxidative stress, along with potential LH production [136]. Furthermore, the adult DBP exposure resulted in increased levels of Sertoli cell markers, vimentin and SOX9, germ cell marker DAZL, and Leydig cell marker SULT1E1 (Figure 6). In adult humans and rodents, mature Sertoli cells are static nonproliferating or slowly dividing [137]. The increased levels of Sertoli cell markers in the 10 mg/kg/day DBP-treated mice indicates that Sertoli cell is yet another important target for DBP testicular toxicity. Sertoli cells play a vital role in spermatogenesis and their number in the seminiferous tubules is linked to the rate of spermatozoa production. During fetal development, gestational exposure to 500 mg/kg/day DBP has been reported to disrupt vimentin cytoskeleton organization and led to abnormal contact between Sertoli cells and gonocytes [65]. The observed increase of DAZL, a master post-transcription regulator of specific mRNAs essential for germ cell survival and differentiation, is likely to affect germ cell maturation and meiosis [138, 139]. In contrast to our findings, two weeks of exposure to the DBP metabolite, MBP, at a concentration of 200 mg/kg downregulated Dazl and Sox9 mRNA expression, as well as induced oxidative damage in adult mice [140]. This discrepancy between DBP and MBP may be attributed to the fact that we measured the protein levels one week after the exposure ended to reflect more persistent effects. In addition, it is possible that the differences in the doses used and the duration of exposure may have contributed to the varying observed effects.
Figure 5. Summary of the effects on the steroidogenic pathway in Leydig cells demonstrated after five weeks of oral DBP exposure in adult male mice (Paper I). The enzymes responsible for each step are displayed in green. Arrows in red color indicate significantly decreased or increased levels. Figure created with BioRender.com
Figure 6. Immunofluorescence analysis of testicular cell markers DAZL (green; germ cells), vimentin (purple; Sertoli cells), and SULT1E1 (red; Leydig cells) in DBP-exposed mice and the control. (a) Representative immunofluorescence images of testis cross-sections. (b-d) Quantification of fluorescence intensity. Data are represented as mean ± SD. Statistically significant differences compared to control are indicated as follows: * p < 0.05 and ** p < 0.01 (Kruskal–Wallis test followed by Dunn’s test). Scale bar: 50 µm [141].
4.1.2. 3D testicular co-culture for screening of DBP reprotoxicity (Paper II)

A 3D heterogenous testicular co-culture derived from neonatal mouse tissue was developed to study the effects of DBP (1nM - 100µM) on the main testicular cell types. DBP concentrations as low as 1 nM affected Leydig cell steroidogenic function. Consistent with our in vivo model based on adult oral exposure to DBP (Paper I), the in vitro production of testosterone in the co-culture was decreased in a concentration-dependent manner following seven days of exposure to 10 µM, 50 µM and 100 µM DBP (Figure 7). This consistency, confirms that DBP has an endocrine-disrupting activity and acts as anti-androgen. However, it is important to note that the regulation of testosterone production varies between neonates and adults due to developmental differences in hormonal regulation [142, 143]. Mechanistically, the in vitro DBP exposure induced a significant decrease of upstream targets in the testosterone biosynthesis pathway, particularly the steroidogenic regulator StAR and the steroidogenic enzyme CYP11A1, across all tested DBP concentrations (1 nM to 100 µM). StAR is essential for transporting cholesterol to the inner mitochondrial membrane in Leydig cells, a rate-limiting step in steroidogenesis [144]. The DBP-induced decrease in the number of Leydig cells (CYP11A1-positive cells) may also contribute to the decrease in testosterone levels.

![Figure 7](image-url)

*Figure 7. Decreased testosterone production after DBP exposure in the 3D testicular co-culture (Paper III). The testosterone concentrations in culture media was measured using ELISA. Values indicate mean ± SEM from three experiments. Statistically significant differences compared to control are marked as * p < 0.05 (one-way ANOVA followed by Dunnett’s multiple comparison test).*
Importantly, by using the established 3D testicular co-culture, it was also possible to study the effects of DBP exposure on the development of Sertoli cell tight junction proteins, which constitute the basis of the blood-testis barrier [145]. Fewer studies have explored the impact of chemicals, including DBP, on Sertoli cell features, such as Sertoli cell number, cytoskeleton structure and tight junctions, compared to Leydig cell steroidogenesis. Thus, it is important to develop suitable methods for studying such features. In our model, DBP exposure did not alter the number of Sertoli cells or the levels of N-cadherin and Zonula occludens protein 1 (ZO-1), which are among the primary components of Sertoli-Sertoli junctions. In contrast to the DBP-induced increase of DAZL levels in testis demonstrated in our animal model (Paper 1), exposure to 10 nM DBP decreased the DAZL levels in germ cells. Several studies have revealed that disruption in Dazl expression can negatively affect the differentiation of germ cells. Dazl knockout mice have been shown to be infertile and exhibit germ cell apoptosis [138, 139]. The discrepancy in DAZL levels between our in vitro and in vivo models may be attributed to differences in testicular germ cell regulation at different developmental stages, as the 3D system model newborn mice compared to fully mature adult mice. Furthermore, in the in vivo study, the protein levels were measured one week after the exposure concluded, which may also explain the different effects.

Although the cells in this 3D co-culture grow without forming structures identical to the seminiferous tubule architecture, the cell-to-cell interactions between the heterogenous testicular cell types are beneficial for promoting cell proliferation and differentiation in vitro. The model can also recapitulate aspects of testicular physiology, such as cell signalling pathways and testosterone production. This is not possible with the widely used mouse tumor Leydig cell line MA-10, which mainly produces progesterone instead of testosterone [125]. The use of high-throughput analysis in a 96-well plate format have further optimized this 3D co-culture model for mechanistic screening of reproductive toxicants. The testicular cells were obtained from seven-day-old neonate mice, which are expected to be in critical developmental and differentiation phases. In mice, Leydig cells typically begin to differentiate approximately PND 10-11 from mesenchymal cells into Leydig cell progenitors [146]. Meanwhile, the male gonad contains no meiotic germ cells, only a mixture of undifferentiated and differentiating spermatogonia cells are present [147]. Sertoli cells remain proliferative to increase their number until around PND 10-15, when they stop dividing and become terminally differentiated. This is followed by functional formation of the blood-testis barrier around PND 15-16 [145, 148]. Using the current 3D testicular co-culture model, it is possible to characterize the effects of DBP exposure on testicular cell differentiation and maturation, and the potential functional implications.
4.2. Effects of DBP on the gut microbiota (Paper III & IV)

The effects of five weeks oral exposure to DBP on the gut microbiota of adult male mice were examined in paper III and IV. The fecal microbiota was characterized using two different DNA sequencing approaches, 16S sRNA (Paper III) and shotgun metagenomics (Paper IV). The 16S analysis demonstrated that DBP-exposed mice had a distinct microbial community compared to the control group, indicated by beta diversity (Figure 8). DBP exposure led to a trend of less diverse gut microbiota. Differential analysis of the ASVs revealed large number of significant differentially abundant ASVs at the genus level. Compared to the controls, the group that received 10 mg/kg/day DBP had 63 more abundant and 65 less abundant ASVs, whereas the group that received 100 mg/kg/day DBP had 60 ASVs with increased abundance and 76 ASVs with decreased abundance.

The DBP-treated groups both showed a higher abundance of ASVs assigned to Desulfovibrio, Enterorhabdus and Lachnospiraceae UCG-001 genera. Two genera were linked to inflammation in the intestine, including Desulfovibrio and Enterorhabdus. Desulfovibrio is a diverse group of sulfate-reducing bacteria that produces hydrogen sulphide. Overgrowth of Desulfovibrio has been reported in biopsies of patients with active ulcerative colitis [149]. In particular, higher levels of D. desulfuricans and D. vulgaris were consistently associated with ulcerative colitis [149, 150]. Increased hydrogen sulfide levels can inhibit butyrate metabolism, which is an important source of energy for colonocytes [150]. Furthermore, hydrogen sulfides can disrupt the mucus barrier, which leads to closer contact between bacteria and the epithelium barrier, thereby contributing to colonic inflammation [150]. The Enterorhabdus genus is a relatively novel genus that includes pathogenic bacteria such as E. mucosicola Mt1B87. This bacterium was first isolated from inflamed ileal mucosa in the TNFdeltaARE mouse model of intestinal inflammation [151]. A second species, E. caecimuris, was isolated from a mouse model of spontaneous colitis. The DBP-treated groups also had decreased abundance of ASVs belonging to Parabacteroides, Lachnospiraceae UCG-006, Ruminiclostridium 6 and Lachnoclostridium compared to the controls.
Interestingly, our group has previously shown that DBP induces immune system effects in the same in vivo DBP mouse model. DBP exposure resulted in an immunosuppressed phenotype with decreased total number of leukocytes, classical monocyte, and T helper (Th) cell count, and increased non-classical monocytes in blood [51]. Immunofluorescence analysis in the spleen showed reduced levels of CD3+ (a marker of total T cells) and CD4+ (a marker of Th cells), while cell-markers of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), and non-classical monocytes increased. The plasma levels of interleukin (IL)-21 and monocyte chemoattractant protein 1 (MCP-1) were also decreased [51]. To investigate potential crosstalk between the DBP-induced immune system alterations and the differentially abundant ASVs we conducted correlation analysis. The results revealed that an ASV from Lachnospiraceae UCG-001 was positively correlated with increased circulating non-classic monocytes in the DBP-exposed mice. In addition, an ASV belonging to Ruminiclostridium 6 was negatively correlated with increased circulating non-classic monocytes. The non-classical monocytes are mostly viewed as an anti-inflammatory subset of monocytes that play a role in maintaining immune homeostasis. However, several studies have shown that this monocyte class can also have a pro-inflammatory effect and may contribute to the development of certain diseases [153]. This indicates that oral
exposure to DBP resulted in gut dysbiosis, which may be linked to the DBP-induced immune toxicity. It remains to be elucidated whether the observed gut dysbiosis is a cause or a secondary consequence of the immune dysfunction in DBP-exposed mice. A bidirectional influence cannot be excluded.

It is possible that changes in gut microbiota also could affect the testicular function and androgen levels [116, 117]. This potential interplay has been scarcely studied. We therefore investigated if there was a relationship between the gut dysbiosis and testicular toxicity caused by DBP. However, in the present in vivo DBP mouse model, there were no correlations between microbiota changes and DBP-induced decreased testosterone levels and altered testicular cell-markers tested in paper1. Limitation of this study include the incomplete taxonomical resolution of the 16S sequencing technique, which hampers the identification of species-level information, and the lack of functional insights. Therefore, and due to heterogeneity of the ASVs genera and the sometimes conflicting literature concerning the effects of some taxa on the host health and disease, a more in-depth analysis was conducted using shotgun sequencing. The aim was to characterize the changes at the bacterial species and identify the functional capacity of the microbiome following DBP exposure.

The shotgun sequencing data (Paper IV) aligned with the 16S sequencing findings and revealed that oral exposure to DBP (10 mg/kg/day and 100 mg/kg/day) for five weeks resulted in a similar trend of less diverse microbial community compared to the control. Taxonomic composition analysis recovered species-level resolution and showed several differential MAGs and species in the DBP treatment groups compared to control. The DBP-exposed groups had higher abundance of multiple species, including Lachnospiraceae bacterium COE1, Bacteroides caecimuris, Duncaniella muris and Adlercreutzia mucosicola. In contrast, the beneficial mucin-degrading bacterium, Akkermansia muciniphila, as well as Alloprevotella tannerae and Bacteroides uniformis were less abundant in the DBP exposed mice. Consistent with the 16S sequencing study, there was an increased abundance of Enterohabdus ASV (Adlercreutzia genus previously known as Enterohabdus), and the shotgun microbiota analysis resolved the species to the pathogenic Adlercreutzia mucosicola. This species was initially isolated from inflamed ileal mucosa in a mouse model of intestinal inflammation [151]. This suggests that DBP exposure may lead to overgrowth of A. mucosicola, which could likely trigger inflammation in the intestine. The UniRef assignments at the assembly level also captured other microbiome components, such as viruses, archaea and eukaryotes in addition to bacteria. However, bacteria constituted the vast majority in all samples.

The function composition analysis revealed that DBP treatment resulted in increased abundance of genes involved in important environmental signaling
and cellular processes, including in quorum sensing and environmental adaptation and CRISPR-associated protein Csx3, which is involved in type III CRISPR-Cas prokaryotic defense mechanism. Interestingly, quorum sensing has been shown to regulate gene expression in response to cell population density and is usually involved in pathogenicity [154]. It is possible that DBP altered the structure of the gut bacteria, leading to transcriptional changes that increase the expression of virulence factors. However, the functional implications of these genes need further investigation. In addition, DBP exposure resulted in a higher abundance of antimicrobial resistance genes (Figure 9). This included genes involved in aminoglycosides antibiotics class resistance.

Figure 9. KEGG Ortholog abundance analysis of the DBP-treated mice and controls. (a) Biplot for the two components of the mixOmics model. (b and d) Loadings plot from mixOmics model. (c and e) Heatmaps of the DESeq2 analysis Log2 fold change. The color of the dots refers to the assigned functional category, while shape of the dots refers to the treatment group (C: control, L: 10 mg/kg/day, and H: 100 mg/kg/day) with the highest mean value of the KEGG Ortholog.
5. Concluding remarks and future perspectives

Exposure to DBP has been linked with adverse reproductive outcomes in males. This thesis demonstrated that adult oral exposure to 10 and 100 mg/kg/day DBP for five weeks resulted in testicular toxicity in mice, which persisted for at least one week after the exposure ended (Paper I). The observed testicular toxicity of DBP is likely due to multi-level crosstalk between testicular steroidogenesis and oxidative stress, with a potential connection to the HPT axis. Because the testosterone levels were significantly reduced even at the lowest DBP dose used (10 mg/kg/day), the no observed adverse effects level (NOAEL) could not be determined. Additional long-term studies using lower doses, such as 1, 0.5 and 0.1 mg/kg/day are needed to determine the NOAEL after adult exposure. When comparing our DBP dose in the animal model of adult exposure to the estimated DBP levels from biomonitoring studies, it is important to consider the differences in pharmacokinetics between humans and mouse and apply uncertainty factors to infer the corresponding dose in humans [155]. The lowest dose used in the mouse model is likely a few times more than the highest exposure estimated in some biomonitoring studies. Also, the fact that DBP has a short half-time but still can be detected reflects the widespread distribution of DBP and that people are continuously exposed to this contaminant. Further mechanistic studies are warranted to investigate whether the persistent DBP testicular effects could be mediated via epigenetic modifications. Future studies should also include investigation of mixture effects, as evidence from epidemiological studies has shown associations between exposure levels of multiple phthalates and negative reproductive outcomes. This could reveal if exposure to a phthalate mixture has a cumulative adverse reproductive impact.

The results of this thesis have demonstrated that the developed *in vitro* 3D cell model (Paper II) can recapitulate testicular physiology and produce testosterone. Concomitant with the significant need for efficient alternative testing methods for screening reprotoxic effects, particularly during early postnatal development, it was shown that the 3D testicular co-culture could be used to model known *in vivo* testicular toxicity pathways of DBP in high throughput format. Consistent with the *in vivo* findings (Paper I), *in vitro* exposure to DBP decreased the levels of testosterone in the culture media. This confirmed
that DBP acts as anti-androgen and has an endocrine-disrupting activity following exposure to broad range of concentrations (1 nM – 100 µM). DBP concentrations as low as 1 nM affected Leydig cell steroidogenic function, demonstrated by decreased levels of steroidogenic regulator StAR and the steroidogenic enzyme CYP11A1. In addition, the relatively small number of animals required to generate the primary co-culture helps in minimizing the use of laboratory animals, thereby align with the principles of the 3Rs (replacement, reduction, and refinement) for more humane use of laboratory animals. The current 3D testicular co-culture could be used to identify potential reptotoxic chemicals that interfere with testosterone production. Further studies are required to understand the molecular mechanism behind the DBP-induced downregulation of StAR and CYP11A1. This includes, for example, the potential role of the nuclear receptor COUP-TFII/NR2F2 in modulating the expression of important targets in testosterone synthesis pathway during development. The established in vitro model could also be used to characterize the effects of DBP and its metabolite on testicular cell differentiation and maturation (in combination with factors needed for proliferation, like LH) and the potential functional implications. This includes the possibility to study the gene expression regulation using the advances in single-cell transcriptomics and epigenomics technologies.

Two approaches were used to characterize the gut microbiota composition of 10 and 100 mg/kg/day DBP-exposed mice (Paper III and IV). Both 16S rRNA sequencing and shotgun metagenomics showed that adult oral DBP exposure for five weeks resulted in a trend of a less diverse microbiome. The DBP exposure significantly altered the abundance of multiple ASVs (Paper III), as well as species and MAGs at the metagenomic level (Paper IV). This indicates that the DBP exposure altered the microbiota taxonomic composition at low taxonomic ranks, mainly species and subspecies. Interestingly, the microbial profile of DBP-exposed mice points toward an increased abundance of taxa associated with inflammation in the intestine. It was also found that specific microbial changes correlated with the immunosuppressive phenotype observed in the DBP-exposed mice (Paper III). This indicates a plausible relationship between gut dysbiosis and immune system toxicity in the DBP mouse model. Differential analysis of metabolic KEGG functional categories revealed that genes involved in quorum sensing, which is linked with virulence, as well as genes for prokaryotic defense mechanisms were more abundant in mice exposed to DBP. Genes known to be important for antimicrobial resistance were also largely abundant in the DBP-exposed mice. This indicates a higher resistance to antibiotic drugs due to DBP exposure. Taken together, the results in this thesis indicate that oral DBP exposure had a negative impact on the composition and functional profile of the gut microbiota. These effects may play a potential role in initiating or exacerbating DBP-induced toxicity. Future studies need to investigate the potential effects of DBP exposure on the
human gut microbiome. It is also important to consider integrating omics data, such as metagenomics and metabolomics, to provide better insights into the contributing role of gut dysbiosis in host metabolome and phenotypes. Moreover, studies in germ-free mice are warranted to better understand the causal relationship between gut microbiota and DBP-induced toxicity. For example, this will demonstrate whether DBP immune effects are mediated through gut microbiota or not. Overall, this thesis highlights the importance to further consider the potential health impact of the interplay between the two exposome components, chemical exposure and gut microbiota.
Populärvetenskaplig sammanfattning

De möjliga hälsoeffekterna av exponering för tillverkade kemikalier har väckt stor oro över hela världen. Ftalater är en grupp av kemikalier som främst används i plastindustrin och har förknippats med flera skadliga effekter på människor. Di-n-butylftalat (DBP) är en de dominerande ftalaterna som föröver vår miljö och kan påverka männens testosteronnivå och fertilitet. Många studier har undersökt de hormonstörande effekterna efter exponering för DBP under tidig utveckling. Tidigare studier av dess effekter på det vuxna reproduktionsystemet och tarmmikrobiotan är dock begränsade.

Resultaten från den här avhandlingen visade ihållande effekter av DBP på det vuxna manliga reproduktionssystemet. Hanmöss som exponerats oralt för DBP under fem veckor (10 eller 100 mg/kg/dag) hade lägre mängder av testosteron i testiklarna jämfört med kontrollgruppen. Nivåerna av proteiner som spelar en avgörande roll för testikelfunktioner som testosteronproduktion och spermieproduktion påverkades också. Även en ökad oxidativ stress observerades i testiklarna. Ett in vitro screeningverktyg som rekapitulerar in vivo testikelmikromiljön utvecklades för att snabbare kunna identifiera reproduktionstoxiska ämnen samt studera effekterna av DBP i en cellmodell med odlade testikelceller. Med hjälp av denna tredimensionella cellmodell härledd från musvävnad bekräftades det att DBP-exponering minskar testosteronproduktion och påverkar nivåerna av proteiner viktiga för flera testikelfunktioner.


Sammanlagt visar denna avhandling de antiandrogena effekterna av DBP såväl som möjliga underliggande mekanismer för testikeldysfunktion. Tarmmikrobiotan påverkades också av DBP-exponering, vilket kan spela en potentiell roll för att initiera eller förvärra den DBP-inducerade toxiciteten. Detta belyser vikten av att studera den potentiella hälsoeffekten av sampelet mellan de två exposomkomponenterna kemikalieexponering och tarmmikrobiota.
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Appendix - Contribution

The contributions of the author to the papers included in this thesis are highlighted below.

**Paper I.** Contributed to planning and performing experimental work with co-authors. The primary responsibilities were cryosectioning and immunofluorescence data acquisition and analysis. Contributed to writing, reviewing and editing the manuscript (co-first author).

**Paper II.** Participated in formulating the research hypothesis, designing and planning experiments. Conducted a major part of the experimental work and data analysis under guidance from supervisors. The primary author responsible for writing reviewing, and editing the manuscript (first author).

**Paper III.** Participated in formulating the research hypothesis, designing and planning experiments. Performed DNA extraction and PCR validation. Contributed to the planning of data analysis. The primary author responsible for writing, reviewing, and editing the manuscript (first author).

**Paper IV.** Participated in formulating the research hypothesis, designing and planning experiments. Performed DNA extraction. Contributed to the planning of data analysis. The primary author responsible for writing, reviewing, and editing the manuscript (first author).
References

10. (ECHA), E.C.A., List of substances included in Annex XIV of REACH ("Authorisation List").


