**INSTITUTO POLITÉCNICO DE LISBOA**

**ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA**

# Implementação de um teste de microbioma intestinal em cancro

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Orientador: Maria José Rego de Sousa, Centro de Medicina Laboratorial Germano de Sousa
Co-Orientador: Joana Rita Mendes Cardoso Vaz, Centro de Medicina Laboratorial Germano de Sousa e Ophiomics-Precision Medicine

Mestrado em Tecnologias Moleculares em Saúde

(esta versão não incluiu as críticas e sugestões feitas pelo júri)

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# Dedicatória

Aos meus pais, por me apoiarem incondicionalmente.

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# Resumo

O microbioma humano representa um conjunto de microrganismos que habitam o corpo, incluindo bactérias, fungos, arqueias e vírus. Esses microrganismos vivem em equilíbrio com o hospedeiro e são essenciais para um grande número de funções, incluindo absorção de nutrientes, regulação do sistema imunitário, síntese de vitaminas, entre outras. A rutura dessa homeostase conduz a disbiose, afetando a diversidade e a riqueza de microrganismos, e está relacionado a várias patologias, incluindo o cancro. Evidências acumuladas apontam que o microbioma pode afetar o risco de desenvolvimento de cancro, a resposta à terapêutica e o desenvolvimento de complicações associadas à terapia. O estudo do microbioma é de particular interesse para cancros com altas taxas de mortalidade e diagnóstico difícil. Este é o caso do cancro de ovário, que é um dos cancros ginecológicos mais mortais a nível mundial. Considerando a potencial contribuição do microbioma para o desenvolvimento e progressão do cancro, o estudo dos microrganismos associados ao cancro do ovário pode proporcionar uma mudança de paradigma no diagnóstico do cancro do ovário e na terapêutica precoce. O presente trabalho tem como objetivo avaliar se a microbiota intestinal e vaginal desempenham um papel no desenvolvimento e na terapêutica do cancro do ovário, identificando com base na pesquisa bibliográfica as implicações de microrganismos específicos e desenvolver um protótipo de um teste baseado no microbioma direcionado ao cancro do ovário.

Palavras-chave: microbioma; cancro do ovário; terapêutica do cancro; microbiota intestinal, microbiota vaginal

# Abstract

The human microbiome represents a consortium of microorganisms inhabiting the body, including bacteria, fungi, archaea and viruses. These microorganisms live in balance with the host and are essential for a wide number of functions, including nutrient absorption, regulation of the immune system, vitamin synthesis, among others. The disruption of this homeostasis leads to dysbiosis, affecting the diversity and richness of microorganisms, which is linked to several pathologies, including cancer. Evidence is accumulating that the microbiome can affect the risk of cancer development, response to anticancer therapy and development of therapy associated complications. Studying the microbiome is of particular interest for cancers with high mortality rates and difficult diagnosis. This is the case of ovarian cancer, which is one of the deadliest gynecological cancers in the world. Considering the potential contribution of the microbiome to cancer development and progression, studying the microorganisms associated with ovarian cancer could provide a turning point in ovarian cancer diagnosis and early therapy. The present work aims to perform a preliminary assessment whether the gut and the vaginal microbiota play a role in ovarian cancer development and therapy. Based on a literature search, we aim to identify the implications of specific microorganisms and to develop the prototype of a test based on the microbiome, directed to ovarian cancer.

Keywords: microbiome; ovarian cancer; anticancer therapy; gut microbiota; vaginal microbiota

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# List of abbreviations

CRC – Colorectal cancer

CTLA-4 – Cytotoxic T lymphocyte-associated protein 4

CTX – Cyclophosphamide

FMT – Fecal microbiota transplant

GALT - Gut-associated lymphoid tissues

HGSOC – High grade serous ovarian cancer

HMP – Human Microbiome Project

HIV - Human immunodeficiency virus

IARC – International Agency for Research on Cancer

IBD – Inflammatory bowel disease

ICI – Immune checkpoint inhibitor

IgA – Immunoglobulin A

LGG - *Lactobacillus rhamnosus* GG

MALT - Mucosal associated lymphoid tissues

NCBI - National Center for Biotechnology Information

NGS – Next generation sequencing

OTT - Open Tree of life Taxonomy

Pap smear – Papanicolaou smear

PARP – Poly (ADP-ribose) polymerase

PD-1 – Programmed cell death 1

PID – Pelvic inflammatory disease

RDP – Ribosomal Database Project

SCFA – Short chain fatty acids

TLR4 – Toll-like receptor 4

Treg – Regulatory T cells

# 1. Introduction

The human microbiome represents a consortium of microorganisms inhabiting the body in homeostasis, including mainly bacteria, but also fungi, archaea and viruses. The two predominant bacterial phyla are *Firmicutes* and *Bacteroidetes,* representing approximately 90% of the gut microbiota (1). These microorganisms live in balance with the host and are essential for a wide number of functions, including nutrient absorption, regulation of the immune system, vitamin synthesis, among others (2–4). The disruption of this homeostasis leads to dysbiosis, affecting the diversity and richness of microorganisms, which is linked to several pathologies, including cancer.

Evidence is accumulating that the microbiome can affect the risk of cancer development, response to anticancer therapy and development of therapy associated complications, as cancer patients often report side effects that can be debilitating and even life-threatening (5). Studying the microbiome is of particular interest for cancers with high mortality rates and difficult diagnosis. This is the case of ovarian cancer, which is one of the deadliest gynecological cancers in the world, without specific associated symptoms nor early-detection specific biomarkers (6,7). Thus, most women are diagnosed with ovarian cancer at advanced stages, decreasing the success of the anti-cancer therapy and survival. Recent studies demonstrated that not only the gut microbiota is related to cancer development or associated risk factors, but also the vaginal microbiota, particularly to ovarian cancer (8–10). The vaginal microbiota, unlike the gut microbiota, is beneficial when in low diversity, particularly when dominated by *Lactobacillus spp*., preventing bacterial infections and potentially reducing the risk of ovarian cancer development (11).

Altogether, the potential contribution of the microbiome to cancer development and progression, as well as its role in anticancer therapy, could result in a paradigm shift in ovarian cancer diagnosis and early therapy, exploiting the role of specific microorganisms participating in these processes. Thus the goals of this work contemplate a collaboration in laboratorial intestinal microbiota test optimization, literature search into investigating the intestinal microbiota features in cancer development and therapy, and proposal of alterations in the current microbiome report in cancer risk prevention context, improvement to anticancer therapy response and minimization of side effects from anticancer therapy.

# 2. Theoretical contextualization

This chapter gathers information from literature search in the microbiome and cancer context, featuring the gut and vaginal microbiota role in cancer development and anticancer therapy, particularly the ovarian cancer, defining the state of art and contributing to the development of prototypes tests for microbiome and cancer.

## 2.1 The microbiome

Technologic advances in culture-independent molecular methods allowed investigators to profoundly understand the diversity and functions of the human gut microbiota, especially since the Human Microbiome Project (HMP) in 2012, which helped characterize the microbiome of several body sites within healthy adults in the United States (12,13). The gut microbiota generally refers to the heterogeneous population of commensal microbial, such as bacteria, fungi, archaea, viruses and protozoa that colonize the gastrointestinal tract (2), while the gut microbiome is defined as the whole genome of the host's gut microbiota. The gastrointestinal tract is the most impactful site of host-microbiome interaction (3), particularly along the intestine, where the gut microbiota encodes crucial functions for the host, not only maintaining the host health and microbial balance, but also sustaining the immune response, metabolization of dietary compounds, vitamin and hormone production and is as well implicated in cancer prevention and modulation of the efficacy and toxicity of cancer therapy, including chemotherapy and immunotherapy (2–4,14). A healthy and balanced microbiome contributes to disease prevention but in the case of dysbiosis serious diseases can be developed.

### 2.1.1 Healthy gut microbiota

Environmental conditions along the human gastrointestinal tract are not identical bur diverge considerably between the stomach and colon. Overall, the small intestine is characterized by a less diverse and temporary variations in the microbial community than the large intestine (15,16). The large intestine is the most microbial populated site of the body, essentially due to the absorption of water and ions in the colon, giving the microorganisms more time to proliferate. The two most abundant phyla in the large intestine are *Firmicutes* and *Bacteroidetes,* followed by *Actinobacteria*, *Proteobacteria* and *Fusobacteria*, differing in their relative contribution to bacterial cells in the microbiota

(3,7,16). It has been suggested that a specific group of microorganisms are present in every human being, denominated as a “core microbiome”, involving the most abundant phylotypes that maintain the functional stability and homeostasis towards optimal health, specifically at the gene level, coding for specific metabolic functions and pathways (16).

Overall, the gut microbiota is similar at the phylum level (dominated by *Firmicutes* and *Bacteroidetes* - 90%), but diverse in species and variable between individuals (1). Several factors can influence this diversity in the composition of the gut microbiota, such as genetics, age, diet, lifestyle, gender, body mass index, drugs and circadian rhythms (17). The microbial diversity is acquired by vertical transmission after birth and changes accordingly to the individual’s exposure (18). It is very likely that the microbial exposure begins in utero (placenta, amniotic liquid and umbilical cord blood). The delivery mode affects the microbiota composition, as vaginal delivered babies are exposed to *Lactobacillus* and *Prevotella* spp. and when born via C-section are exposed to skin microbes (such as *Staphylococcus* spp.). The gut microbiota of vaginally delivered babies is actually remarkably similar to the mother’s microbiota, when compared to C-section delivered babies, which present a reduced resemblance (14,19). Although at the age of 2-3 the gut microbiota resembles that of an adult, during infancy and through adolescence, the composition of the gut microbiota experiences a few changes, attributable to hormones, diet, physical activity and antibiotics use. This process of microbiome maturation takes place in parallel with development of host organs, including the intestine, which elongates with age, providing additional niches for the microbiome to expand in number and diversity (20). Elders gut microbiota demonstrates a reduction in commensal bacteria, such as *Bifidobacteria* and *Bacterioides*, which is associated with reduced life quality when increased *Enterobacteriaceae*. (13).

### 2.1.2 Dysbiosis of the gut microbiota

The term dysbiosis is used to describe the disruption of the gut microbiota homeostasis, leading to deviations from normal or healthy gut microbiota and to an imbalanced microbial community (21,22). Changes in the gut microbiota leading to dysbiosis might be caused by many factors, such as antibiotic treatment, physical or psychological stresses, radiation and dietary shifts (16).

The gut dysbiosis increases the risk to develop a wide range of diseases, including metabolic and immune disorders and several forms of cancer (23). Dysbiosis is characterized by low microbial diversity supporting the outgrowth of opportunistic pathogens and depletion of commensals (5), leading to changes in the metabolic and immunologic activity (16). This changes may be responsible for the development of

intestinal disorders such as inflammatory bowel disease (IBD), which is characterized by a decrease of *Clostridia* abundance and overall bacterial diversity reduction; asthma; obesity; type 1 diabetes; cardiovascular disease; metabolic syndrome and neurological disorders (21,22,24,25). Considering the most serious consequences of dysbiosis, cancer is one distressing outcome. It is estimated that 20% of cancers worldwide are induced by a microbial agent (23), possibly due to immune dysregulation leading to hyper inflammation, dysplasia, proliferation, prevention of apoptosis and ultimately cancer development (7). The presence of oncogenic viruses, bacteria and parasites in the dysbiotic microbiota could directly cause cellular transformation by encoding certain oncoproteins or effector molecules leading to genomic instability and dysregulated cell growth (7,26). Continuous study in this field would help us understand the etiologies of diseases, specially cancer, and possibly use the microbial associations to help diagnose and treat this disease.

### 2.1.3 Microbiome and cancer

It has been recognized that the microbiome and its variations is closely related to the development of a variety of diseases, including several types of cancers. In the presence of a dysbiotic microbiota, some microorganisms can be cancer promoters, providing toxic metabolites or carcinogenic products and also inducing inflammation or immunosuppression (18). A wide variety of microorganisms have and still are continuously studied in order to understand the specific mechanisms by which they may induce cancer development as well as cancer prevention, given that in some cases the presence of specific microorganisms in the microbiome can have a protective effect in disease development.

The International Agency for Research on Cancer (IARC) has classified so far 11 microorganisms as carcinogens for their strong association with cancer development, including 7 viruses, 3 parasites and 1 bacterium (last update march 2020) (27). Among a few well known examples, *Helicobacter pylori* is a carcinogenic gram negative bacteria with a strong association with gastric adenocarcinoma development, infecting the stomachs of half the world's population (7,28). The stomach pH is typically 1-2, disfavoring the growth of ingested microbes and thus a reduced abundance of microorganisms, containing a community dominated by the *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (15,16). Previous to cancer development, the presence of the bacteria induces changes in the stomach microbiota, leading to dysbiosis, inflammation and epithelial injury that ultimately contributes to carcinogenesis (20). *Helicobacter pylori* has a genotoxic effect that directly promote changes in gastric

mucosa's crucial intracellular signaling pathways, disrupting autophagy and apoptosis pathways as well as oncogenic signaling pathways, that regulate the proliferation and growth of the mucosal cells (5,29).

Another example of a microorganism associated to cancer is *Fusobacterium nucleatum*, a gram negative bacteria commensal of the oral cavity microbiota (30). Several studies describe the abnormal presence of this bacteria in colorectal cancer (CRC) and colon adenomas, often over-represented and increased in CRC patients, promoting intestinal inflammation that originate alterations in signaling pathways and promoting carcinogenesis (14,20,29). *Fusobacterium nucleatum* encodes several adhesion factors, including the most studied FadA, that is capable to bind and invade the colonic epithelial cells, promoting bacterial attachment with signaling pathways activation resulting in oncogenic transcriptional changes (5,7,29).

Evidence is accumulating that the microbiome can affect the risk of cancer development, response to anticancer therapy and development of therapy associated complications. Studying the microbiome is of particular interest for cancers with high mortality rates and difficult diagnosis, which is case of ovarian cancer, which is one of the deadliest gynecological cancers in the world. It is becoming more and more clear that significant perturbations in the microbiome may be directly associated with specific cancers, creating unique microbiome signatures that in the future may be used for early diagnosis and to increase the effectiveness of cancer therapy.

## 2.2 The ovarian cancer

The ovarian cancer is one of the most common and deadly gynecologic cancers, with over 238,000 new cases diagnosed each year (31,32). The European continent has the major incidence and in Portugal each year about 474 new cases are discovered, representing 18% of all gynecologic tumors (31). Overall survival of ovarian cancer depends on tumor staging, killing per year 152,000 women (32).

The ovarian cancer is acknowledged as "silent killer" because is usually detected at advanced stages due to the lack of specific symptoms at initial stages, resulting in a high mortality rate (6). The absence of specific and sensitive biomarkers for ovarian cancer is also an obstacle in screening, prevention and early detection. CA125 is a biomarker used by clinicians to monitor women presenting with high risk to ovarian cancer, but cannot be used as biomarker to early detection due to lack of sensibility and specificity (33). CA125 is expressed in the majority of High grade serous ovarian

carcinomas but only in 60% of the mucinous and clear cell subtypes, making it a weak biomarker that cannot be used as an universal biomarker for ovarian cancer (7).

The etiology of ovarian cancer is still not completely understood, although some theories have been postulated. Considering the incidence and mortality of ovarian cancer, establishing risk and protective factors is essential to improve overall survival and early diagnosis.

### 2.2.1 Risk and protective factors

Several theories have been postulated to explain the etiology of ovarian cancer.

The “incessant ovulatory theory” suggests that ovarian cancer risk increases trough repetitive ovulation, damaging the ovarian and fallopian epithelium, increasing aberrant DNA repair, inactivation of tumor suppressor genes and mutagenesis that might promote carcinogenesis (7,34). Thus, multiparity, breastfeeding and oral contraceptive use may reduce the ovarian cancer risk by suppressing ovulation and have a protective effect, while late menopause may increase the risk due to prolonged ovulation cycles (6,7).

The “pituitary gonadotropin hypothesis” proposes that high levels of estrogen and gonadotropins would over stimulate the ovarian epithelium and lead to malignant transformation(35). It is likely that the use of hormone replacement therapy with estrogens for 10 years or more is associated with increased risk for ovarian cancer (6). A few studies suggest that infertility treatments increase the risk as well since the drugs have elevated gonadotropin levels, leading to superovulation, even though it’s still controversial (35).

The “inflammation hypothesis” indicates that endometriosis, pelvic inflammatory disease and other inflammatory conditions may stimulate carcinogenesis by the persistent immune cells surrounding the epithelium, damaging DNA through release of reactive oxygen species or producing cytokines that promote proliferation (7).

Family history is the most important risk factor for ovarian cancer, either personal or family member such as mother, sister and daughter (36). Hereditary factors are responsible for 10-15% of ovarian cancers. Women with *BRCA1* or *BRCA*2 mutations are at significantly higher risk to develop epithelial ovarian cancer, as mutations in these genes fail to regulate cell death and uncontrolled cell growth, promoting carcinogenesis (34).

Other risk factors to considerate include lifestyle and nutrition. Some studies suggest that obesity is a predisposing factor for ovarian cancer because adipocytes play an important role in tumor initiation, growth and metastasis (6,32,34). In the presence of cancer cells, adipocytes can be reprogrammed into cancer-associated adipocytes, stimulating adhesion, migration and invasion of tumor cells (7).

### 2.2.2 Histological subtypes

Ovarian tumors are classified according to the cell type from where they originate and according to architectural features. The majority of ovarian cancers (90%) have epithelial origin, 5% originate from germ cell and 5% have stromal origin. Germ cell tumors can occur at any age yet are more prevalent in younger ages, including teratomas, dysgerminomas and endodermal sinus tumor (37). Stromal tumors (sex cord-stromal tumors) usually present at younger ages, although it as a wide age range (38).

Epithelial ovarian cancer is the most frequent and is classified into five histologic subtypes: high-grade serous (70%), endometrioid (10%), clear cell (10%), mucinous (3%) and low-grade serous carcinomas (<5%) (6,39). Serous carcinomas are classified in two separate subtypes – high grade and low grade - according to architecture, potential involvement of a serous borderline component, lack of diffuse p53 mutation and BRAF/KRAS mutation (40,41). High grade serous ovarian carcinoma (HGSOC) is the most frequent and lethal subtype, likely originating from the epithelial layer of the neighboring fallopian tube fimbriae (7,42).

According to architectural features, ovarian tumor can also be classified in histological grades: well differentiated, moderately differentiated, poorly differentiated and undifferentiated (34).

### 2.2.3 Therapy

There is not a wide range of ovarian cancer treatments. The first-line and main treatment is surgery, which can be: 1) unilateral, removing one ovary and the attached fallopian tube, applied whenever possible in woman at reproductive age; 2) bilateral, removing both ovaries and fallopian tubes; 3) total (hysterectomy), removing the uterus, including the cervix (43). Chemotherapy is most often used after primary treatment with surgery, usually with platinum agents. Platinum agents, including cisplatin, oxaliplatin and carboplatin, induce apoptosis of tumor-forming cells, damaging DNA and leading to the activation of pro-apoptotic pathways (43). Another treatment option is targeted

therapy, which uses drugs to target a specific or unique feature of cancer cells, inhibiting their growth. Three main drugs are used in ovarian cancer targeted therapy: bevacizumab, which is an angiogenesis inhibitor; PARP inhibitors, which target proteins involved in DNA damage repair; and tyrosine kinase inhibitors (TKIs), which target proteins involved in cell growth, survival and death pathways. Another treatment is immunotherapy, specifically used in patients with recurrent ovarian cancer. This therapy increases the immune system activity, usually with immune checkpoint inhibitors (ICI), such as pembrolizumab (43).

The American Cancer Society states that only approximately 20% of ovarian cancers are detected at an early stage, meaning fewer women can have an effective treatment. Despite some advances in treatment, if not detected in a very early stage it is often fatal, thus the need for new ways to detect ovarian cancer. The human microbiome has been exceptionally studied in the past decade, as well as its association with cancer, either in causation, progression or treatment, and it may help to better understand the development of the ovarian cancer or even contribute to early detection or therapy improvement.

## 2.3 The ovarian cancer microbiome

The work by Banerjee *et al.* (44) deeply studied the ovarian cancer microbiome, as they designated the ovarian cancer “oncobiome”. This relevant work aimed to understand the alterations present in the ovarian microbiota of cancer patients. In the study they used 99 ovarian cancer samples, 20 matched (non-tumor ovarian tissue) and 20 non-matched controls. Several differences between matched and non-matched samples were found, with two major bacterial phyla detected in ovarian cancer samples: *Proteobacteria* (52%) and *Firmicutes* (22%) and minority represented *Actinobacteria*, *Chlamydiae*, *Fusobacteria*, *Spirochaetes*, *Bacteroidetes* and *Tenicutes*. *Shewanella* signatures were detected with the highest prevalence in 91% of the cancers, which is the first specific microorganism directly associated with epithelial ovarian cancers. The results indicated that the majority of the bacterial signatures detected in the cancers had high prevalence, with no common bacteria between all three types of samples and 52 unique bacterial agents were detected predominantly only in the cancer (44). Fungal, viral and parasitic signatures in ovarian cancer were also identified. Overall this work not only defined the ovarian cancer microenvironment microbiome but also concluded that

the microbiome of ovarian tissues is different from its surrounding non-cancerous tissue and very different from ovarian tissue that has never been near a tumor (44).

With data accumulating, more and more microorganisms are being directly or indirectly associated with ovarian cancer. Table 2.1 summarizes the most relevant studies that have associated changes to microbiome species with ovarian cancer.

Two microorganism have been suggested to be indirectly associated with ovarian cancer: *Mycoplasma genitalium* and *Chlamydia trachomatis*, however it still is controversial. *Mycoplasma genitalium* (*M. genitalium*) is a sexually transmitted pathogen with potential to malignant transformation and chromosomal instability when the infection is prolonged. *Chlamydia trachomatis* (*C. trachomatis*) is also a sexually transmitted pathogen with a controversial role in ovarian cancer. A study by Shanmughapriya *et al.* (45) showed that in a cohort of 39 women with ovarian cancer, 70% of tumor ovarian tissue were infected with *Chlamydia*, while control women were not. Another study (46) reported an association between plasma cHSP60-10 IgG, which is an anti-apoptotic factor expressed by Chlamydial bacteria, and ovarian cancer, but not with *C. trachomatis* IgG nor IgA. This anti-apoptotic effect suggests that the survival of the bacteria within the host is facilitated as well as the survival of DNA-damaged cells, which may lead to an increasing risk for carcinogenesis (46). Besides the anti-apoptotic hypothesis, it is suggested that *Chlamydia* could be involved in the development of cancer via production of reactive oxygen species causing DNA damage or disrupting junctions structures thus increasing susceptibility to other infections (45). Nevertheless, the involvement of these pathogens in ovarian cancer carcinogenesis is still conjectural. *Chlamydia trachomatis* is the most common cause of pelvic inflammatory disease (PID) (47), an inflammation of the upper genital track, which includes the uterus, the fallopian tubes and the ovaries, caused by an infection derived from the lower genital track (48) and has been linked with ovarian cancer risk (49). PID appears to be a polymicrobial disease regardless of the initiation agent (50), associated with bacterial vaginosis, which constitutes itself a risk for infectious diseases. Repeated infections with *Chlamydia trachomatis* caused by vaginal microbiota dysbiosis constitutes a risk for PID, which may trigger carcinogenesis caused by a chronic upper genital tract infection and inflammation (50). Rasmussen *et al*. conducted two studies on Danish women to find an association between PID and ovarian cancer: in the first study they found that women with history of PID had an increased risk for borderline ovarian tumors, but not invasive ovarian cancer (51); in the second study they found that PID was associated with risk of serous ovarian cancer, but not any other histotypes (48). A similar study was conducted by Lin *et al* on Taiwanese women with history of PID and they found an association between ovarian cancer and PID, which

was slightly higher in women younger than 35 years old (52). These results suggest that there might be an increased risk for ovarian cancer in women with PID, although possibly not every histotypes. More studies need to be conducted in order to verify if this association constitutes a higher risk for ovarian cancer development. Nonetheless, considering that a dysbiotic vaginal microbiota facilitates infectious diseases that may lead to PID and rise cancer-promoting virulence factors (53), restoring the vaginal eubiosis could represent a preventive matter for bacterial infections and ultimately lower the risk for ovarian cancer.

Just as the gut microbiota could influence the development of cancer, recent studies suggest that alterations in the vaginal microbiota can also play a role in cancer development and have similar effects on immune regulation and oncogenesis. The healthy vaginal microbiota has been characterized and is generally populated by the *Firmicutes* phylum, dominated by *Lactobacillus* species, such as *Lactobacillus cripatus, Lactobacillus gasseri, Lactobacillus iners and Lactobacillus jensenii*, that may have a protective effect and therapeutic potential (54). A typical *Lactobacillus* dominated microbiota is essential to maintain the pH <4.5, which is well tolerated by these species and, more importantly, it is inhibitory to the development of several other bacterial types and appears to reduce the risk of Human immunodeficiency virus (HIV) transmission. Whenever the pH is altered, for instance is higher, the microbiota is no longer dominated by *Lactobacillus* but instead is characterized with a higher bacterial diversity with prevalence of anaerobic species (11). These alterations in the vaginal microbiota may often lead to inflammatory conditions, such as bacterial vaginosis and candida vulvovaginitis. Specifically, in bacterial vaginosis is verified a reduction in *Lactobacillus* and an increase in abundance of *Prevotella* and other bacteria. The healthy vaginal microbiota can be restored after vaginal infections with application of live lactobacilli and oestriol using vaginal suppositories (55).

Ravel *et al*. (56) characterized the vaginal microbiota of 396 asymptomatic women and described 5 different bacterial communities groups. Four groups were dominated by *Lactobacillus*, either *L. iners, L. crispatus, L gasseri or L. jensenii*, and one group was more heterogeneous, with higher propositions of strictly anaerobic organisms. Based on the community groups described by Ravel J. *et al*, Nené N. *et al*. (10) studied a group of women with ovarian cancer and women with the BRCA1 mutation, which is a known risk factor to ovarian cancer. They found that women with ovarian cancer and women with risk factors to ovarian cancer (*BRCA1* mutation or 50+ years old), women with ovarian cancer and less than 50 years old (ovarian cancer and -50 years old) and women without cancer with *BRCA1* mutation less than 50 years old (*BRCA1* mutation

and -50 years old) were significantly associated with a heterogeneous cervicovaginal community type, (e.g. a non-lactobacilli-dominated cervicovaginal microbiota). They specifically studied the cervicovaginal microbiota because that area serves as barrier for ascending infections. Despite the fact that we still lack sufficient knowledge on how a non-lactobacilli-dominated vaginal microbiota could be used as a novel way to predict or early diagnosis of ovarian cancer, these findings may be an important step towards a novel way to early diagnosis of ovarian cancer. Overall, the dysbiotic vaginal microbiota may contribute indirectly to ovarian cancer development via inflammation or modulation of local immunosurveillance, as it is associated with a higher pH, consequence of less lactic acid production.

*Table 2.1 - Relevant microbiome associated ovarian cancer studies*

| **Cancer** | **Microbiome specimen** | **Microbiome evaluation** | **Microbial change** | **Year** | **References** |
|---|---|---|---|---|---|
| Ovarian cancer | Ovarian cancer tissue | PCR-ELISA | Association between *Mycoplasma* and ovarian cancer | 1996 | Chan *et al*. (57) |
| Epithelial ovarian cancer | Blood samples | Serologic testing for IgG antibodies | ↑*Chlamydia trachomatis* | 2003 | Ness *et al.* (58) |
| Ovarian cancer | Ovarian tissue | Real-time PCR | None of the tissue samples were positive for *Mycoplasma genitalium* | 2010 | Idahl *et al.* (47) |
| Ovarian cancer | Ovarian tissue | Nucleic acid amplification | None of the ovarian tissue samples were positive for *Chlamydia trachomatis* RNA | 2010 | Idahl *et al.* (47) |
| Ovarian cancer | Blood samples | Serologic testing for IgG and IgA antibodies | Chlamydial HSP60-1 IgG antibodies were associated with type II ovarian cancer | 2011 | Idahl *et al*. (46) |
| Borderline ovarian tumor | Blood samples | Serologic testing for IgG antibodies | *Mycoplasma genitalium* IgG antibodies were associated with borderline ovarian tumors | 2011 | Idahl *et al*. (46) |

| | | | | | |
|---|---|---|---|---|---|
| Epithelial ovarian cancer | Fresh ovarian tissues | Nested PCR-based assay | ↑*Chlamydia trachomatis* | 2012 | Santhanam *et al.* (45) |
| Ovarian cancer | Ovarian cancer tissues | PathoChip Array | ↑*Proteobacteria, Firmicutes, Brucella, Chlamydia, Mycoplasma* | 2017 | Banerjee *et al.* (44) |
| Ovarian cancer | Blood samples | Multiplex, fluorescent bead-based assay | ↑*Chlamydia trachomatis* | 2018 | Trabert *et al.* (49) |
| Epithelial ovarian cancer | Cervical smear samples | 16S rRNA sequencing and qPCR | ↓*Lactobacillus* spp | 2019 | Nene et al. (10) |
| High-grade serous ovarian cancer | Fresh ovarian cancer tissues | 16S rRNA sequencing and qPCR | ↑ *Proteobacteria/Firmicutes* racio increased<br>↓Decreased bacterial diversity | 2019 | Zhou *et al.* (59) |

## 2.4 The role of gut microbiota in cancer treatment

In addition to its role in carcinogenesis, the gut microbiome plays a key role on response to cancer therapy, not only mediating the response but also modulating the toxicity effects of the cancer therapy. The gut microbiota has an impact on response to cancer therapy and, interestingly, the cancer therapy itself impacts the microbiota. One can assume that, in order to have an effective therapeutics and successful response, the gut microbiota must be in a state of eubiosis (balanced), as the commensal microbiota is actually crucial in the fight against cancer. The cancer therapies that affect the gut microbiota include chemotherapy and immunotherapy (29,60). The gut microbiota is indeed affected by these therapies, however, it plays a main role modulating the toxicity following the therapy. Besides the involvement of gut microbiota in cancer therapy, it is probable that the tumor microenvironment itself confer some effect as well, since those bacteria are metabolically active can modify the structure of common used chemotherapeutic agents, changing their activity, by either increasing or decreasing it, and consequently their effective local concentration (29). Considering this, it is

expectable that novel therapeutic approaches will also focus on microbial intervention and will take advantage of microbiome functions, aiming to achieve better performances and outcomes of cancer therapy as well as to reduce toxicity.

### 2.4.1 Chemotherapy

Human studies regarding the impact of chemotherapy on gut microbiota and the effect in modulating the therapy are still scarce, although relevant data from animal studies are already available.

Cyclophosphamide (CTX) is an alkylating agent used for chemotherapy to treat advanced, recurrent and/or persistent ovarian cancer, usually used in combination with other anticancer molecules (20,61), as it has been demonstrated to be modulated by commensal bacteria. A study performed by Viaud S. *et al.* (62) demonstrated the CTX alters the gut microbiota in mice in germ-free mice and antibiotic treated mice. Using selective antibiotics on Gram positive and Gram negative bacteria, they found that the efficacy of the CTX was reduced in mice with Gram positive selective antibiotics, resulting in the disruption of intestine's epithelial barrier and a relative decrease of *Lactobacilli* and *Enterococci* abundance, suggesting that these specific Gram positive bacteria play a role in the regulation of CTX antitumor efficacy, facilitating the translocation of certain bacteria across the gut epithelium (62). Within 48h of treatment these Gram positive bacteria were detected in mesenteric lymph nodes and the spleen, translocating through the intestinal epithelial barrier, stimulating the immune response. This resulted in a significant increase of Th17 and Th1 cells, thus facilitating a systemic antitumor effect of CTX (60,62). In addition, they found that germ-free mice were significantly less responsive to CTX, displaying a reduction in T cell activation (62). In summary, this study demonstrated the impact of the gut microbiota on chemotherapy effectiveness, revealing the consequences in gut microbiota's perturbations and also the influence of antibiotic treatment during chemotherapy and their negative impact on the outcomes of cancer therapy with cyclophosphamide.

Besides the treatment with cyclophosphamide, oxaliplatin and cisplatin are commonly used in platinum delivered chemotherapy. These drugs induce apoptosis of tumor-forming cells, damaging DNA and leading to the activation of pro-apoptotic pathways. A study by Iida N *et al*. (63) demonstrated that antibiotics reduce the therapeutic efficacy of platinum compounds against tumors, as in antibiotic treated mice the expression of pro-inflammatory genes was downregulated, had significantly reduced tumor regression and survival (63). In summary, this results suggest that the commensal

bacteria in the gut microbiota affect the inflammatory responses required for platinum therapy to be effective, denoting that the modulation of the gut microbiota may improve the cancer therapy. The most common side effect of chemotherapy include diarrhea, fatigue, loss of appetite and anemia. Severe cases of toxicity could lead to mucositis and cachexia.

### 2.4.2 Immunotherapy

Immunotherapy has been very successful in the treatment of several malignancies, such as advanced melanoma, renal cell cancer and lung cancer. Immune checkpoint inhibitors (ICI) are promising novel immunotherapy agents. ICI include programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), which are monoclonal antibodies to specific cell membrane receptors able to block the signaling pathways which negatively modulate the immune system. ICIs binding can transmit co-inhibitory signals to restore the exhausted T cells and activate the immune system to promote tumor cells destruction. Antibodies against CTLA-4 block the interaction of CTLA-4 with its ligands, thus releasing the checkpoint inhibition and promoting the T cell proliferation (60). The simultaneous use of these two classes of antibodies results in blocking two signaling pathways of ICIs (PD-1/PD-L1 and CTLA-4), which has been demonstrated to have more successful outcomes (60,61,64,65). Considering the role of the gut microbiota on immune response modulation, studies have evaluated the role of the gut microbiota in ICI cancer therapy. Some studies have demonstrated that the diversity of the gut microbiota was increased in patients responding to PD-1 therapy with specific microorganism such as *Clostridiales*, *Ruminococcaceae* and *Faecalibacterium*, and in non-responding patients showed lower microbial diversity, with higher Bacteriodales abundance. These results reveled that patients responding to PD-1 therapy had a specific favorable gut microbiota, when compared to non-responding patients (18). Although not all studies detected an association between antibiotic use and response to anti-PD-1, some have revealed that patients receiving antibiotics during ICI treatment had lower microbial diversity and altered gut microbiota composition, suggesting gut dysbiosis induced by antibiotic may negatively affect the anti-PD-1 response (5). A study held by Sivan A. et al. (66) revealed an unexpected role of commensal *Bifidobacterium* in anti-PD-1 therapy, enhancing the anti-tumor immunity. They suggested that *Bifidobacterium* colonizes specific gut compartments, allowing the bacteria to interact with host cells and thus modulating the immune response (66). *Bifidobacterium* has been proved to upregulate gene transcription of dendritic cells, associated with interaction of

cytokines, maturation of dendritic cells and activation of lymphocytes, thereby contributing to tumor regression (66). Another study showed that bacteria such as *Bacteroidetes* and *Bifidobacterium* may be protective against toxicity to cancer immunotherapy and *Firmicutes* bacteria is associated with favorable response as well as toxicity (29). The most common side effect of immune therapy include diarrhea, loss of appetite and fatigue.

### 2.4.3 PARP inhibitors

BRCA proteins participate in the repair of double strand breaks via the homologous recombination DNA repair machinery (67). Mutated BRCA proteins are unable to repair the break and thus in order for cells to survive, an alternative DNA repair pathway where PARP proteins participate is recruited. Poly (ADP-ribose) polymerase (PARP) constitute a family of enzymes with an important role in DNA repair pathways, particularly in single strand breaks. BRCA genes participate in double strand breaks. When PARP proteins are pharmacologically inhibited in the presence of mutated BRCA proteins, this can lead to cell death based on the principle of deficiencies of two genes (e.g. PARP and BRCA) leads to cell death (68). PARP inhibitors are used as a therapy to kill ovarian cancer cells in women with *BRCA1* or *BRCA2* mutations or with tumors also harboring somatic *BRCA1* or *BRCA2*, a phenomenon known as “BRCAness” phenotype (67,69). “BRCAness” ovarian tumors are sensitive to both platinum agents and PARP inhibitors (67), and have overall higher survival rates compared to non-“BRCA-ness” ovarian tumors (69). Due to this improvement in survival, PARP inhibitors are currently used as standard maintenance therapy for ovarian cancer management (70). While the relation between PARP inhibitors and the gut microbiome is still poorly understood, as so far there is no evidence that PARP inhibitors are able to influence the gut microbiome, Nene *et al*. (10) found that ovarian cancer patients with BRCA1 mutation are more likely to have a significant decrease of Lactobacilli population in cervicovaginal microbiota. Since the most common side effect of PARP inhibitors include diarrhea, constipation, fatigue, loss of appetite, anemia and muscle and joint pain (70), it is likely that the gut and other microbiomes may be altered by PARP inhibitors.

### 2.4.4 How to improve cancer therapy within the gut

Since accumulating data is pointing to gut microbiota as a likely player in carcinogenesis and/or in cancer prevention/killing processes, it is of major importance to

understand the role of gut microbiota in the regulation of cancer therapy because by correctly manipulating it we may be able to enhance the immune response and the management of the symptoms such as side effects. Aiming for this goals, probiotics, prebiotics and diet are often manipulated in order to restore a healthy gut microbiota.

Probiotics are cocktails of bacteria or combination of live bacteria that confer a health benefit to host when consumed in adequate amounts capable to alter the host's microbiota, either by implantation or colonization (5,29,54). Probiotics are composed by potentially good strains of bacteria, generally including *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus*, and *Enterococcus* as well as the yeast genus *Saccharomyces* (71). They appear to have several advantages, such as balancing the gut microbiota, reinstating microbiome homeostasis, restoring of the gut microbiota after antibiotic therapy, counteracting the pathogenic activities and increasing efficient activity of the immune system (71). The use of probiotics during anticancer therapy is still controversial, while many studies reveal the importance and contribution of probiotics supplements (72,73), others do not find any relevant positive associations (74). The scientific community is still apprehensive on the potential interactions of metabolites that may result from probiotics intake with the anticancer therapy, with still unclear consequences. For example, one study exposed an increased tumor penetrance upon probiotics intake, although it is theorized that the differences rely on timing of probiotic administration (29). On the positive side, several studies are showing the benefits of the usage of probiotics. For example, a study on the influence of probiotics intake during cyclophosphamide chemotherapy revealed that probiotics containing a specific set of Gram positive bacteria would enhance the immune response (71). This set of bacteria includes *Lactobacillus johnsonii, Lactobacillus murinus*, and *Enterococcus hirae* and is essential in the mediation and response of specific T lymphocytes (Th17 and Th1) (71). Several other studies linked the probiotic use and chemotherapy driven symptoms, revealing that probiotics may mitigate the chemotherapy and radiotherapy induced symptoms, such as diarrhea, in gynecologic cancer patients (54). Probiotics are of particular interest for battling the toxicity driven by these therapies. One example of this, involves one of the most studied and well characterized probiotic, the *Lactobacillus rhamnosus* GG (LGG). LGG has anti-inflammatory properties, which may be involved in ameliorating the anti-cancer therapy-related side effects through restoration of the gut microbiota balance (75). Additionally, LGG also seems to have anti-proliferative and anti-metastatic effect, as revealed by an in-vitro model study of several cancers, including ovarian cancer and can also influence the host immune system, through the enhancement of the host ability to eliminate cancer cells (2).

On the other hand, prebiotics are nonviable food components that offer the host health benefits through microbiota modulation and can be supplementary to probiotics. Prebiotics can be found in natural products, such as berries, bananas, asparagus, green vegetables, wheat and other foods, which are enriched with prebiotics, facilitating the colonization and relative expansion of particular bacteria (71), even though the role of prebiotics depends on the particular bacteria already present in the host (61). Synbiotics have properties of both prebiotics and probiotics and might constitute a promising novel approach to improve the anticancer treatment and/or stabilize the therapy driven symptoms (71).

Another way to manipulate the gut microbiome is through fecal microbiota transplant (FMT). FMT consists on the transplantation of fecal bacteria from a healthy donor in order to restore the gut microbiota of a dysbiotic individual. FMT has been widely studied in dysbiotic gastrointestinal diseases, specifically in the treatment of *Clostridium difficile* induced diarrhea, demonstrating promising outcomes (29). However, the major challenge in FMT is the optimal donor selection, which should comprise a wide variety of microbial composition, especially the ones that have been proven to be favorable, including *Bifidobacteria* spp., *E. hirae* and *Bacteroides spp* (5).

Other factors that can regulate the gut microbiota composition include diet, sleep cycles, exercise and medications.

Thus there are multiple possibilities to modulate the gut microbiota in order to potentially influence anti-cancer therapy outcomes.

# 3. Materials and Methods

## 3.1 Literature search

A comprehensive literature search was performed using PubMed to search for articles reported in English and favoring those published within the last 10 years. A combination of search terms included “ovarian cancer”, “human microbiome”, “risk factors”, “gut microbiota”, “vaginal microbiota”, “dysbiosis”, “anticancer therapy”, “side effects”, “chemotherapy”, “immunotherapy”, “PARP inhibitors” and “probiotics”. Review articles were considered for inclusion and studies with human subjects were favored over animal models.

## 3.2 Patient samples

To study the vaginal microbiota, vaginal swabs will be obtained from healthy donors and ovarian cancer patients during routine screening for healthy donors and visits to the oncologist in the case of ovarian cancer patients. These same patients will be used to study the gut microbiota. From the same patients, stool samples will be obtained from healthy donors and ovarian cancer patients. Vaginal and Stool samples will be collected into a vaginal or stool collection device containing DNA/RNA Shield (Zymo Research) to preserve the genetic integrity and species profiles of samples at ambient temperatures. Sample collection will comply to Ethical commission approval and to patient informed consent.

## 3.3 DNA extraction and quantification

Extracting bacterial DNA from vaginal or fecal specimens for molecular diagnostics will be performed using the semi-automatic DNA extraction system easyMag (R) (BioMerieux) according to manufacturer’s specifications (DNA extraction process were performed by the robot according to protocol A and eluted in 110 µL easyMag® elution buffer). DNA quantification and quality profile assessment will be performed on an Agilent 2200 TapeStation System (Agilent) using an Agilent Genomic DNA ScreenTape (Agilent).

## 3.4 NGS libraries preparation and sequencing

Next Generation libraries will be prepared from vaginal or fecal DNA samples, using the Ion 16S™ Metagenomics Kit (ThermoFisher) according to manufacturer's protocol. This kit includes two primer sets that are used to amplify seven hypervariable regions of the 16S rDNA gene in bacteria (primer set 1 includes V2, V4 and V8 regions, primer set 2 includes V3, V6, V7 and V9 regions). Template preparation and chip loading will be performed on an Ion Chef™ System (ThermoFisher) and the sequencing procedure will be carried on using semiconductor technology on an Ion S5™ next-generation sequencing system (ThermoFisher), both according to manufacturer's protocol and aiming for a minimum of 100000 reads/sample.

## 3.5 NGS data analysis

Bam files obtained for each sample will have the taxons present identified using programs such as Kraken (76) and improved by Braken (77). Several parameters will be compared against freely available main databases such as Greengenes, Ribossomal Database Project (RDP), Silva, Open Tree of life Taxonomy (OTT) and National Center for Biotechnology Information (NCBI) which contain a large collection of data from healthy individuals. To analyze gut microbiomes, data will be compared against gut databases such as the American Gut Project (78) and to analyze the vaginal microbiota, data will be compared against databases such as the Vaginal 16S rDNA Reference Database (79). For specific taxons detected in the literature important for cancer screening and for therapy decision, if poorly represented in the databases but they will be added to the list of taxons to be compared. Analysis pipelines will be mainly based on Python (80) and R (81) programming languages.

## 3.6 Report design

In order to better understand the nature of the test, we developed a prototype of a report simulated for both situations (ovarian cancer screening and ovarian cancer therapy management). Complete reports in appendixes.

### 3.6.1 Microbiome test for ovarian cancer screening

The aim of this test is to evaluate the vaginal microbiota community, knowing that a homogeneous microbiota dominated by *Lactobacillus* is more beneficial and healthier than a more heterogeneous community, no longer dominated by *Lactobacillus* and prone to bacterial infections due to higher pH. This test will also determine the presence of microorganisms associated with bacterial vaginosis infections.

### 3.6.2 Microbiome test for ovarian cancer therapy management

The aim of this test is to provide information on the intestinal microbiota status during ovarian cancer therapy, identifying microorganisms that may potentiate the anticancer therapy efficacy, microorganism responsible for side effect from therapy and the microorganisms with probiotic potential, microorganisms with anti-inflammatory properties and those participating in immune regulation, along with recommendations for microorganisms potentially beneficial in case of inadequate range levels. The report is essentially constituted by two sections: the first directed to anticancer therapy evaluation and side effects, and the second part with detailed results, including diversity, pathogens, probiotic bacteria and immunity and inflammation indicators. The first section displays microorganisms detected in the gut microbiota sample of the patient undergoing ovarian cancer therapy. Their detection could provide information on anticancer therapy efficacy, identifying potential options to enhance the performance. This section also include the display of shows microorganisms detected in the gut microbiota sample of the patient presenting with side effects from anticancer therapy. Their detection identifies the source of the symptoms, along with microorganisms potentially able to revert or attenuate the symptoms. If the test identifies microorganisms associated to a specific side effect that currently the patient isn’t demonstrating, it could as well be a preventive measure. More studies are required to define the range at which the presence of the microorganisms is indicative of an anticancer therapy driven side effect.

# 4. Results

This chapter mainly gathers information from literature searches on context of microbiome and cancer. By featuring the gut and vaginal microbiota role in cancer anticancer therapy and side effects management, particularly in ovarian cancer, the herein presented report prototypes may contribute to define and characterize relevant parameters that should be present in laboratorial tests evaluating cancer-related microbiomes.

## 4.1 Microbiome test for ovarian cancer diagnosis

Up to this day, there is no available test on the market based on gut microbiome able to diagnose cancer. Several ongoing studies have demonstrated the gut microbiome potential as a novel diagnostic tool, for some specific cancers (82–85). However, in the ovarian cancer setting, no data nor studies focusing on microbiome based biomarkers associated with ovarian cancer are available, therefor making the attempt to develop a diagnosis test based on available data not yet possible. The only information available on ovarian cancer is on the tumor microenvironment and its microbiome, where viral, bacterial, fungal and parasitic signatures were found (44). While we have evaluated this available cancer microenvironment microbiome-based data as a potential start point for the development of a new diagnosis test, given the invasive nature to acquire tumor microenvironment-based data, which we want to avoid, we did not consider it further. In addition, we did not find any information on ovarian cancer-associated gut or vaginal microbiota with the potential to support a credible test for ovarian cancer early diagnosis.

## 4.2 Microbiome test for ovarian cancer screening

While the available information in the literature is insufficient to support the conception and development of an early diagnosis test for ovarian cancer, some data can aid and support the development of a screening test for ovarian cancer, instead of a diagnostic procedure. Given the weight of a diagnosis test, which must be clinically useful and robust enough to dethrone the current standard-of-care diagnostic procedures, developing an ovarian cancer, microbiome-based screening test is more easily achievable and can aid in guiding current diagnosis. This screening test would be directed to high risk women, such as post-menopause individuals or women with a family history of ovarian/breast/colorectal cancer and/or who inherited mutations in specific genes (e.g. BRCA1, BRCA2, MLH1, etc.) (86). On the current available data, despite the

absence of ovarian cancer related information on gut microbiota, some studies have found associations between vaginal microbiota and ovarian cancer (10). Thus if moving forward with a vaginal microbiota test, the aim of such screening test will be to evaluate the microbial community of the vaginal microbiota, knowing that a homogeneous microbiota is beneficial and healthier than more heterogeneous community, no longer dominated by *Lactobacillus* and prone to develop and bacterial infections due to higher pH (87). One strategy to implement a screening test could be to use the combination of a pap smear with a vaginal microbiota test. The Papanicolaou (Pap) smear is intended to screen cervical cancer and for the presence of Human Papilloma Virus (HPV), thus due to its low diagnostic sensitivity for ovarian and endometrial cancers, these cancers are infrequently detected via this cervical cytology alone. In the recent years, at least one group developed a mutation-screening based test (PapSeek) for the early detection of ovarian cancer from Pap smears (88) proving the usefulness of using Pap smears beyond cervical cancer detection. The combination of a Pap smear and/or mutational screenings with a vaginal microbiota has the potential to provide more information regarding ovarian cancer development, to increase the sensitivity of the detection, to deliver a less invasive screening method and perhaps to allow for pre-cancer interventions in women at risk. For example, not only a combined screening test could pinpoint women with recurrent vaginal infections (a potential risk factor to develop ovarian cancer, especially if the woman is already at risk) but also, if these woman presents a heterogeneous vaginal microbiota, the physician could prescribe probiotics, rich in *Lactobacillus*, rectifying the dysbiosis and perhaps minimizing the risk.

## 4.3 Microbiome test for ovarian cancer therapy management and evaluation

Once an ovarian cancer is diagnosed, the treatment efficacy and the reduction of side effects is also a major issue. Following surgery, cycles of chemotherapy are the standard of care, but in recent years there is an increasing interest in also using immunomodulators given that relapse rates are high in this malignancy and occur around every 2-years (89). Immunotherapy comprehends various methods to enhance the immune system. Given that the gut microbiota participates in the immune system regulation, modulating the gut microbiota is a potential novel approach for cancer treatment, especially because of the high relapse rates and associated resistance to therapy, driven by molecular changes in ovarian cancer cells (89).

Developing a microbiome test for ovarian cancer therapy management could provide information on the health gut status and evaluate its efficacy. Such test could provide information on the intestinal microbiota status during therapy, and if combined with selected clinical parameters could make a direct link between the gut health and the cancer therapy. The development of this test comprehends three major goals: minimize therapy-related side effects, monitor the gut health and increase the immune response to enhance the therapy. In order to achieve these goals, we would study the gut microbiome of healthy individuals and ovarian cancer patients undergoing therapy to detect the healthy/dysbiotic signatures and evaluate which microorganisms participate directly or indirectly in therapy management. To characterize the dysbiosis, the test would focus on important microbiota features, such as inflammation; immunity and intestinal mucosal barrier; presence of pathogen microorganisms; and probiotic bacteria. Further, to analyze the gut microbiota response to cancer therapy, the test would focus on: identifying microorganisms responsible for gut-directed side effects; measuring pathogenic bacteria but also the pro-inflammatory microorganism and methane production; the synthesis of important vitamins (e.g. B12) and also identifying which microorganisms are able to enhance or to be detrimental for the therapy, previously described in the literature. By gathering all this information in a comprehensive report, the physician could potentially prescribe a treatment for the dysbiosis (e.g. with prebiotics and probiotics) and also determine if the therapy is effective or if another approach is necessary.

### 4.3.1 Inflammation

Some microorganisms have anti-inflammatory activity and directly intervene in inflammatory processes. In the context of cancer, maintaining the gut functionality and integrity with its anti-inflammatory microorganisms seems to be crucial to achieve better outcomes. Short chain fatty acids (SCFA) are originated in the gut from intestinal microbial fermentation and constitute the main energy source of colonocytes (90). The most common SCFA are butyrate, acetate and propionate and have several physiological activities, strengthening the epithelial barrier function, vitamin synthesis, immunomodulatory functions and maintaining the intestinal homeostasis trough anti-inflammatory activities in the colon mucosa (91–93). Butyrate is mainly produced by *Faecalibacterium praunsnitzii, Ruminococcus, Roseburia, Blautia, Eubacterium hallii* and *Anaerostipes*. Acetate and propionate are mainly produced by *Bacteroides, Akkermansia muciniphila* and *Alistipes.* Butyrate generates the anti-inflammatory effects inducing the regulation of T cells, downregulation of pro-inflammatory cytokines and the

Toll-like receptor 4 (TLR4) (94). Considering that butyrate is involved in anti-inflammatory processes, is likely that the butyrate producing bacteria have anti-inflammatory activity, therefore impacting the physiologic function and intestinal homeostasis (95). Reduction of butyrate-, acetate- and propionate-producing bacteria reduce SCFA production, thus reducing the regulation of inflammatory processes contributing to inflammation (96). Principal SCFA microorganism producer are represented in table 3.1.

*Table 4.1 - Principal SCFA microorganism producers*

| | **Microorganisms** | **SCFA production** | **References** |
|---|---|---|---|
| SCFA producers | *Faecalibacterium praunsnitzii* | Butyrate | (95,97–104) |
| | *Ruminococcus* | Butyrate | (98) |
| | *Roseburia* | Butyrate | (97,99–102) |
| | *Blautia* | Butyrate | (98,105) |
| | *Eubacterium hallii* | Butyrate | (97–102,104) |
| | *Anaerostipes* | Butyrate | (97,99,100,102) |
| | *Bacteroides* | Acetate/propionate | (97,99,100) |
| | *Akkermansia muciniphila* | Acetate/propionate | (90,100) |
| | *Alistipes* | Acetate/propionate | (96,100) |

### 4.3.2 Immunity and mucosal barrier integrity

The intestinal mucosal barrier plays a crucial role in intestinal immune regulation. Intestinal mucus is a glycan produced by secretory cells of the epithelial layer and constitutes a barrier against gut microbiota, creating an interface between the host cells and the microorganisms, although the gut bacteria actively contributes to the intestinal mucus and to its functions (106). In homeostasis, the intestinal barrier integrity is modulated through constant mucin degradation and mucin production by gut microbiota bacteria. Microorganisms responsible for mucin degradation include *Akkermansia muciniphila, Ruminococcus gnavus* and *Bifidobacterium bifidus;* mucin production is mainly performed by *Akkermansia muciniphila* and butyrate producing bacteria (91,94,102,104,107–110). Intestinal barrier disruption leads to bacterial translocation (103,111). The integrity of the intestinal barrier is so crucial for the host health that disruptions or defects on the mucosal layer can eventually contribute to intestinal infection and inflammation, although it may not be sufficient to cause disease by itself.

SCFAs play as well an important role in intestinal immune regulation, regulating the intestinal mucosal immunity through mucin segregation, regulation of tight junctions and also the generation of regulatory T cells (Treg). Furthermore, SCFAs participate in

intestinal Immunoglobulin A (IgA) production of B cells and inhibits carcinogenesis, promoting apoptosis and suppressing tumor cells proliferation (98). Under physiologic conditions, IgA controls bacterial translocation and neutralizes bacterial toxins at the intestinal mucosal surface (112). The majority of immunoglobulin A (IgA) plasma cells are derived from B-cell activation in mucosal associated lymphoid tissues (MALT) (112). The gut is the major producer of immunoglobulins in the body, and as the majority of MALT is located along the gut, it is therefore designated gut-associated lymphoid tissues (GALT) (112). The mucosal immune system key function is the production of secretory IgA antibodies, which confers protection against infections and establishes a healthy microbiota. The strict relation between IgA and the microbiota is evident, as on one hand the composition of the microbiota affects the IgA composition, and on the other hand the composition of the microbiota is influenced by IgA production (113). Secretory IgA antibodies also play a role preventing the systemic spread of commensal gut bacteria (114). Considering this thigh relation, dysfunctions in this balance may lead to dysbiosis and inflammation in the gut. For example, in germ free mice poor quantities of IgA are produced, which rapidly reverts upon bacterial colonization (115). Secretory IgA antibodies production can be stimulated in the in the gut by SCFA producing bacteria (116).

Considering the importance of intestinal barrier integrity, targeting the gut microbiota to prevent intestinal disease and promote homeostasis is a promising pathway to develop novel treatment or preventive options for dysfunctional mucus layer related diseases.

### 4.3.3 Microorganisms associated with cancer therapy

The gut microbiota not only plays a role in carcinogenesis but also participates in cancer therapy, influencing the response and the toxicity of several therapies, including chemotherapy, radiotherapy, immunotherapy and immune checkpoints inhibitors. Thus the gut microbiome impacts the therapy but the therapy deeply influences the microbiome as well. These impacts are visible in the form of dysbiosis, triggered by the therapy but also by adjuvant administrated drugs (e.g. antibiotics) deeply affecting the gut microbiota composition (29). Chemotherapy affects both the alfa (species richness) and beta diversity (diversity in the microbial community) of the microbiome, when compared with samples without chemotherapy (117). Common chemotherapy for ovarian cancer includes platinum compounds (e.g. cisplatin), taxanes and cyclophosphamide. Cyclophosphamide induces damage in the intestinal mucosal barrier and the proliferation of Gram negative bacteria, including *Barnesiela intestinihominis*,

and of the Gram positive bacteria *Enterococcus hirae* that enhances effector T cells which in turn promote a pro-inflammatory tumor environment creating an anticancer immune response (118). Both bacteria are regulated by intestinal NOD2 receptors and constitute positive predictors of progression-free survival in ovarian cancer patients treated with chemotherapy. Administration of E. *hirae* in antibiotic treated mice restored the efficacy of cyclophosphamide, demonstrating that its efficacy relied on the gut microbiota (118). Another study (62) suggested the importance of *Lactobacillus johnsonii, L. murinus* and *Enterococcus hirae*, for the systemic antitumor effect of cyclophosphamide through microbiome: the translocation of these bacteria across the intestinal barrier to secondary lymph nodes results in an increase of Th17 and Th1 cells, facilitating a systemic antitumor effect of cyclophosphamide. These studies demonstrate de potential role of *Lactobacillus johnsonii, L. murinus*, *Enterococcus hirae and Barnesiela intestinihominis* into enhancing cyclophosphamide chemotherapy. Their presence in the gut microbiota of ovarian cancer patients could represent a novel tool for therapy management, predict a successful outcome and survival and a possibility to modulate the microbiota in non-responding patients. Table 4.2 includes relevant studies with microorganisms associated with therapy response and table 4.3 represents the parameters to develop the test report.

*Table 4.2 - Microorganisms associated with cancer therapy*

| **Therapy** | **Microorganism** | **Effect** | **References** |
|---|---|---|---|
| Cyclophosphamide (CTX) | *Enterococcus hirae* *Barnesiella intestinihominis* | *E. hirae* and *B. intestinihominis* enhanced cognate anticancer immune responses<br>*E. hirae* and *B. intestinihominis* are regulated by intestinal NOD2 receptors<br>CD4+ T cell responses against *E. hirae* are associated with survival in cancer patients<br>*E. hirae* restored the efficacy of CTX in antibiotics- treated mice | (118) |
| | *Lactobacillus johnsonii, L. murinus, Enterococcus hirae* | Commensal bacteria cross the intestinal barrier to enter secondary lymphoid nodes | (62,118) |

| | | | |
|---|---|---|---|
| | *Lactobacillus* | Gram-positive commensals mediate accumulation of TH17 and TH1-cell response | (62) |
| Immunotherapy (PD-1/PD-L1) | *Bifidobacterium* | Enhance anti-PD-L1 efficacy by reactivating dendritic cells that boosted CD8-positive T cell responses to defeat tumors<br>Tumor-specific T-cell induction and increased T cells in tumor microenvironment | (119) |
| Immunotherapy (CTLA-4) | *Bifidobacterium longum, B. breve*<br>*Akkermansia muciniphila* | Enhance the efficacy of this therapy in tumor-bearing mice | (120) |
| | *Bacteroidales* | Decreased activation of splenic effector CD4+ T cells and tumor-infiltrating lymphocytes in mice | (121) |
| Platinum compounds (cisplatin) | *Lactobacillus acidophilus* | Restored the anti-tumor effects of cisplatin in a murine lung cancer mode (after antibiotic intake) | (122) |

*Table 4.3 - Report parameter: Microorganisms associated with cancer therapy*

| **Therapy** | **Microorganism positively associated** | **Result** | **Microorganism negatively associated** | **Result** |
|---|---|---|---|---|
| Cyclophosphamide | *Enterococcus hirae*<br>*Barnesiella intestinihominis*<br>*Lactobacillus johnsonii, L. murinus* | Positive Or Negative | N/A | Positive Or Negative |
| Cisplatin | *Lactobacillus acidophilus* | Positive Or Negative | N/A | Positive Or Negative |
| PD-1/PD-L1 | *Bifidobacterium longum, B. breve*, and *Akkermansia muciniphila* | Positive Or Negative | N/A | Positive Or Negative |

| | | | | |
|---|---|---|---|---|
| CTLA-4 | *Bacteroidales* | Positive Or Negative | N/A | Positive Or Negative |

### 4.3.4 Microorganisms associated with cancer therapy side effects

The most frequent side effects of cancer therapy are diarrhea, constipation, anemia, cachexia and mucositis/enteritis. This side effects reduce life quality, are debilitating to the patients and in severe cases are life threatening. Chemotherapy-induced diarrhea is a severe condition frequent in advanced stage cancer patients which can lead to infections and potentiate a life threatening situation. Constipation is associated with methane production in the gastrointestinal tract. Anemia is a side effect that arises due to changes in erythropoiesis, which could be attenuated with Vitamin B12 supplements. Cachexia is a severe therapy side effect comprehending fatigue, appetite and weight loss and could represent a cause of premature death, related to advanced cancer and metastasis. Severe mucositis is associated with a higher mortality in cancer patients. Table 4.4 summarizes alterations in the gut microbiome and microorganisms associated to common side effects.

*Table 4.4 - Alterations in the gut microbiome and microorganisms associated to side effects*

| Side effect | Microorganism | Effect | References |
|---|---|---|---|
| Chemotherapy induced Diarrhea | *C. difficile* *Salmonella*, *Campylobacter*, and *Shigella* | Most frequent cause of bacterial diarrhea | (123,124) |
| Mucositis /enteritis | *Enterobacteriaceae* | ↓Bifidobacterium<br>↓Faecalibacterium prausnitzii<br>↑Enterobactereaceae<br>↑Bacteroides | (125) |
| Cachexia | Enterobacteriaceae | ↓Lactobacillales | (126) |
| Constipation | *Methanobrevibacter* *Methanobacterium* | Methane producing archaea resulting in constipation | (127) |
| Anemia (vitamin B12 synthesis) | *Lactobacillus reuteri* | Vitamin B12 biosynthesis | (128) |

### 4.3.5 Probiotic bacteria

The gut microbiota comprises probiotic bacteria, that not only intervene in gut homeostasis maintenance but also has implications in cancer therapy, enhancing the immune response and restoring the microbial balance. Thus, modulation of the gut microbiota by probiotic species administration could improve the efficacy of cancer therapy. The most important probiotic bacteria are *Bifidobacterium* and *Lactobacillus* species. Probiotic species of *Lactobacillus* may improve gastrointestinal barrier function by the proliferation of some harmful bacteria (129). *Bifidobacterium* can also inhibit harmful bacteria, improve gastrointestinal barrier function, and suppress pro-inflammatory cytokines. Table 4.5 summarizes the effect of probiotic bacteria in ameliorating cancer therapy side effects and table 4.6 represents the parameters to develop the test report.

*Table 4.5 - Effects of probiotics in cancer therapy side effects*

| Side effect | Microorganism | Effect | References |
|---|---|---|---|
| Chemotherapy induced Diarrhea | *Lactobacillus* supplementation | Reduced the frequency of grade 3 or 4 diarrhea; less abdominal discomfort | (130) |
| | *L. casei*, *L. rhamnosus* and *B. bifidum* | Attenuate chemotherapy-associated diarrhea in a mouse model through the inhibition of Tnf, Il1b and Il6 mRNA expression | (131) |
| Mucositis /enteritis | *Bifidobacterium longum, B. breve, B. infantis Lactobacillus acidophilus* | Reduce incidence rates and ameliorate symptoms | (132) |
| Cachexia | *Lactobacillus reuteri* | Reduced effect of cancer associated cachexia in mice | (133) |
| Constipation | *B. lactis* | Reduced constipation, improving stool frequency and consistency | |
| | *L. reuteri* | Efficacy with chronic constipation increasing bowel movement | (134) |

*Table 4.6 - Report parameter: Microorganisms associated with side effects from anticancer therapy*

| **Side effect** | **Microorganism positively associated** | **Result** | **Microorganism negatively associated** | **Result** |
|---|---|---|---|---|
| Chemotherapy induced diarrhea | *Lactobacillus - L. casei*, *L. rhamnosus* Bifidobacterium *bifidum* | Positive Or Negative | *C. difficile* | Positive Or Negative |
| Mucositis /enteritis | *Bifidobacterium – B. longum, B. breve, B. infantis* *Lactobacillus acidophilus* | Positive Or Negative | *Enterobacteriaceae* | Positive Or Negative |
| Cachexia | *Lactobacillus reuteri* | Positive Or Negative | Enterobacteriaceae | Positive Or Negative |
| Constipation | *Bifidobacterium lactis* *Lactobacillus reuteri* | Positive Or Negative | *Methanobrevibacter* *Methanobacterium* | Positive Or Negative |
| Anemia (vitamin B12) | *Lactobacillus reuteri* | Positive Or Negative | N/A | Positive Or Negative |

# 5. Discussion and Concluding remarks

With the current work we have attempted to design a gut and a vaginal microbiome test for ovarian cancer therapy management and the respective report that can be used to be validated in a patient cohort. Given the information that we have found by surveying the scientific literature, the test and the report should account for key aspects related to the interaction between the gut and/or vaginal microbiome and therapy, such as: mechanisms to enhance therapy, evaluation of anticancer therapy efficacy and information on microorganisms associated with anticancer therapy side effects, for potential management of the symptoms. To maximize the performance of the test, a few clinical parameters must be provided: 1) history of recent antibiotic therapy; 2) history of recent probiotic use; 3) ovarian cancer classification; 4) ongoing therapy; 5) therapy side effects (specifying the symptoms). These parameters will help in developing a patient-tailored report, and in the setting of a validation cohort, with more information being accumulated, will allow to better fine tune the recommendations on how to adjust the gut and/or the vaginal microbiota profile for each specific patient.

On the antibiotic therapy usage issue and as expected, we have found that antibiotics are mostly non beneficial for ovarian cancer patients under therapy (63,135,136), as they deeply affect the gut microbiota composition. Such disequilibrium could be reverted with probiotic supplements. Probiotic bacteria from the *Lactobacillus* and *Bifidobacterium* genus are acid lactic producers and have intestinal restoring properties. Their usage has the potential to attenuate side effects from anticancer therapy, however they must be further tested to prove this. Probiotic *Lactobacillus* species include *L. casei*, *L. rhamnosus*, *L. acidophilus* and *L. reuteri*, commonly found in standard probiotic supplements and dairy foods (e.g. milk, cheese, yogurt) (137). In the same category as *Lactobacillus*, *Bifidobacterium* species include *B. bifidum, B. longum, B. breve, B. lactis* and *B. infantis*, which are found in probiotic supplements, dairy products, vegetables, fruits, cereal, nuts and seeds.

On the issue of microorganisms with potential to enhance anticancer immune response we have previously mentioned, *Enterococcus hirae* and *Barnesiella intestinihominis.* These were identified as microorganisms with potential to enhance anticancer immune response of patients with ovarian cancer, treated with cyclophosphamide chemotherapy (118). Both microorganisms could represent a novel tool to evaluate and enhance the cancer therapy, considering that their presence seems to increase therapy efficacy and to predict longer progression-free survival.

Considering that *Enterococcus* are lactic-acid bacteria, they can be found in fermented foods, such as milk and cheese, and raw meat, or administrated as probiotic supplements (138). *Barnesiella* can be found in milk oligosaccharides (139). However, because the currently available in the information is still insufficient, the identification of these bacteria in a gut microbiome test and its subsequent usage to boost the cyclophosphamide efficacy still depends on future tests using cohorts of cancer patients.

Related to platinum compounds, another bacteria, *Lactobacillus acidophilus, was* shown to restore the antitumor effects of cisplatin platinum chemotherapy in a lung cancer mouse model (122). Even though it constitutes a promising candidate, more in-vivo studies are needed to determine if the microorganism has the same potential in ovarian cancer patients. Overall, further studies are needed to identify novel gut microorganism able to enhance the or that negatively affect platinum chemotherapy performance.

On the PD-1 immunotherapy efficacy, this could be enhanced with supplementation of *Bifidobacterium longum, B. breve* and *Akkermansia muciniphila*, but again, its usage still requires further investigation in the context of the microbiome of ovarian cancer patients. Another important microorganism in the context of PD-1 immunotherapy is *Akkermansia muciniphila*. Though it is still unclear which foods increase its abundance, prebiotic foods such as omega-3 fish oils and oligofrutose are candidates to increase of *Akkermansia muciniphila* in the gut microbiome (140).

Regarding CTLA-4 immunotherapy, although not specific for ovarian cancer, a study (121) demonstrated the potential benefit of *Bacteroides thetaiotaomicron*, *B.fragilis* and *Faecalibacterium prausnitzii* to enhance the CTLA-4 anticancer therapy. *Bacteroides thetaiotaomicron* and *B.fragilis* are commonly found in the gut microbiota as commensals, and even though they are both opportunistic pathogens, lines of research are currently leaning on specific strains with potential to be used as probiotics (141).

The clinical information of each ovarian cancer patient represents potential valuable data to be used in combination with microbiota data. However, with our literature survey we detected that we still face major difficulties regarding the standardization of information about the gut and/or vaginal microbiota of ovarian cancer patients associated with high risk factors, such as age, family history of ovarian/breast/colorectal cancer and *BRCA1* and *BRCA2* mutations. The major problem to develop a microbiome test is the absence of a comprehensive database of ovarian cancer with intestinal and vaginal microbiome sequences complemented with crucial clinical/personal parameters that may constitute risk factors for ovarian cancer poorer outcomes (e.g. age +/- 50 years; mutated

or wildtype for BRCA1/2 genes; undergoing therapy or no; the presence of a bacterial vaginosis, in the case of vaginal microbiome; etc.). All or some of these parameters may contribute to the differences within the gut and/or vaginal microbiota between women harboring distinct clinical factors. For example, age is an essential factor to consider, as postmenopausal women (over 50 years) present a more heterogeneous vaginal microbiota, when compared with younger women. The same profile is present in women with *BRCA1* or *BRCA2* mutations and women with ovarian cancer. All these factors must be considered when developing a risk prediction indicator for a vaginal microbiome-based ovarian cancer screening report.

Although many studies have focused in decipher the colorectal cancer relationship with the gut microbiota, the overall research of the microbiome and cancer is still in its infancy and must be expand to other cancer pathologies. This need is evident in more complex cancers, with unclear ethology and difficulties in early diagnosis, such as the ovarian cancer. More studies are needed regarding several aspects of this work. First, the need to further demonstrate in human subjects the mouse model findings, according to anticancer treatment and the cancer pathology, in order to potentiate future clinical uses. Second, the need for epidemiologic studies to further assess the microbiome as a risk factor. Nonetheless the already available knowledge regarding the interaction of the microbiome and the host, the turning point that implies strong clinical validation for its potential usage as a biomarker to detect increased risk for a specific oncological disease is still unclear. Third, further research is warranted in prebiotics/probiotics field, as they may potentially improve the gut and vaginal health, used to prevent cancer and to stabilize the gut and/or vaginal microbiota before and during anticancer therapy, and even though many results have been published, the overall data is still contradictory.

Particularly for ovarian cancer, there is a need to identify microorganisms capable to enhance anticancer therapy efficacy, considering the high mortality rates, relapses and resistance to therapy development, aiding therapy improvement, the development of novel less toxicity associated-agents and personalized therapy according to patient's microbiota. Further, the vaginal microbiota requires more attention to better comprehend its relationship with gynecological cancers, as the majority of recent studies focus essentially in gastrointestinal microbiota (e.g. colorectal cancer and gut microbiota relationship is well documented).

With this work, based in literature searches, we made an attempt to develop a prototype of a potentially promising novel approach to test and manage ovarian cancer. We strongly believe that a collective of more studies aiming for the tight relationship

between the microbiome and cancer can ultimately constitute a paradigm shift in modern medicine, regarding early diagnosis and therapy management.

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## 7.1 Appendix 1 - Report simulation: vaginal microbiota test for ovarian cancer screening

**Report – Vaginal microbiota test for ovarian cancer screening**

**REPORT Unique ID: XXXX**

| **Personal information** | **Request** |
|---|---|
| Name: N.E. BODY | Date: |
| Age: 43 | Clinician: |
| Gender: F | Hospital/Clinic: |

**Clinical information**

History of recent vaginal infections: No

History of recent antibiotic therapy: No

History of recent probiotic use: No

pH: 5,7

Risk factors: Family history of ovarian/breast cancer

**Main Result**

We have detected changes in the vaginal microbiota compared with healthy individuals.

**Results**

The analysis of your vaginal microbiota sample has revealed infection with *Gardnerella vaginalis* and *Prevotella bivia*, which is associated with bacterial vaginosis. Your vaginal microbiota needs improvement.

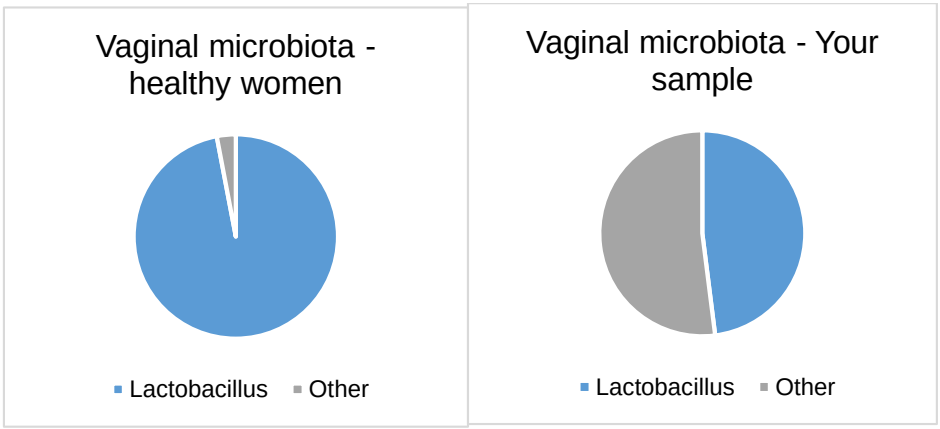
**DETAILED RESULTS**

**pH –** 5.7

Significance: your vaginal pH is **high**, consequence of not being dominated by *Lactobacillus* species, which is an indicator of poor vaginal health and prone to infections.

**Vaginal microbiota diversity**

Representation of a hypothetical vaginal microbiota diversity.




Significance: the analysis of the vaginal microbiota sample revealed a dysbiotic profile, not dominated by Lactobacillus species, potentially resulting in higher pH and infections.

**Bacterial vaginosis infectious agents**

*Bacteroides fragilis* – undetected

*Streptococcus agalactiae* – undetected

*Staphylococcus aureus* – undetected

*Mycoplasma genitalium* – undetected

*Chlamydia trachomatis* – undetected

*Prevotella bivia* – **detected**

*Gardnerella vaginalis* – **detected**

Significance: the analysis of the vaginal microbiota sample revealed infection with *Prevotella bivia* and *Gardnerella vaginalis,* which is related to lower abundance of *Lactobacillus* and higher pH. Treating the infection could potentially restore the vaginal microbiota eubiosis, combined with *Lactobacillus* rich probiotics.

**Methods**

The sample was sequenced with NGS (Next Generation Sequencing), using the Ion 16S™ Metagenomics Kit (ThermoFisher). Taxonomy was identified with Kraken program and improved with Bracken.

## 7.2 Appendix 2 - Report simulation: intestinal microbiota test for ovarian cancer therapy management

**Report – Intestinal microbiota test for ovarian cancer therapy management**

**REPORT Unique ID: XXXX**

**Personal information**

Name: N.E. Body

Age: 43

Gender: F

**Request**

Date: _____

Clinician: _____

Hospital/Clinic: _____

**Clinical information**

History of recent antibiotic therapy: No

History of recent probiotic use: No

Cancer pathology: Epithelial ovarian cancer

Ongoing therapy: Cyclophosphamide chemotherapy

Side effects from therapy: Constipation

**Main Result**

We have detected changes in the intestinal microbiota compared with healthy individuals.

**Results**

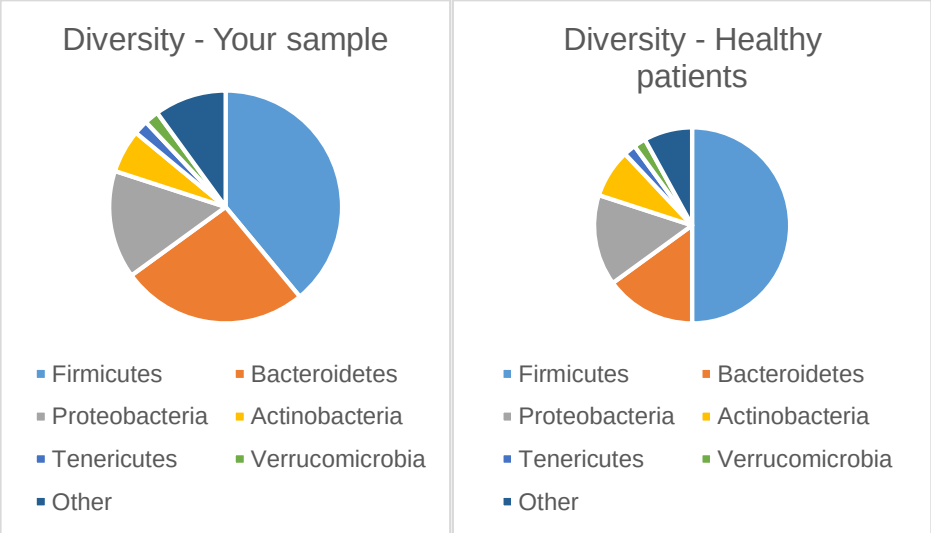
The analysis of your gut microbiota sample has revealed a potential to enhance the cyclophosphamide chemotherapy, based on literature findings. According to literature, overall performance of cyclophosphamide chemotherapy needs improvement.

| **Evaluation of cyclophosphamide chemotherapy efficacy** |
| --- |
| Detected **low** levels of *Enterococcus hirae*  <br><br>Significance: Based on literature, *Enterococcus hirae* has potential to enhance anticancer immune response. Considering the low levels of this microorganism in your sample, probiotic supplements rich in *Enterococcus hirae* or a diet richer in fiber is more likely to favor the cyclophosphamide chemotherapy outcome. |
| Detected **average** levels of *Barnesiella intestinihominis*  <br><br>Significance: Based on literature, *Barnesiella intestinihominis* has potential to enhance anticancer immune response. Considering the average levels of this microorganism in your sample, the cyclophosphamide chemotherapy outcome is more likely to be favorable. |
| **Undetected** levels of *Lactobacillus murinus*  <br><br>Significance: Based on literature, *Lactobacillus murinus* has potential to enhance anticancer immune response. Considering the low levels of this microorganism in your sample, probiotic supplements rich in *Lactobacillus murinus* or a diet richer in dairy products is more likely to favor the cyclophosphamide chemotherapy outcome. |
| Detected **low** levels of *Lactobacillus johnsonii*  <br><br>Significance: Based on literature, *Lactobacillus johnsonii* has potential to enhance anticancer immune response. Considering the low levels of this microorganism in your sample, probiotic supplements rich in *Lactobacillus johnsonii* or a diet richer in dairy products is more likely to favor the cyclophosphamide chemotherapy outcome. |

| **Symptoms associated with side effects from therapy** |
| --- |
| Detected **high** levels of *Methanobrevibacter*  <br><br>Significance: According to literature, *Methanobrevibacter* is a methane producing microorganism associated with bloating and slower transit time, resulting in constipation. |
| Detected **high** levels of *Methanobacterium*  <br><br>Significance: According to literature, *Methanobacterium* is a methane producing microorganism associated with bloating and slower transit time, resulting in constipation. |
| Detected **average** levels of *Bifidobacterium lactis*  <br><br>Significance: According to literature, *Bifidobacterium lactis* is a microorganism associated with a regular transit time, improving stool frequency and consistency, regulating constipation. |
| Detected **low** levels of *Lactobacillus reuteri*  <br><br>Significance: According to literature, *Lactobacillus reuteri* is a microorganism associated with a regular transit time and increased bowel movements, regulating constipation. Considering the low levels of this microorganism in your sample, probiotic supplements rich in *Lactobacillus reuteri* or a diet richer in dairy products may reduce the constipation symptoms. |

**Detailed results**

**Diversity**




Significance: the analysis of the gut microbiota sample revealed a dysbiotic profile, differing from a healthy sample.

**Pathogens**

No pathogens detected

**Immunity and inflammation**

Detected **average** levels of *Faecalibacterium praunsnitzii*

Detected **average** levels of *Ruminococcus*

Detected **low** levels of *Roseburia*

Detected **low** levels of *Blautia*

Detected **low** levels of *Anaerostipes*

Detected **average** levels of *Eubacterium hallii*

**Undetected** levels of *Akkermansia muciniphila*

Significance: the analysis of the gut microbiota sample indicates room for improvement regarding bacteria positively associated with immunity and inflammation. Considering the low levels of the microorganisms *Roseburia, Blautia, Anaerostipes* and *Akkermansia muciniphila,* probiotic supplements or a diet richer in fiber and omega-3 fish oils may enhance the immunity and reduce inflammation in the gut.

**Probiotic bacteria**

Detected **average** levels of *Bifidobacterium spp.*

Detected **low** levels of *Lactobacillus spp.*

Significance: According to literature, probiotic bacteria may have the potential to improve gastrointestinal barrier function by the proliferation of some harmful bacteria. Considering the low levels of Lactobacillus, probiotic supplements or a diet richer in dairy products may enhance probiotic bacteria in the gut microbiota.

**Methods**

The sample was sequenced with NGS (Next Generation Sequencing), using the Ion 16S™ Metagenomics Kit (ThermoFisher). Taxonomy was identified with Kraken program and improved with Bracken.