



**CYTOTOXIC EFFECTS OF *Lactiplantibacillus plantarum* I-UL4 POSTBIOTICS  
CULTIVATED IN DIFFERENT MEDIA AGAINST MCF-7 HUMAN BREAST  
CANCER CELLS**

**By**

**YAP HAN YUN**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in  
Fulfilment of the Requirements for the Degree of Master of Science**

**July 2023**

**FBSB 2023 20**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**CYTOTOXIC EFFECTS OF *Lactiplantibacillus plantarum* I-UL4 POSTBIOTIC CULTIVATED IN DIFFERENT MEDIA AGAINST MCF-7 HUMAN BREAST CANCER CELLS**

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**July 2023**

**Chair : Prof. Foo Hooi Ling, PhD**  
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Postbiotics are receiving vast attention for their potential benefits in cancer therapy. The cytotoxicity of postbiotics produced by *Lactiplantibacillus plantarum* I-UL4 have been demonstrated on breast cancer cells and were affected by the components of the selective de Man Rogosa Sharpe (MRS) growth medium. The aim of this study was to compare the cytotoxic activities of postbiotics produced by *L. plantarum* I-UL4 on breast cancer cells using different media formulated by Plackett Burman statistical design (PBD). This study prepared 16 types of postbiotics in two forms: freeze-dried (FP) and liquid postbiotics (LP). The bacteriocin inhibitory activities of LP were determined by agar-well diffusion assay using *Pediococcus acidilactici* 4-46 as an indicator for quality assessment prior to cytotoxicity determination. The cytotoxicity of FP was subsequently determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay using MCF-7 breast cancer cells and MCF10A non-malignant breast epithelial cells as a control, respectively. The effects of medium components on the bacteriocin inhibitory and cytotoxic activities of postbiotics were determined by PBD. The cytotoxicity mechanism on MCF-7 breast cancer cells was elucidated with Annexin V Fluorescein-5-isothiocyanate assay for the selected postbiotics. The protein concentrations of selected liquid and freeze-dried media and their respective postbiotics were compared by Bradford and Modified Lowry assays.

Different bacteriocin inhibitory activities were detected for different postbiotics produced by *L. plantarum* I-UL4 cultivated in different media. The highest bacteriocin inhibitory activity was achieved by the reported LPRMRS postbiotic. Yeast extract from all the formulated media contributed to the highest percentage of bacteriocin inhibitory activity of postbiotics (80.42%). Similarly, the results of cytotoxic activities indicated clearly that the medium composition and the MTT incubation time greatly influenced the cytotoxic profile of FP on MCF-7 cells. Yeast extract only demonstrated a positive effect on the

cytotoxic activities of FP at 72 hr of incubation. Most minerals contributed to the cytotoxic activities of FP throughout the incubation period. FM9 and FM12 media and their respective FP were selected for the subsequent study of postbiotic cytotoxicity mechanisms on MCF-7 cells due to their potent cytotoxicity. FP12 induced higher apoptotic MCF-7 cells as compared to FP9 from 24 hr to 72 hr. In comparison with the reported control media, minute toxicity was observed for all FP, except for FPRMRS. All FP induced lower toxicity towards MCF10A cells from 24 hr to 72 hr of incubation as compared to the cytotoxic activities against MCF-7 cells, The reported freeze-dried FM (FFM) media and its respective FP (FPFM) induced higher percentage of late apoptotic MCF-7 cells as compared to MCF10A cells at 48 hr and 72 hr. Different protein concentrations were detected for the selected liquid and freeze-dried media and their amino acids. In conclusion, the medium composition greatly affected the cytotoxic activity of postbiotics produced by *L. plantarum* I-UL4.

**Keywords:** Cytotoxic, Different media, Human breast cancer cells, *Lactiplantibacillus plantarum*, Postbiotic

**SDG:** GOAL 3: Good Health and Well-being.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**KESAN SITOTOSIK POSTBIOTIK *Lactiplantibacillus plantarum* I-UL4  
DITUMBUHKAN DALAM MEDIA BERBEZA TERHADAP MCF-7 SEL  
KANSER PAYUDARA MANUSIA**

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Postbiotik menerima perhatian yang luas untuk potensi manfaatnya dalam terapi kanser. Sitotoksiti postbiotik yang dihasilkan oleh *Lactiplantibacillus plantarum* I-UL4 telah ditunjukkan pada sel-sel kanser payudara dan dipengaruhi oleh komponen medium pertumbuhan de-Man Rogosa Sharpe (MRS) terpilih. Tujuan umum kajian ini adalah untuk membandingkan aktiviti sitotoksik postbiotik yang dihasilkan oleh *L. plantarum* I-UL4 dengan menggunakan media yang dilaporkan dan pertumbuhan media berbeza yang direkabentukkan oleh statistik Plackett Burman (PBD). Kajian ini menyediakan 16 jenis postbiotik dalam dua bentuk: postbiotik sejuk beku (FP) dan postbiotik cecair (LP). Aktiviti inhibitori bakteriosin LP ditentukan melalui ujian resapan telaga agar menggunakan *Pediococcus acidilactici* 4-46 sebagai penunjuk untuk penilaian kualiti sebelum penentuan sitotoksiti. Sitotoksiti FP kemudiannya ditentukan oleh ujian 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromida (MTT) dengan menggunakan sel kanser payudara MCF-7 dan sel epithelium payudara bukan malignan MCF10A sebagai kawalan, masing-masing. Kesan komponen media terhadap aktiviti inhibitori dan sitotoksik postbiotik ditentukan oleh PBD. Mekanisme sitotoksiti pada sel kanser payudara MCF-7 telah dijelaskan ujian Annexin V Fluorescein-5-isothiocyanate untuk postbiotik terpilih. Kepekatan protein bagi media cecair dan sejuk beku terpilih dibandingkan dengan ujian Bradford dan Modified Lowry masing-masing.

Aktiviti inhibitori bakteriosin yang berbeza telah dikesan untuk postbiotik berbeza yang dihasilkan oleh *L. plantarum* I-UL4 yang dikultur dalam media yang berbeza. Aktiviti bakteriosin inhibitori tertinggi dicapai oleh postbiotik LPRMRS. Ekstrak yis daripada semua media yang dirumus menyumbang kepada peratusan tertinggi aktiviti perencatan bakteriosin postbiotik (80.42%). Begitu juga dengan keputusan aktiviti sitotoksik, ia menunjukkan dengan jelas bahawa komposisi medium dan masa inkubasi ujian MTT sangat mempengaruhi profil sitotoksik FP pada sel kanser payudara MCF-7. Ekstrak yis

hanya menunjukkan kesan positif ke atas aktiviti sitotoksik FP pada jam 72. Kebanyakan mineral menyumbang kepada aktiviti sitotoksik FP sepanjang tempoh inkubasi. Media FM9 dan FM12 telah dipilih untuk kajian seterusnya untuk ujian mekanisme sitotoksik postbiotik pada sel MCF-7 kerana sitotoksik mereka lebih kuat. FP12 mendorong sel MCF-7 apoptosis yang lebih tinggi berbanding FP9 dari jam 24 hingga jam 72. Berbanding dengan media kawalan yang dilaporkan, ketoksikan sedikit diperhatikan untuk semua FP kecuali untuk FPRMRS. Semua FP menyebabkan ketoksikan yang lebih rendah terhadap sel MCF10A dari jam 24 dan 72 berbanding dengan aktiviti sitotoksik terhadap sel MCF-7. Media sejuk beku FM (FFM) yang dilaporkan dan postbiotik sejuk beku masing-masing (FPFM) mendorong peratusan sel MCF-7 apoptosis lewat yang lebih tinggi berbanding sel MCF10A pada jam 48 dan 72. Kepekatan protein yang berbeza telah dikesan untuk media cecair dan sejuk beku dan postbiotik masing-masing yang terpilih oleh ujian Bradford dan Modified Lowry disebabkan oleh komposisi asid amino. Kesimpulannya, komposisi medium sangat mempengaruhi aktiviti sitotoksik postbiotik yang dihasilkan oleh *L. plantarum* I-UL4.

**Kata Kunci:** Sitotoksik, Media berbeza, Sel Kanser payudara, *Lactiplantibacillus plantarum*, Postbiotik

**SDG:** Matlamat 3: Kesihatan yang Baik dan Kesejahteraan.

## ACKNOWLEDGEMENTS

I would like to express my gratitude to those who are so generous and willing to give a hand during my Master Science study.

First and foremost, I would like to attribute my deepest appreciation to my supervisor, Prof. Dr. Foo Hooi Ling who has guided me along my studies. She provided professional guidance in my academic studies and good personality formation which is practical. She gets my highest amount of praise for her professionalism and dedication in the role of supervisor.

Besides, I would also extend my gratefulness to my co-supervisor, Associate Prof. Dr. Chia Suet Lin for his support and valuable discussions in this research. His caring and easy-going characteristic makes me feel comfortable to approach him.

Not to be forgotten, I expressed my appreciation to Dr. Federick Leong for his generosity in teaching and guiding in flow cytometry.

Finally, I expressed my sincere thanks to all my lab mates and friends in Universiti Putra Malaysia for helping, supporting, and caring for me in my journey of studies. Special thanks to Mei Zhi, Zahiah, Umar, Li Chin, Ming Yue, Bei Ru, Mei Ling, Hui Ting and Yem Sim, my friends in high place.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science Biotechnology. The members of the Supervisory Committee were as follows:

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## LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µg/µL	Microgram per millilitre
µL	Microliter
µm	Micrometre
ACE	Angiotensin-converting enzyme
Akt	Serine/threonine kinase
ATCC	American Type Culture Collection
AU	Arbitrary unit
Bcl-xL	B-cell lymphoma-extra large
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
BAK	Bcl-2-homologous antagonist/killer
BAX	Bcl-2-associated X protein
CDK	Cyclin-dependent kinase
c-FLIP	Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
cIAP1	Cellular inhibitor of apoptosis protein 1
DAMP	Damage-associated molecular pattern
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
Dpp	Dipeptide
ERα	Oestrogen receptor alpha

ERE	Erythromycin esterase
ERK1/2	Extracellular signal-regulated kinase ½
FasL	Fas cell surface death ligand
FasR	Fas cell surface death receptor
FDA	Food and drug administration
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GSK3	Glycogen synthesis kinase 3
HER2	Human epidermal growth factor receptor 2
HMGB1	High mobility group box 1 protein
HPLC	High-pressure liquid chromatography
hr	Hour
IAPs	Inhibitors of apoptosis proteins
IC <sub>50</sub>	Inhibition concentration of 50% growth
INPP4B	Inositol polyphosphate-4-phosphatase type II B
IRS	Insulin receptor substrate
IL	Interleukin
JNK	c-Jun N-terminal kinase
LAB	Lactic acid bacteria
MAPK	Mitogen-activated protein kinase
MAU	Modified arbitrary unit
MCF-7	Primary epithelial breast cancer cells
MCF10A	Non-malignant epithelial breast cells
MEK	Mitogen-activated protein kinase kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
N. D	Not detected
NFκB	Nuclear factor kappa B
NSPase	Non-starch polysaccharide degrading enzymes
p-AKT	Protein kinase B
PAR-4	Prostate apoptosis response 4
PARP	Poly (ADP-ribose) polymerases
PBD	Plackett-Burman Design
PES	Polyethersulfone
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PS	Phosphatidyl serine
PTEN	Phosphatase and tensin homolog
Raf protein	Rapidly accelerated fibrosarcoma protein
Ras protein	Rat sarcoma virus protein
RB pathway	Retinoblastoma tumour suppressor pathway
RPMI	Rosewell Park Memorial Institute
SEM	Standard error of mean
TNF	Tumour necrosis factor
TNM	Tumour (T), Nodes (N), Metastases (M)
TRAIL	TNF-related apoptosis inducing ligand
Trk	Tropomyosin receptor kinase
TSC	Tuberous sclerosis protein

Wnt pathway

Beta-catenin pathway

w/v

Weight per volume



## CHAPTER 1

### INTRODUCTION

Postbiotics are the metabolites produced by probiotics lactic acid bacteria (LAB), which contain a complex mixture of soluble bioactive compounds (Tsilingiri et al., 2012; Cicienia et al., 2014). They are known to exert many beneficial health effects on livestock (Loh et al., 2014; Humam et al., 2019) and humans (Wang et al., 2012; Cao et al., 2018). Short-chain fatty acids, bacteriocins, vitamins, exopolysaccharides, and many proteinaceous compounds are some of the major bioactive compounds frequently reported for most LAB postbiotic (Zolkiewicz et al., 2020). Postbiotics have been extensively reported to improve intestinal tissue barriers, reduce inflammation, intestinal pathogens, and blood cholesterol concentrations, while increasing LAB counts in the intestine of rats (Tsilingiri et al., 2012; Wang et al., 2012; Cao et al., 2018; Gao et al., 2019; Foo et al., 2003). The bioactive compounds in postbiotics such as the short-chain fatty acids, vitamins, and proteinaceous bioactive compounds have been demonstrated to reduce inflammation caused by pathogenic bacteria (Nakkarach et al., 2020), enhance cellular activities (LeBlanc et al., 2011), reduce high blood pressure (Vallabha & Tiku, 2014) and maintain gut homeostasis (Al-Hassi et al., 2014) in human.

Recently, there has been a growing interest in studying the cytotoxic activities of postbiotics produced by LAB on cancer cells due to its probiotic-like properties (Kothari et al., 2019) and the advantages over viable probiotic cells. For instance, numerous studies have documented the potential anticancer properties of the probiotic viable cells, demonstrating their efficiency in addressing various types of cancer, such as colorectal (Raftar et al., 2007), bladder (Seow et al., 2002), liver (Li et al., 2016), breast (Maroof et al., 2012), stomach (Tong et al., 2007), and cervix cancers (Nami et al., 2014). However, the use of living probiotic cells raises concerns of the risk of antibiotic resistance and virulence gene transfer between probiotic and human gut microbiota and the possibility of contracting diseases from the living probiotic cells (Drago et al., 2011; Egervarn et al., 2010; Kothari et al., 2019). Thus, the non-living LAB postbiotics hold promise as a safer and more efficient alternative for cancer therapy.

Breast cancer was the most frequently diagnosed cancer and the top five cancer that caused death among women worldwide in year 2020 (Global Cancer Observatory [GLOBOCAN], 2020). Breast cancer cases and mortality rates are witnessing a worrisome surge, with projected numbers estimated to rise to 13.8 thousand and 6.4 thousand deaths respectively from the most recent figures of 8,418 cases and 3,503 deaths by the year 2040 (GLOBOCAN, 2020b). Medical treatments for breast cancer are costly (Greenup et al., 2019) and unfortunately, still fall short of guaranteeing complete recovery. LAB postbiotics and some of the bioactive compounds of the postbiotics have been reported to induced anticancer activity on breast cancer in in-vitro and in-vivo studies. This includes Laterosporulin10 produced by *Brevibacillus* sp. strain SKDU10 (Baindara et al. 2017) and short-chain fatty acids from intestinal *E. coli* strain (Nakkarach et al., 2020), which had demonstrated to induced cytotoxic effect on MCF-7 cells while exerting low toxicity on normal cells. In an in-vivo study, a delay in tumour growth was demonstrated in mice fed with kefir cell-free fraction obtained from milk

fermented by *L. helveticus* (LeBlanc et al., 2006) via modulating the immune response and hormones such as interleukin-10 and interleukin-6 involving in mammary cell growth.

The previous studies had demonstrated the cytotoxic activities of postbiotics produced by *L. plantarum* on various cancer cells was strain dependent (Chua et al. 2019) whereas the cytotoxic activities of postbiotics produced by *L. plantarum* I-UL4 on MCF-7 breast cancer cells was affected by the medium composition of media (Tan et al. 2015). Although there were many studies on optimising media for postbiotics' antimicrobial activity (Todorov & Dicks, 2004; Todorov & Dicks, 2007; Ooi et al., 2015; Ooi et al., 2021), documentation on the effects of different growth media on the cytotoxic activities of postbiotics is very limited. Thus, the present study aimed to investigate the cytotoxic effects of postbiotics on MCF-7 breast cancer cells produced by probiotic *L. plantarum* I-UL4 cultivated in different media.

MCF-7 breast cancer cells and MCF10A non-malignant breast epithelial cells were employed in this study for the cytotoxicity determinations. The specific objectives of this study were:

- i) To determine the bacteriocin inhibitory activities of liquid postbiotics produced by *L. plantarum* I-UL4 cultivated in different media
- ii) To determine the cytotoxicity effects of freeze-dried postbiotics produced by *L. plantarum* I-UL4 cultivated in different media
- iii) To determine the cell death mechanism exerted by selected freeze-dried postbiotics produced by *L. plantarum* I-UL4

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