THERAPEUTIC POTENTIAL OF *LACTIPLANTIBACILLUS PLANTARUM* PROBIO87 IN MODULATING HPV VIRAL LOAD, VAGINAL HEALTH, AND IMMUNE RESPONSE IN HPV-POSITIVE WOMEN

XU PEI

UNIVERSITI SAINS MALAYSIA

2024

THERAPEUTIC POTENTIAL OF *LACTIPLANTIBACILLUS PLANTARUM* PROBIO87 IN MODULATING HPV VIRAL LOAD, VAGINAL HEALTH, AND IMMUNE RESPONSE IN HPV-POSITIVE WOMEN

by

XU PEI

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

December 2024

ACKNOWLEDGEMENT

I thank my primary supervisor, Professor Dr. Liong Min Tze, for her exceptional supervision, advice, and guidance throughout my research journey. It has been an honour to complete my PhD under her mentorship. I am also grateful to my co-supervisors, Associate Professor Dr. Oon Chern Ein, Dr. Engku Husna Binti Engku Ismail, Dr. Mohamad Hafizi Abu Bakar and Associate Professor Dr. Tan Cheng Siang from Universiti Malaysia Sarawak, for their invaluable contributions, support, and insights that helped me navigate challenges during my fieldwork. I want to thank Dr. Salina Binti Sany, Associate Professor Dr. Jun-Jie Tan from IPPT Universiti Sains Malaysia and Dr. Shandra Devi Balasubramaniam from AIMST for their outsourcing (gratis) assistance during my research study. I extend my sincere gratitude to Dr. Sarah Binti Samsudin of Hospital Seberang Jaya, Dr. Muhammad Nashrig Kadir of HUSM, Dr. Nurul Izza Binti Mohamed Rusdi of Hospital Raja Perempuan Zainab II, and Dr. Abigail Rembui Anak Jerip of Universiti Malaysia Sarawak (UNIMAS) for their invaluable expertise, guidance, and assistance in the human clinical study.

I would also like to express my gratitude to my seniors and laboratory members, including Uma Mageswary, Azka Ainun Nisaa, Deepa Rajendran, and Yi-jer Tan, for their guidance, advice, and encouragement, as well as for fostering a supportive and friendly working environment. I am also thankful to Mr. Azmaizan Yaakob and Mdm. Najmah Hamid from the Bioprocess Lab for their technical assistance.

Lastly, my deepest thanks to my beloved family members for their unconditional love and support, which gave me the strength and determination to persevere, even when I felt discouraged and wanted to give up.

Xu Pei Date: 2/10/2024

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF PLATES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

1:3	Dilution 1 in 3
$1x10^9$ CFU/mL	$1x10^9$ colony forming units/mL
5-HT	5-hydroxytryptamine
AIDS	Acquired immunodeficiency syndrome
AIMST	Asian Institute of Medicine, Science and Technology
ANOVA	Analysis of Variance
ANG-2	Angiopoietin-2
AP-1	Activator protein 1
API	Analytical Profile Index
ARF-BP1	ARF-binding protein 1
ATCC	American Type Culture Collection
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3-related
BDNF	Brain-derived neurotrophic factor
BHI	Brain Heart Infusion
BHIs	BHI supplemented with other ingredients
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
BSH	Bile salt hydrolase
BV	Bacterial vaginosis
CD4	Cluster of Differentiation 4
cDNA	Complementary deoxyribonucleic acid
CFS	Cell-free supernatant
CFU	Colony forming unit

CIN	Cervical intraepithelial neoplasia
CREB	cAMP Response Element-Binding protein
CSCC	Cutaneous squamous cell carcinoma
Ct	Cycle threshold
CXCR5	C-X-C chemokine receptor type 5
DBH	Dopamine β-Hydroxylase
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EFSA	European Food Safety Authority
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
ERα	Estrogen receptor α
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FOS	Fructooligosaccharide
GAD	Glutamate decarboxylase
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GIT	Gastrointestinal tract
Glu	Glucose
GM	Gut microbiota
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid

HGF	Hepatocyte Growth Factor
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HUSM	Hospital Universiti Sains Malaysia
ICC	Invasive cervical carcinoma
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IL-10	Interleukin-10
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IST broth	Iso-Sensitest Broth
kb	kilobases
kDa	kiloDaltons
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
LSM	LAB susceptibility test medium
M	Molar (mol/L)
Mdm2	Mouse Double Minute 2
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
MRS	De Man-Rogosa-Sharpe
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen

NCBI	National Center for Biotechnology Information
NFκB	Nuclear factor kappa B
nm	nanometers
NMDS	non-metric multidimensional scaling
℃	Degree Celsius
O & G	Obstetrics and Gynaecology
OD	Optical density
OD 600 nm	Optical density measured at 600 nanometers
OTU	Operational Taxonomic Unit
PCoA	Principal coordinates analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
qPCR	Quantitative polymerase Chain Reaction
RNase	Ribonuclease
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SCC	Squamous cell carcinoma
SCFAs	Short-chain fatty acids
SOD	Superoxide dismutase
STIs	Sexually transmitted infections
TDO	Tryptophan 2,3-Dioxygenase
TGFβ	Transforming Growth Factor Beta
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TSB	Tryptic Soy Broth
μL	Microliter
μM	Micromolar
UV	Ultraviolet

USM	Universiti Sains Malaysia
VAS	Vaginal assessment scale
VC	Vehicle control
VEGF	Vascular Endothelial Growth Factor
VM	Vaginal microbiota
VSQ	Vulvovaginal symptom questionnaire
VuAS	Vulvar assessment scale
VVC	Vulvovaginal candidiasis
VVI	Vulvovaginal infection
WHO	World Health Organization
YEPD	Yeast extract peptone dextrose broth

LIST OF APPENDICES

POTENSI TERAPEUTIK *LACTIPLANTIBACILLUS PLANTARUM* PROBIO87 DALAM MEMODULASI BEBAN VIRUS HPV, KESIHATAN FARAJ, DAN RESPON IMUN PADA WANITA POSITIF HPV

ABSTRAK

Jangkitan HPV berisiko tinggi merupakan punca utama kanser serviks, kanser keempat paling kerap berlaku dalam kalangan wanita. Pilihan rawatan semasa adalah terhad, dan kadar berulang masih tinggi. Pemberian probiotik menawarkan strategi yang menjanjikan, selamat, dan kos efektif untuk menguruskan jangkitan HPV. Sifat probiotik *Lactiplantibacillus plantarum* Probio87 memenuhi keperluan untuk probiotik, termasuk penggunaan karbohidrat, toleransi terhadap asid dan garam hempedu, keupayaan antimikrobial, sifat simbiosis, penggunaan prebiotik, lekatan mukin, hemolisis, pengeluaran hidrogen peroksida, dan profil rintangan antibiotik. Kesan penghambatan supernatan bebas sel (CFS) *L. plantarum* Probio87 telah diuji secara *in vitro* pada garis sel kanser serviks menggunakan ujian MTT (3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazolium bromida), ujian angiogenesis, dan ujian Cignal Finder Reporter & Dual-Glo® luciferase. Keputusan menunjukkan bahawa CFS daripada *L. plantarum* Probio87 secara signifikan ($\rho < 0.05$) menghalang pertumbuhan garis sel kanser serviks yang dimediasi oleh HPV (HeLa dan CaSki) berbanding dengan kumpulan kawalan negatif. Walau bagaimanapun, pada sel C-33A (bukan berkaitan HPV), tidak terdapat perbezaan yang ketara ($\rho > 0.05$). Selain itu, probiotik ini menghalang pembentukan promoter angiogenesis padat dalam ketiga-tiga garis sel kanser tersebut. Ia juga menyokong laluan yang dikaitkan dengan perencatan pertumbuhan sel atau apoptosis dalam sel HeLa dan CaSki. Kajian manusia berikutnya melibatkan tempoh pengambilan selama 12 minggu sama ada probiotik atau plasebo,

selepas itu ekspresi gen dalam darah dianalisis menggunakan Transkripsi Balikan kuantitatif PCR (RT-qPCR) yang menunjukkan peningkatan ekspresi gen pro-radang IL-1beta (p = 0.006) dan IFN-gamma (p = 0.028), gen pengawalan sel T CD44 (p = 0.008), CXCR5 (p = 0.040), dan CD4 (p = 0.016), serta gen pengawalan neural CREB (p = 0.019), IDO (p = 0.095), DBH (p = 0.069), BDNF (p = 0.049), dan TDO (p = 0.077) dalam kumpulan plasebo. Ini mencadangkan pengurangan keradangan, tindak balas imun, dan tekanan neuron dalam kumpulan probiotik. Penemuan ini selaras dengan hasil daripada soal selidik kesihatan faraj, yang menunjukkan bahawa probiotik tersebut mengurangkan gejala fizikal dan isu psikologi yang berkaitan dengan interaksi sosial dan kesan terhadap kehidupan. Selain itu, kumpulan probiotik menunjukkan pengurangan yang signifikan dalam skor Nugent (p < 0.001) dan kehadiran HPV (p = 0.001). Profil mikrobiota faraj mendedahkan bahawa kumpulan plasebo mempunyai kepelbagaian alfa dan beta yang lebih tinggi, mencadangkan bahawa *L. plantarum* Probio87 membantu mengekalkan komuniti mikroba faraj yang stabil. Tambahan pula, analisis mikrobiota najis menunjukkan kepelbagaian alfa dan beta yang lebih tinggi dalam kumpulan probiotik, menunjukkan bahawa probiotik ini menyokong keadaan usus yang sihat. Kajian *in vivo* ini menekankan potensi besar *L. plantarum* Probio87 dalam mengurangkan beban virus HPV, meningkatkan kesihatan faraj, mengurangkan keradangan kronik, dan mengurangkan simptom psikologi dalam kalangan wanita yang positif HPV.

THERAPEUTIC POTENTIAL OF *LACTIPLANTIBACILLUS PLANTARUM* PROBIO87 IN MODULATING HPV VIRAL LOAD, VAGINAL HEALTH, AND IMMUNE RESPONSE IN HPV-POSITIVE WOMEN

ABSTRACT

High-risk HPV infection is the primary cause of cervical cancer, the fourth most common cancer among women. Current treatment options are limited, and recurrence rates remain high. Probiotic administration presents a promising, safe, and cost-effective strategy for managing HPV infections. The probiotic properties of *Lactiplantibacillus plantarum* Probio87 fulfilled the requirements for probiotics, including its carbohydrate utilization, acid tolerance, bile tolerance, antimicrobial ability, symbiosis properties, prebiotic utilization, mucin adhesion, haemolysis, hydrogen peroxide production, and antibiotic resistance profile. The inhibitory effects of *L. plantarum* Probio87's cell-free supernatant (CFS) were tested *in vitro* on cervical cancer cell lines using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test, the angiogenesis assay, and the cignal finder reporter & Dual-Glo® luciferase assay. The results demonstrated that the CFS of *L. plantarum* Probio87 significantly ($\rho < 0.05$) inhibited the growth of HPV-mediated cervical cancer cell lines (HeLa and CaSki) compared to the negative control group. However, in C-33A (non-HPV related) cells, there was no obvious difference ($\rho > 0.05$). Furthermore, this probiotic inhibited the generation of solid angiogenesis promoters in all three cancer cell lines. It supported pathways linked to cell growth arrest or apoptosis in HeLa and CaSki cells. Subsequent human studies involved a 12-week consumption period of either the probiotic or placebo, after which Reverse Transcription quantitative PCR (RT-qPCR) analyzed blood gene expression indicated higher upregulation of pro-

inflammatory genes IL-1beta (p = 0.006) and IFN-gamma (p = 0.028), T-cell regulation genes CD44 (p = 0.008), CXCR5 (p = 0.040), and CD4 (p = 0.016), and neural regulation genes CREB (p = 0.019), IDO (p = 0.095), DBH (p = 0.069), BDNF (p = 0.049), and TDO (p = 0.077) in the placebo group. This suggested reduced inflammation, immune responses, and neuronal stress in the probiotic group. These findings were consistent with results from vaginal health questionnaires, indicating that the probiotic alleviated physical symptoms and psychological issues related to social interactions and life impact. Moreover, the probiotic group showed a significant reduction in the Nugent score (p < 0.001) and HPV abundance (p = 0.001). Vaginal microbiota profile revealed that the placebo group had higher alpha and beta diversity, suggesting that *L. plantarum* Probio87 contributed to maintaining a stable vaginal microbial community. These clinical trials underscored the considerable potential of *L. plantarum* Probio87 in reducing HPV viral load, enhancing vaginal health, mitigating chronic inflammation, and alleviating psychological symptoms in HPV-positive women.

CHAPTER 1

INTRODUCTION

1.1 Background

Human papillomavirus (HPV) is a double-string DNA virus. Although most infections with HPV resolve spontaneously and cause no symptoms, persistent infection causes 95% of cervical cancers. This cancer is the fourth most common cancer in women. In 2022, an estimated 660,000 women were diagnosed with cervical cancer worldwide, and about 350,000 women died from the disease (World Health Organization, 2024). The rate of new cases is increasing rapidly. In Malaysia, every year, 1740 women are diagnosed with cervical cancer, and 991 die from this disease (HPV Information Centre, 2023).

Persistent infection with high-risk HPV (hr-HPV) of mucosal HPV is the primary factor for the development of cervical cancer and its precursor lesions (Nunes et al., 2018). However, only 10-15% of high-risk HPV infections cannot recover spontaneously and consequently promote the progression of precancerous cervical intraepithelial neoplasia (CIN) to invasive cervical carcinoma (ICC) (Shulzhenko et al., 2014). That means there are other cofactors required for the development of cervical cancer (Łaniewski et al., 2020). More and more research show that the local microenvironment in the vagina is the cofactor of cervical carcinogenesis, which is essential for the control of persistent HPV infection and disease pathogenesis (Egawa et al., 2015).

Probiotics are live microorganisms that have positive health effects when consumed in sufficient doses (FAO/WHO, 2006). *Lactobacillus* and *Bifidobacterium* are two dominant microorganisms used in the probiotic industry over a century ago, even nowadays (Metchnikoff, 2016; Vivek et al., 2023). Administration of the probiotic dairy-based food significantly increased the number of vaginal *Lactobacilli* (Bolton et al., 2008; Nishijima et al., 2005). Several studies have shown that various *Lactobacillus* probiotic strains can restore vaginal microecology by lowering the amount of pathogenic bacteria, preserving the acidic microenvironment, and suppressing the immune system (Han & Ren, 2021). Research shows that a healthy microbiome may help reduce the risk of HPV-related carcinogenesis and speed up the removal of HPV infection (Serrano-Villar et al., 2017).

1.2 Problem Statement

Cervical neoplasms are treated chiefly with non-specific interferon and surgery. However, recurrence is relatively common (Yang et al., 2019). The primary treatment for early-stage, low-risk malignant tissue is surgery, which is essential for long-term survival (Fader, 2018). Advancements in radiotherapy technology, such as intensity-modulated radiotherapy, have decreased the acute and late toxicity associated with locally progressed diseases (Vavassori et al., 2019). For patients with recurring or metastatic illness, the prognosis is still not good generally (Koh et al., 2018). Additionally, the significant cytotoxicity of chemotherapy causes side effects most of the time and makes it impossible to distinguish between cancerous and healthy tissue

(Lorusso et al., 2014; Kitagawa et al., 2015). Consuming a probiotic milk product with 8×10^9 CFU *L. casei Shirota* per 2.7-ounce bottle was linked to increased HPV viral clearance, according to a controlled trial (Verhoeven et al., 2013a). To determine whether probiotic administration has the potential to serve as an efficient, safe, and cost-effective treatment for HPV infection, further research is needed. Current studies on this topic remain limited, and the methodologies employed in existing research often lack sufficient rigor.

1.3 Aims and Objectives

The study aimed to assess the efficacy of oral administration of *Lactiplantibacillus plantarum* Probio87 at 9 log CFU/day for 12 weeks in modulating HPV viral load, vaginal health, and immune response in HPV-positive women in Malaysia, compared to a placebo.

1.4 Specific Objectives

1. To evaluate the probiotic properties of *L. plantarum* Probio87.
2. To evaluate the inhibitory properties of this probiotic against HPV-related cervical cancer cell lines.
3. To evaluate the efficacy of this probiotic compared to a placebo after consuming 12 weeks, focusing on its impact on reducing HPV viral load, attenuating inflammation and mental stress, and modulating the vaginal microbiota in HPV-infected women.

CHAPTER 2

LITERATURE REVIEW

2.1 Human Papillomavirus

The human papillomavirus, or HPV, is a sexually transmitted virus that can lead to genital warts and many malignancies, including cervical cancer. There are currently more than 200 varieties of this sort of non-enveloped double-stranded circular DNA, which has a length of about 8 kb. (HPV and Cance, 2019) The early, late, and long control regions are represented by the three zones of the viral DNA: the E, L, and LCR regions. It is within each location's power to manage the virus's survival and dissemination (Warowicka et al., 2022). The viral proteins in Table 2.1 are essential and intimately connected to the dissemination and growth of HPV (Balasubramaniam et al., 2019).

Table 2.1 Viral proteins of HPV and their primary functions.

Viral Proteins	Functions
E1	Viral DNA replication and transcription
E2	Viral DNA replication, apoptosis, transcription repressor of E6/E7
E4	Viral DNA replication
E5	Immune recognition (major histocompatibility complex, MHC)
E6	p53 degradation, alteration of cell cycle regulation, apoptosis resistance
E7	Retinoblastoma protein (pRb) degradation, re-entry into S phase cell cycle, p16 overexpression
L1	Major viral capsid protein
L2	Minor viral capsid protein

The risk associated with virus kinds varies depending on the categorisation. Some low-risk HPV strains only cause warts that eventually vanish (Zheng et al., 2022). In contrast, high-risk strains can cause tumors, including oropharyngeal,

cervical, and penile malignancies (Łaniewski et al., 2019). Research indicates that HPV is the primary cause of over 95% of cases of invasive cervical carcinoma (ICC) (Mattoscio et al., 2018; Nicolò et al., 2021). ICC is the fourth most common type of cancer in women, with over 500,000 new cases annually (Global Burden of Disease Cancer Collaboration, 2019), approximately 90% of which occur in low- and middle-income countries (*Donors Making a Difference*, 2022). In addition to ICC, HPV is known to cause various related diseases, accounting for 5% of all cancer cases globally (Kaczmarek et al., 2022). The prevention and treatment of HPV can improve people's health and save many lives, especially for women in low-income nations.

2.2 HPV Infection Process

HPV life cycle and keratinocyte epithelial development are closely related. The latter process includes binding host transcription factors to LCR in the viral genome, chromatin remodelling, histone changes, and DNA methylation (Gutiérrez-Hoya & Soto-Cruz, 2020; Morgan et al., 2020). The productive phase of the viral life cycle is activated upon epithelial differentiation, leading to viral genome amplification, high levels of late gene expression, and the assembly of virions that are shed from the epithelial surface. HPV establishes infection in the proliferating basal keratinocytes of the stratified epithelium. (Moody, 2022)

HPV infection can lead to lesions or cancer (Figure 2.1), known as precancerous cervical intraepithelial neoplasia (CIN) and invasive cervical carcinoma (ICC), often requiring persistent infection over several years to develop (Łaniewski et

al., 2019). The integration of the HPV genome into the host chromosomes, which can result from persistent infection with high-risk HPV, can lead to the unregulated expression of the E6 and E7 viral oncoproteins and the development of cervical cancer. (Miao et al., 2020; Xiong et al., 2021; Meng et al., 2022). More than 80% of cervical malignancies have HPV integration. Throughout the early stages of the growth of the cervical lesion, there is an increase in both the quantity and frequency of HPV integrations (Balasubramaniam et al., 2019; Meng et al., 2022).

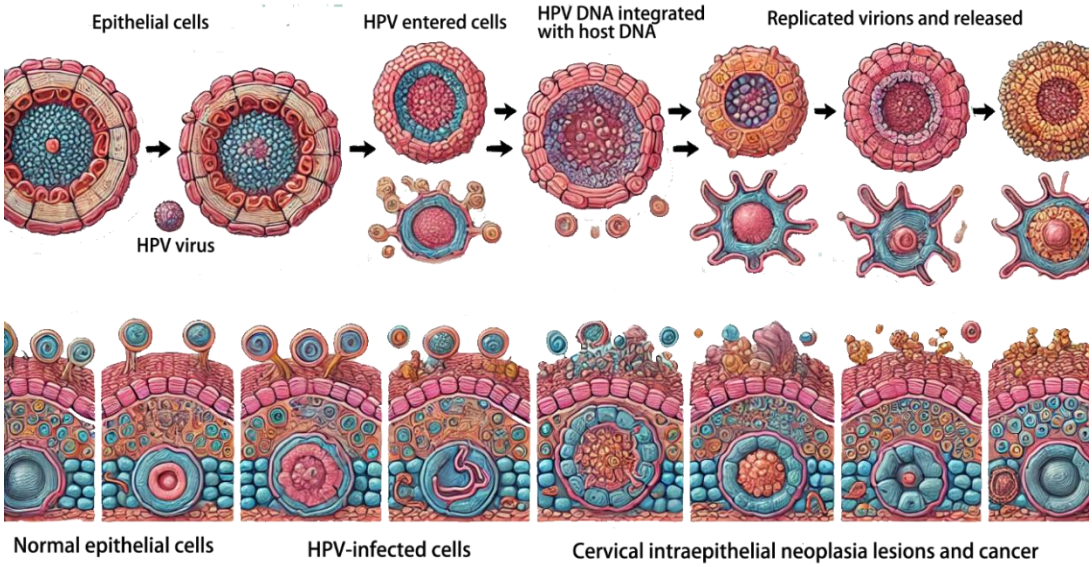

Figure 2.1 Distribution of standard and HPV-infected squamous epithelial cells in cervical tissue, progressing from ordinary through precancerous lesions (mild, moderate, and severe dysplasia; CIN 1, CIN 2, and CIN 3, respectively) to cervical cancer. HPV enters basal epithelial cells through micro-wounds in the epithelial layer. Once inside, the virus integrates its genome into the host genome within the nucleus, facilitated by breaks in the nuclear envelope. After integration, HPV takes control of the host genome, promoting its replication and spreading throughout the epithelium. This leads to irregular and disorganised growth of host cells compared to normal cells. Eventually, the host epithelium's virions and dead squamous cells are shed, facilitating further transmission.

Persistent infection with HPV can occur due to various cofactors, such as smoking, age of sexual debut, high parity, long-term use of contraceptives, hormone

treatment and co-infections with sexually transmitted pathogens, which are associated with the progression of cervical neoplasia among HPV-infected women (Castle et al., 2001; Lehtinen et al., 2011; Mhatre et al., 2012). An effective immune system is essential for eradicating HPV infections. Nonetheless, immune system abnormalities, immunosuppressive circumstances and medicines can reduce the immune response and increase the likelihood of HPV persistence. (Lechien et al., 2019)

2.3 Changes after HPV Infection

2.3.1 Immunomodulation

Malignancies linked to HPV infection arise from the modulation of the immunological milieu by HPV infection, which results in a pro-tumorigenic state characterized by immune evasion and suppression (Shamseddine et al., 2021). HPV uses several immune-evading strategies and modifies the antigen-processing machinery to alter the detectability of infected cells, expediting the process from infection to cancer by causing chronic dysplasia (Franciosi et al., 2020; Vanajothi et al., 2022). Both innate and adaptive immunity respond to HPV; CD8+ T lymphocytes, dendritic cells, and macrophages can all be seen in the tumor microenvironment. These interactions result in a microenvironment that is pro-inflammatory and pro-tumorigenic. (Subbarayan et al., 2019) HPV focuses on the pathways involved in innate immunity and DNA damage repair to facilitate chronic infection (Gusho & Laimins, 2021). HPV infection is common, and most people clear it within two years.

However, a malfunctioning immune response causes the infection to persist in roughly 10% of women, raising the risk of cervical cancer (Koshiol & Butsch, 2012).

2.3.2 Inflammation

As inflammation is known to cause reactive oxygen and nitrogen species to damage DNA, it may play a role in the development of cancer (Lee & Park, 2015; Ahechu et al., 2018). The development of several cancers has been associated with chronic inflammation, which fosters all phases of carcinogenesis by generating an inflammatory milieu around the tumor (Lee & Park, 2015; Greten & Grivennikov, 2019). Research has investigated the role of inflammasome genetics in susceptibility to HPV infection and cervical cancer development (Pontillo et al., 2016), as well as the expression of miR-34a and miR-125b in HPV infection and cervical cancer development (Ribeiro et al., 2015). Pro-inflammatory cytokine levels are elevated in persistent HPV infection, which encourages cell proliferation and inhibits apoptosis to accelerate the growth of tumors (Hibma, 2012).

Research has determined that several biomarkers serve as predictors of the risk of cervical cancer. Pro-inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), are among them. Additionally, genes like COX-2 and p16INK4a are linked to aberrant cervical cells that have the potential to develop into cancer. IL-6 is crucial in tumor progression, promoting cell proliferation and inhibiting apoptosis (Caetano et al., 2016). During cervical carcinogenesis, IL-6 shows a strong positive correlation with VEGF and HG (Yu et al., 2015; Łaniewski et al., 2019). IL-6 was produced by the tumor vasculature,

suggesting a paracrine role for IL-6 in prostate cancer (Hao et al., 2018). By controlling the NFκB/miR-29b pathway, IL-6 production in tumor-associated macrophages contributed to tumor IL6/STAT3 signaling and the advancement of breast cancer (Vlaykova et al., 2020). Additionally, a study on oesophageal cancer revealed that cancer-associated fibroblasts in the tumor microenvironment affect intratumoral CD8+ and FoxP3+ T cells via IL-6, leading to tumor immunosuppression (Kato et al., 2018).

2.3.3 Hormone Levels

The cervix is hormone-responsive, and female hormones have been implicated in the pathogenesis of cervical cancer. Studies have explored the relationship between estrogen and cervical cancer, including the role of estrogen receptors in cervical cancer pathogenesis (Yu et al., 2018), the effects of 60 kDa prolactin and estradiol on metabolism and cell survival in cervical cancer (Riera-Leal et al., 2018), and the induction of aromatase expression in cervical carcinomas (Nair et al., 2005).

A study found that estrogen attenuates the growth of HPV-positive epithelial cells, supporting the idea that estrogen has the potential as a therapeutic agent for the treatment of HPV-positive cancers (Bristol et al., 2020). However, in other cases, HPV infection can lead to an increase in estrogen production, promoting the growth of cervical cells and potentially leading to the development of precancerous or cancerous lesions (Chung et al., 2010). HPV can alter the expression of estrogen receptors in cervical cells, affecting their response to estrogen (Girón et al., 2009). The estrogen receptor α (ERα) classical pathway is required for estrogen-induced epithelial cell proliferation and carcinogenesis in the cervix of HPV-transgenic mice (Son et al.,

2014). A higher prevalence of HPV DNA in women with higher levels of progesterone in blood serum (Kedzia et al., 2000). These studies underscored the complex interplay between HPV infection and estrogen signaling, highlighting the necessity for further investigation to elucidate their role in cervical cancer progression and to explore potential therapeutic strategies.

2.3.4 Gene Expression

After infection, the viral proteins can influence some genes that help the virus avoid clearance by the immune system (Balasubramaniam et al., 2019). The E5, E6, and E7 proteins are essential for HPV survival and lesion formation, and they can alter the pathways that lead to cell proliferation and, ultimately, cell immortality (Figure 2.2). These oncogenes mainly inflict damage by ubiquitinating the p53 and pRb genes and inhibiting p21, which is essential for apoptosis and functions as a tumor suppressor.(Venuti et al., 2011; Pal & Kundu, 2020)

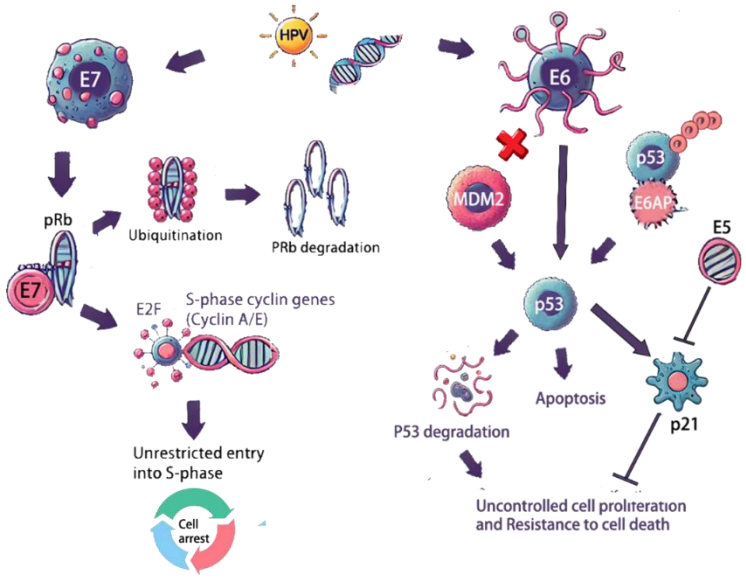

Figure 2.2 The mechanisms of HPV oncoproteins in disrupting cell cycle regulation. E6 ubiquitinates p53, leading to its degradation and resulting in the loss of

p53's ability to induce apoptosis, allowing infected cells to proliferate uncontrollably and resist cell death. E7 binds to pRb, releasing E2F and permitting unrestricted entry into the S-phase. E5 inhibits the function of p21, which usually suppresses cell proliferation.

2.4 Important Cancer Pathways

Numerous critical pathways and biomarkers play significant roles in the progression of cancers and persistent HPV infections. Targeting these pathways could be crucial for developing effective treatments and facilitating HPV clearance.

2.4.1 p53 Signaling Pathway

When significant DNA damage was incurred, p53-induced cell cycle arrest was initiated, allowing for DNA repair and death to halt the proliferation of those cells (Ozaki & Nakagawara, 2011). The p53 pathway is essential for regulating p21 expression. Phospholipid stressors or DNA damage activate p53, which initiates p21 transcription. This activation halts the cell cycle, permitting DNA repair mechanisms to function. (Fischer et al., 2016; Engeland, 2022) This p53-p21 signaling axis is a crucial tumor suppressor mechanism that stops the growth of cells with genetic instability or mutations that could lead to cancer (Lei et al., 2020).

2.4.2 TGFβ Signaling Pathway

In normal epithelial cells, Transforming Growth Factor Beta (TGFβ) triggers a strong antiproliferative response, leading to differentiation and cell death. This highlights TGFβ's role as a tumor suppressor, especially in the early stages of carcinogenesis (Seoane & Gomis, 2017). Nevertheless, it acts as a tumor promoter in

advanced cancer, encouraging the growth and metastasis of the tumor. TGFβ's pleiotropic properties were the cause of its dual effect on cancer (Gu & Feng, 2018).

The presence of p53 was required for TGFβ to fully activate the transcription of the cyclin-dependent kinases inhibitor $p21^{waf1}$ in mammalian cells. The cytostatic response to TGFβ signals was diminished in cells lacking p53. (Cordenonsi et al., 2003) TGFβ and wild type p53 are key tumor suppressors that regulate various biological functions. TGFβ signals partly through the Smad pathway. Direct interaction between wild-type p53 and Smads significantly enhances the transcription of tumor-suppressive genes (Adorno et al., 2009; Elston & Inman, 2012).

TGFβ also inhibited telomerase, which was possibly a factor in the senescence of cancer cells and the shortening of telomeres. Senescence was brought about through TGFβ signaling, which also enhanced β3 integrin expression and the cell cycle inhibitors p16 and p21. (Mikuła-Pietrasik et al., 2022) The stimulation of TGFβ signaling may cause the production of p21, which is required to regulate the cell cycle. This pathway is crucial for many biological processes, especially cell cycle regulation, aligning with TGFβ's ability to inhibit tumor growth in precancerous stages. (Bauer et al., 2015; Jana et al., 2015)

2.4.3 NFκB Signaling Pathway

The role of NFκB (Nuclear Factor-kappa B) in cell apoptosis and cancer progression was controversial (Huang et al., 2015). The activation of NFκB typically suppresses p53 function and vice versa, indicating an antagonistic relationship (Dey et al., 2008). However, their mutual regulation could lead to varied outcomes depending

on the context, as NFκB also played a crucial role in activating p53. Research has shown that p53 regulation required an intact NFκB site in its promoter, and the activation of NFκB by TNF-α or transient expression of the p65 subunit led to the activation of p53 expression (Wu & Lozano, 1994). HDM2 activity was decreased, and NFκB elevated p53 phosphorylation at Ser-20. The upregulation of p53 led to the upregulation of pro-apoptotic genes, including $p21^{waf1}$ and PUMA (Fujioka et al., 2004). Moreover, NFκB activation induces the production of a wide range of pro-inflammatory cytokines, chemokines, and antiviral mediators (Balasubramaniam et al., 2019), which play pivotal roles in orchestrating immune and inflammatory responses. Additionally, under specific circumstances, p53 and NFκB cooperated in regulating apoptosis. The activation of p53 in osteosarcoma Saos-2 cells increased NFκB DNA-binding activity, demonstrating a direct connection between p53-induced apoptosis and NFκB activation (Carrà et al., 2020). Furthermore, after arsenic treatment, NFκB has been shown to trigger apoptosis in both p53-deficient and p53-proficient cells, especially in response to cellular stress (Barkett & Gilmore, 1999; Yin & Yu, 2018).

2.4.4 Myc/Max Signaling Pathway

Studies showed that low c-Myc levels boosted cell proliferation, while high levels inhibited it by activating the ARF/p53 response (Chen et al., 2013). The ARF protein, also referred to as $p14^{ARF}$ in humans, accumulates because of c-Myc overexpression, which inhibits its degradation mediated by Ubiquitin Ligase for ARF (ULF). Under normal conditions, ULF-mediated ubiquitination reduces ARF levels during DNA

damage. However, elevated c-Myc levels block ARF degradation, leading to a significant enhancement of apoptotic responses (Figure 2.3).

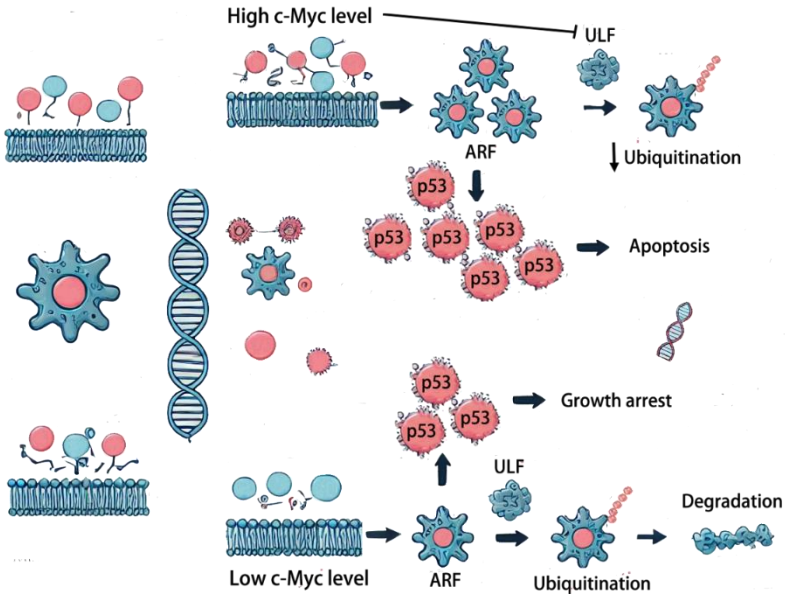

Figure 2.3 Different levels of c-Myc influence p53 expression, resulting in either growth arrest or apoptosis by modulating ARF via ULF-mediated ubiquitination. Under low c-Myc level (normal expression), DNA damage activates p53 with enhanced ARF degradation. In contrast, high c-Myc levels (oncogenic expression) stabilize ARF and further enhance p53 activation.

ARF primarily activates the p53 pathway by inhibiting the E3 ligase activity of Mouse Double Minute 2 (MDM2) or ARF-binding protein 1 (ARF-BP1), preventing p53 degradation (Chen et al., 2013). Additionally, ARF can inhibit cell growth independently by interacting with c-Myc without involving MDM2 or p53 (Datta et al., 2004).

2.4.5 MAPK/ERK Signaling Pathway

ERK1/2, traditionally known for its anti-apoptotic role, was increasingly recognised for its pro-apoptotic functions, especially in tumor suppression and chemotherapy responses (Lu et al., 2020). Enhanced ERK1/2 signaling could induce

cell death in tumor cells under specific conditions (Figure 2.4), highlighting its potential for promoting apoptosis (Sugiura et al., 2021).

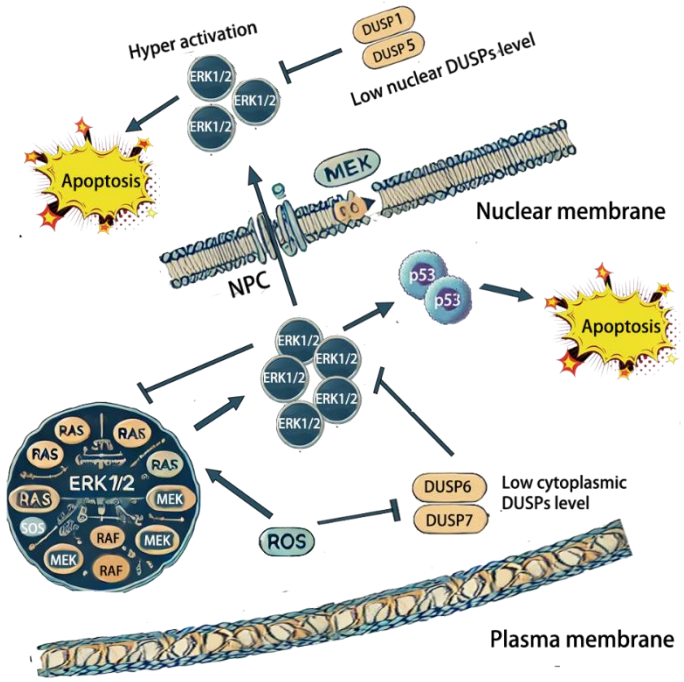

Figure 2.4 Activation of ERK1/2 upstream factors or down-regulation of Dual-specificity phosphatase 6/7 (DUSP6/7) by various stimuli, such as ROS, triggers ERK1/2 activation in the cytoplasm. This activated cytoplasmic ERK1/2 either induce mitochondrial apoptosis or is translocated to the nucleus via the nuclear pore complex (NPC). Similarly, decreased levels of DUSP1/5 due to stimuli like ROS also activate ERK1/2 in the nucleus. Consequently, reduced levels of cytoplasmic or nuclear DUSPs result in the accumulation of active ERK1/2 in the nucleus. Sustained activation of ERK1/2 above a certain threshold activates specific pro-apoptotic targets, leading to apoptosis.

Recent studies underscore the role of dual-specificity phosphatases (DUSPs) in modulating ERK's pro- or anti-apoptotic functions in cancer (Sugiura et al., 2021). Zou et al., (2019) proposed that p53 could regulate the transcription of all nuclear DUSPs (DUSP1/2/4/5). MEK1/ERK signaling drove cell proliferation, whereas MEK2/ERK signaling promoted G1/S cell cycle arrest (Ussar & Voss, 2004). The

Ras/Raf/MEK/ERK pathway induced cellular senescence in various tissues and cell types, relying on p16/INK4A, p21, and p53 integrity. In human primary fibroblasts, inhibiting p16 or p53 alone could not reverse ERK1/2-induced senescence (Ms et al., 2005; Zhuang et al., 2008). ERK1/2 shows diverse phosphorylation targets, independent of cellular location. In the nucleus, it activates transcription factors like CREB (cAMP response element-binding protein), c-Myc (transcriptional regulator), and NFκB, making it a key anti-tumor target (Braicu et al., 2019). ERK signaling enhances p53-mediated apoptosis by stabilizing and activating p53. It phosphorylates p53 at Ser15, preventing its degradation via Mdm2, and at Thr55, boosting p53 activity in apoptosis, such as in doxorubicin-treated cancer cells. Experimental inhibition of p53 further confirms ERK's apoptotic effects depending on p53 function. These findings highlight the interconnected roles of ERK and p53 in cancer cell apoptosis (Sugiura et al., 2021).

2.4.6 Notch Signaling Pathway

The activation of Notch signaling had dual roles, acting either as a tumor suppressor or as an oncogene, depending on the specific cellular context (Ntziachristos et al., 2014). The Notch signaling pathway plays a key role in cancer metabolic reprogramming and shaping the tumor microenvironment, balancing oncogenic and tumor-suppressive effects. Growing evidence indicates that Notch signaling acts as a tumor suppressor in cancers such as squamous cell carcinoma (SCC), hematological malignancies, cervical cancer, and forebrain glioma. Its role as oncogenic or tumor-suppressive appears highly context-dependent (Shi et al., 2024). This suggested a

possible synergy between NOTCH1 and p21 in exerting their tumor-suppressive roles, which was consistent with prior findings in ras-transformed p21 knockout keratinocytes (Missero et al., 1996). Moreover, activation of NOTCH1 in human keratinocytes led to a moderate increase in p21 levels (Nguyen et al., 2006). Notch1 regulates keratinocyte differentiation and acts as a tumor suppressor in the mammalian epidermis (Yugawa et al., 2007). In contrast, other research found that Notch2 expression in colorectal cancer (CRC) inversely correlated with Notch1, with reduced Notch2 predicting poor prognosis. Nuclear Notch3 expression was strongly associated with distant relapse-free survival in stage II CRC. Notch4 expression was decreased in CRC, and its mRNA level served as an independent prognostic marker for disease-free and overall survival. (Q. Shi et al., 2024)

Notch signaling in keratinocytes was suggested to be positively regulated by p53. It had been determined that the NOTCH1 promoter had a p53-responsive element (Figure 2.5). Hence, in both human primary keratinocytes and CSCC cell lines, higher p53 levels caused an increase in NOTCH1 expression. (Cl et al., 2006; K et al., 2007; Yugawa et al., 2007). Notch signaling can regulate p53 activity context-dependently, leading to the upregulation or downregulation of p53 function, which is closely linked to either promoting or inhibiting carcinogenesis. Higher levels of the HPV E6 oncoprotein and lower levels of NOTCH1 have been associated with the progression of cervical cancer. When NOTCH1 is activated in cervical cancer cells, HPV E6 levels—which are controlled by activator protein 1 (AP-1)—drop, leading to an

increase in p53 protein levels and activity. As a result, p53 and NOTCH1 created a positive feedback loop. (Dotto, 2009).

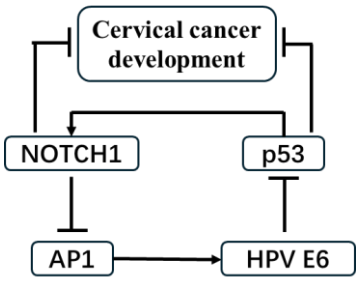

Figure 2.5 A positive feedback loop between p53 and NOTCH1 counters cervical cancer development. Advanced cervical cancer stages involve increased HPV E6 oncoprotein levels and decreased NOTCH1. The activation of NOTCH1 boosts p53 levels and activity in cervical cancer cells by reducing HPV E6 by suppressing AP-1 dependent transcription. Conversely, increased p53 induces NOTCH1, forming a direct positive relationship.

2.4.7 Potential Connections

Based on the correlations mentioned, the probable linkages between these routes are illustrated in Figure 2.6. Important biomarkers such as p53, p21, and ARF regulate biological pathways that stop cancer from spreading by inducing cell cycle arrest or apoptosis. While p53 functions as the main hub that unifies multiple routes, p21 is linked to specific pathways. Moreover, c-Myc-induced ARF is a significant biomarker that functions both independently and in concert with p53.

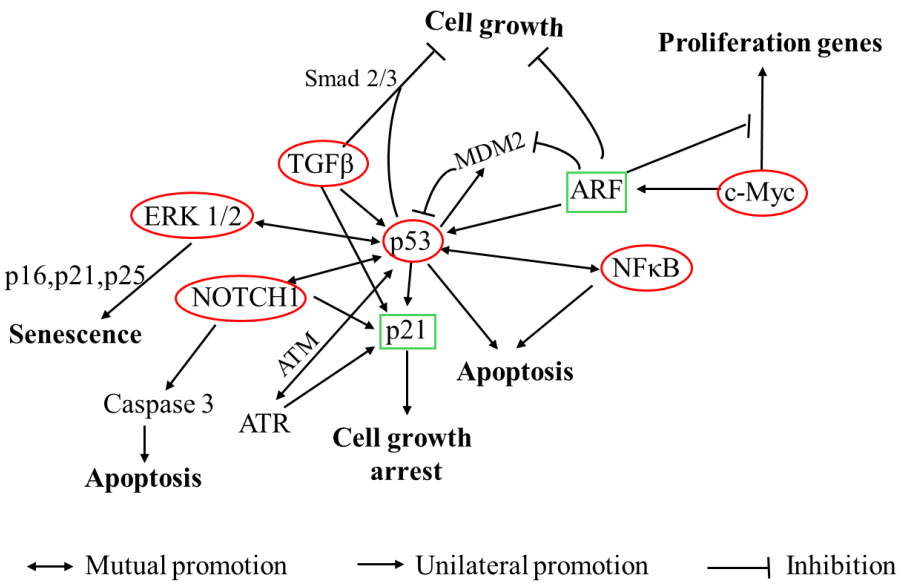

Figure 2.6 The potential interplay of these crucial biomarkers, with p53 assuming a central role. It interacts with the ERK, Notch, and NFκB pathways in a bidirectional manner, where p53 can activate these pathways, and in turn, they can enhance or modulate p53 activity. These pathways can trigger apoptosis or senescence, dependent on or independent of p53. Additionally, c-Myc may induce p53 expression by generating ARF, a robust promoter of apoptosis. TGFβ activation initiates the activation of p53 and p21, which results in cell cycle arrest or apoptosis. Furthermore, TGFβ works synergistically with p53 and Smad 2/3 to inhibit cell growth.

2.5 The Gut-Organ Axis

The gut microbiota (GM) is a collection of microorganisms that live in the human digestive system (Thursby & Juge, 2017). It plays a crucial role in several immune, metabolic, and nutrient absorption integral to the host's survival. The interaction between GM and the host immune system ensures tolerance of commensal bacteria and antigens ingested with food while maintaining the ability to identify and attack potential pathogens and prevent invasion and infection (Yoo et al., 2020; Riazi-Rad et al., 2021). This mechanism can strongly influence human health, like reversing lung inflammation, increasing liver function, strengthening the ageing skeleton, lessening muscle decline and helping prevent diabetes and obesity, etc. (Wang et al., 2021)

The interplay between the GM and the host significantly impacts bodily functions, leading to a close relationship with other organs (Figure 2.7) and ultimately giving rise to what can be termed an "axis" connecting them (Nicholson et al., 2012). The GM is known to connect various organs, including the brain, kidney, liver, bone, skin (Ahlawat et al., 2021), heart (Hardy-Rando & Fernandez-Patron, 2019), and lungs (Enaud et al., 2020), through neural, endocrine, immune, humoral, and metabolic pathways. The endocrine effects of bacteria influence various host responses, including behaviour, metabolism, appetite, and immune responses (Neuman et al., 2015; Parida & Sharma, 2020).

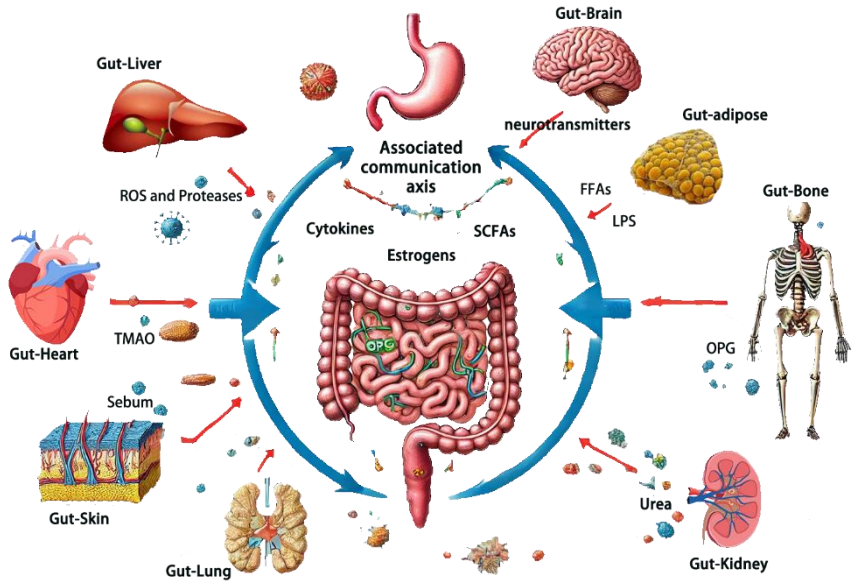

Figure 2.7 Representation of a bi- or multidirectional communication axis between the gut, its associated microbiota, and various organs. TMAO, Trimethylamine N-oxide; LPS, Lipopolysaccharide; FFAs, Free Fatty Acids; ROS, Reactive oxygen species; OPG, Osteoprotegerin.

2.6 The Gut-Vagina Axis

Changes in the GM can influence the VM and vice versa (Yoshikata et al., 2022). This interaction, known as the gut-vagina axis (Figure 2.8), was first described by

Ravel and Brotman in 2016 (Graham et al., 2021). The GM affects vaginal health by serving as a reservoir for pathogens, influencing systemic inflammation, and altering hormone levels such as estrogen (Graham et al., 2021; Thomas-White, 2022). Non-*Lactobacillus* dominant communities and pathogenic bacteria can increase vaginal pH and decrease lactic acid levels, leading to an imbalance that disrupts vaginal health. This can result in persistent infections and an immune response that increases pro-inflammatory chemokines and cytokines (e.g., IL-6, IL-8, IL-1α, IL-1β, TNF-α). Maintaining a healthy VM is essential to prevent pathogenic overgrowth and maintain balance. Studies show that probiotics like *L. crispatus*, *L. delbrueckii*, *L. plantarum* 59, and *L. fermentum* 137 can help prevent or treat vaginal infections by inhibiting pathogen growth, reducing inflammation, and restoring vaginal microbiome balance (Santos et al., 2018; Li et al., 2019; Bakus et al., 2023). Oral intake of probiotics can also affect the vaginal microbiome, as certain probiotic strains can travel from the gut to the vagina via the gut-vagina axis (Amabebe & Anumba, 2020). This approach has been shown to reduce the recurrence of bacterial vaginosis (BV) significantly (Kumherová et al., 2021; Webb, 2021). Therefore, preserving gut health can enhance vaginal health by reducing inflammation and promoting beneficial bacteria (Chandra et al., 2021; Prados, 2023).

The VM is shaped by hormonal changes in women throughout their lives, from pre-puberty to post-menopause (Lehtoranta et al., 2022). The GM can indirectly affect the VM by affecting oestrogen levels. Enzymes produced by specific gut bacteria alter estrogen, causing hormonal abnormalities that affect the composition of the vaginal

mucosa and its epithelium. (Al Othaim et al., 2021; Yang et al., 2022). Estrogen-stimulated vaginal glycogen encourages *Lactobacillus* dominance in the VM (Gliniewicz et al., 2019). Age-related drops in oestrogen lead to a decline in *Lactobacillus* populations, increasing the amount of detrimental bacteria and the microbiome's diversity (Yoshikata et al., 2022). Hormone balance may be impacted by changes in GM composition, which may result in immunological dysfunction and chronic inflammation, further upsetting the balance of hormones (Zhao et al., 2023).

Additionally, GM can affect the VM by producing short-chain fatty acids (SCFAs) like butyrate, propionate, and acetate, which have anti-inflammatory and immunomodulatory effects (Ratanapokasatit et al., 2022). SCFAs are critical energy sources for the host and are produced by GM. The metabolic activity of the GM is linked to the host body's energy homoeostasis through the SCFA receptor GPR43 (Kimura et al., 2013). Moreover, SCFAs enhance the expression of the anti-inflammatory cytokine IL-10 and Foxp3+ CD4+ T cells, which help suppress immune responses. This leads to a reduction in LPS-induced NFκB activation, thereby decreasing NFκB-mediated expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-12p40, IFN-γ, and chemokines like CXCL9-CXCL11. (Parada Venegas et al., 2019).

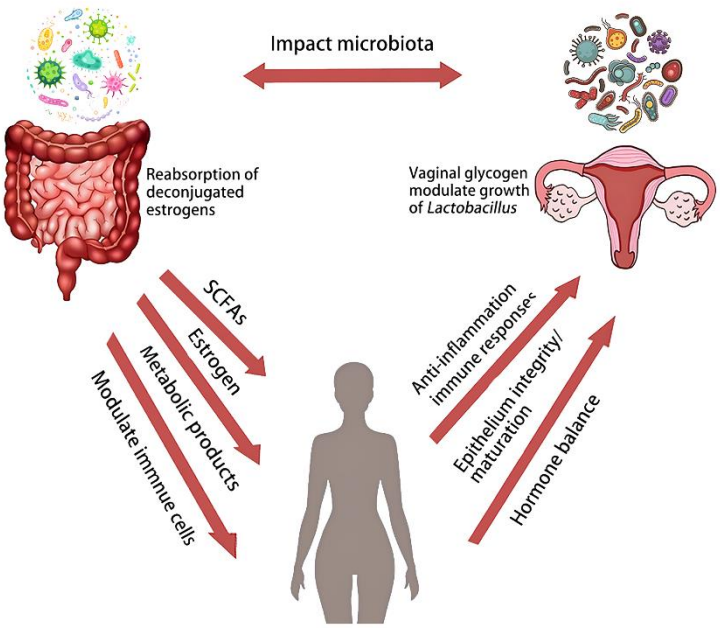

Figure 2.8 The gut-vaginal axis is influenced by immunological cell regulation, estrogen levels, metabolic products and short-chain fatty acids (SCFAs) in the whole body. Through modulating estrogen levels, the integrity and maturation of the vaginal epithelium are influenced, along with the regulation of VM dominated by *Lactobacillus* species and vaginal glycogen synthesis. Reduced production of inflammatory cytokines results in a decreased immune response in the vagina. This equilibrium can be upset by poor gut health, which increases the risk of infections and vaginal dysbiosis. This dysfunction in the vagina also contributes to further deterioration of gut health.

2.7 *Lactiplantibacillus Plantarum* as Probiotics

Probiotics are live microbial feed supplements that can provide beneficial properties to the host when administered adequately (FAO/WHO, 2006). Lactic acid bacteria (LAB) are a group of bacteria that produce lactic acid as a byproduct of carbohydrate fermentation (Wang et al., 2021). They are essential to several food fermentation processes (Soemarie et al., 2021), and many strains are commonly used

as probiotics, including species of *Lactobacillus* and *Bifidobacterium* (Plaza-Diaz et al., 2019).

Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum*) offers numerous health benefits as a probiotic (Table 2.2). They can enhance gut health by strengthening the intestinal barrier, reducing inflammation, improving nutrient absorption, and modifying the microbiota (Chang et al., 2022; Li et al., 2023) These bacteria generate strong bacteriocins (Echegaray et al., 2023), organic acids (Vougiouklaki et al., 2022), and hydrogen peroxide (Ruiz et al., 2023), which target harmful Gram-positive bacteria like *Listeria monocytogenes* and *Staphylococcus aureus*, as well as urogenital pathogens such as *Gardnerella vaginalis* and *Candida* species (Sun et al., 2022). They support the immune system by reinforcing the intestinal barrier, stimulating immunoglobulin production, and enhancing natural killer cell activity (Hussin et al., 2022; Bu et al., 2023; Luo et al., 2023; Hong et al., 2024). Their metabolites exhibit anti-inflammatory properties and could help reduce food allergies (Yamamoto-Hanada et al., 2023). Additionally, they have shown potential in lowering cholesterol and providing antioxidant benefits (Štšepetova et al., 2023; Lee et al., 2024; Padro et al., 2024). Furthermore, their properties may offer