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CHARACTERISATION OF Lactococcus lactis M4 CARRYING DUAL-EXPRESSION PLASMID TO DEMONSTRATE BACTOFECTION OF HUMAN COLON CANCER CELL LINE, SW620

HABIBAH BINTI FAROQUE

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By

HABIBAH BINTI FAROQUE

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August 2017

Chairman : Siti Sarah Othman, PhD
Faculty : Biotechnology and Biomolecular Sciences

Lactic acid bacteria (LAB) such as *Lactococcus lactis* is a well-known food-grade bacterium which is identified as an excellent candidate for delivering DNA vaccine towards colorectal carcinoma cells. Safety usage of *L. lactis* was confirmed by its generally recognised as safe (GRAS) status and the long tradition of use in the food industries. Exploitation of LAB as probiotics also provided an interesting and broad field of possibilities in overcoming boundaries towards biomedicine markets. This is because the production cost is affordable even in large scale production. Implementation of gene therapy for cancer recovery such as immunotherapy has become an attractive and an alternative approach towards classical treatments. Most of the immunotherapeutic studies have been using delivery vectors such as viruses, attenuated pathogens and parasites as they efficiently deliver plasmids or DNA towards various types of cancer cells. However, researcher questioned on the possibility of these vectors to revert back its pathogenicity has become one of the hurdles for this research to be put into practice. Therefore, LAB with its GRAS status has shown to be a better alternative vector for gene delivery. In this study, a local dairy isolate, *L. lactis* M4 was investigated for its ability to be developed as a live delivery vector of plasmid DNA harboring the fluorescent genes towards the human colon cancer cell line, SW620. The interaction mechanisms involved between this LAB strain and SW620 during the interaction assays was analysed. This human colorectal cell was used in this study since it is an epithelial cell which is suitable to be used as an expression host that would allow for the demonstration of the plasmid delivery into the mammalian cells. In addition, the SW620 cells have also been widely used for the application in cancer research involving LAB. It was shown, through the trypan blue exclusion method performed along with the interaction

assays that *L. lactis* M4 has no cytotoxicity effect towards SW620 cells at the multiplicity of infection (MOI) of 250:1 and below. *L. lactis* M4 strain was found to adhere and to internalise into the SW620 cells optimally at two hours post-infection at the MOI 250:1, bacteria per cancer cell and managed to survive intracellularly for 7 hours. When SW620 cells were pre-incubated with Cytochalasin D and Vinblastine drugs (the microfilaments and microtubules destabilisers) before the invasion assays, uptake of the *L. lactis* M4 was blocked indicating that the mode of delivery into the cell was via endocytosis which are dependent on the rearrangement of both microfilament and microtubule. Bactofection mechanism of the SW620 cells by *L. lactis* M4 was demonstrated through the 3D image modeling of the expression of fluorescent reporter proteins from a dual-expression plasmid, pHSR constructed in this study. Viable *L. lactis* M4 was found to express red fluorescent protein (RFP) in the intracellular compartment of the SW620 cells at three hours post-infection. Concurrently, SW620 cells were also observed to express green fluorescent protein (GFP) at the same time. Hence, the success of gene delivery demonstrated based on the expression of RFP and GFP from pHSR have proven that *L. lactis* M4 can be considered as a promising candidate of a live delivery vector of plasmid DNA into the mammalian cells.

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

PENCIRIAN TERHADAP *Lactococcus lactis* M4 YANG MEMBAWA PLASMID DWI-EKSPRESI BAGI DEMONSTRASI BAKTOFEKSI TERHADAP TITISAN SEL KANSER KOLON MANUSIA, SW620

Oleh

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Bakteria Asid Laktik (LAB) seperti *Lactococcus lactis* M4 amat terkenal sebagai bakteria gred-makanan yang merupakan calon terbaik untuk menghantar vaksin DNA kepada sel kanser kolon. Keselamatan penggunaan *L. lactis* telah berstatus umumnya diiktiraf sebagai selamat (GRAS) dan ianya mempunyai tradisi berpanjangan dalam kegunaan industri pemakanan. Eksploitasi LAB sebagai probiotik juga telah memberi kemungkinan yang luas dan menarik terhadap pemasaran bioperubatan. Hal ini berkaitan dengan kos pengeluaran yang mampu ditanggung walaupun dalam pengeluaran berskala besar. Pelaksanaan terapi gen seperti imunoterapi telah menjadi tarikan dan merupakan pendekatan alternatif terhadap rawatan klasik. Kebanyakan kajian imunoterapi telah menggunakan vektor-vektor penghantar seperti virus, patogen teratenuat dan parasit kerana kecekapan vektor tersebut menghantar plasmid dan DNA kepada pelbagai jenis titisan sel kanser. Namun, pengkaji mempersoalkan akan keselamatan penggunaan vektor tersebut yang boleh berbalik kepada keadaan virulen yang menjadi salah satu daripada beban terhadap kajian ini untuk diamalkan. Oleh itu, LAB dengan status GRAS, telah diperlihatkan sebagai vektor penghantar gen alternatif. Dalam kajian ini, satu stren tenusu terasing, *L. lactis* M4 telah dikaji bagi kebolehan terorak sebagai penghantar vektor hidup bagi plasmid DNA pembawaan gen berpendaflour terhadap titisan sel kanser kolon manusia, SW620. Mekanisme interaksi yang terlibat antara stren ini dan SW620 ketika asai interaksi telah dianalisa. Sel kolon manusia ini telah digunakan dalam kajian ini kerana ia merupakan sel epitelium yang sesuai untuk digunakan sebagai hos ekspresi yang membenarkan demonstrasi penghantaran plasmid ke dalam sel mamalia.Tambahan pula, sel SW620 juga telah digunakan secara meluas untuk aplikasi dalam kajian kanser. Kaedah penyisihan tripan biru yang

dilakukan disamping asai interaksi telah membuktikan bahawa *L. lactis* M4 tidak bersifat sitotoksik terhadap sel SW620 pada kegandaan jangkitan (MOI) 250:1 dan ke bawah. Stren *L. lactis* M4 telah ditemui melekap pada dan memasuki sel SW620 secara optimum selepas dua jam pemberian pada MOI 250:1, bakteria per sel kanser dan dapat bertahan untuk hidup 7 jam intrasel. Ketika pra-inkubasi sel SW620 dengan dadah Cytochalasin D dan Vinblastine (penjejas kestabilan mikrofilamen dan mikrotubul) sebelum asai pencerobohan, pengambilan *L. lactis* M4 telah disekat, menunjukkan bahawa mod penghantaran ke dalam sel SW620 adalah melalui endositosis yang bersandarkan kepada penyusunan semula kedua-dua mikrofilamen dan mikrotubul. Mekanisme baktofeksi terhadap sel SW620 oleh *L. lactis* M4 telah ditunjukkan melalui pemodelan gambar 3D melalui ekspresi protein pelapor berpendafluor daripada plasmid dwi-ekspresi, pHSR yang dikonstruk dalam kajian ini. *L. lactis* M4 yang berdaya hidup telah ditemui mengekspresi protein berpendaflour merah (RFP) di dalam ruang intrasel sel SW620 tiga jam selepas infeksi. Seiringan itu, sel SW620 juga telah dicerapi mengekspresi protein berpendafluor hijau (GFP) pada waktu itu. Oleh yang demikian, kejayaan dalam penghataran gen yang ditunjukkan berdasarkan ekspresi RFP dan GFP daripada pHSR telah membuktikan yang *L. lactis* M4 boleh dianggap sebagai calon harapan penghantar vektor hidup untuk plasmid DNA ke dalam sel mamalia.

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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. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

LAB	Lactic acid bacteria
GRAS	Generally Recognised As Safe
MOI	Multiplicity of infection
RFP	Red fluorescent protein
GFP	Green fluorescent protein
LPS	Lipopolysaccharide
US FDA	United States Food and Drug Administration
NCI	National Cancer Institute
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
CLSM	Confocal Laser Scanning Microscope
DAPI	4', 6-diamidino-2-phenylindole
NICE	Nisin Controlled Gene Expression
®	Registered trademark symbol
^{P}170	Prokaryote auto-inducible promoter 170
mRNA	Messenger ribonucleic acid
REED	Reverse Electro Enhanced Dialysis
IARC	Internation Agency for Research on Cancer
ACS	American Cancer Soceity
BCG	Bacillus Calmette-Guérin
HPV	Human papillomaviruses

HBV	Hepatitis B virus
T-VEC	talimogene laherparepvec
CEA	carciembryonic antigen
MVP	major vault protein
LDF	*Lactobacillus delbrueckii* fermentation
FnBPA	fibronectin binding protein A
TEM	Transmission Electron Microscope
h	hours
inlA	internalin A gene
EBL	Embryonic Bovine Lung
HS	Haemorrhagic septicaemia
^{P}sodC	Prokaryote sodC promoter
RE	Restriction enzyme
PCR	Polymerase Chain Reaction
gusA	β-Glucuronidase reporter gene
EGFP	Enhanced Green Fluorescent Protein
MCS	Multiple cloning sites
^{P}CMV	Eukaryote cytomegalovirus promoter
^{P}nisA	Prokaryote nisin A promoter
LB	Luria-Bertani
GM17	M17 media supplemented with glucose
MRS	de-Man, Rogosa and Sharpe media
v/v	volume per volume
1X	One times dilution

PBS	Phosphate Buffered Saline
OD_{600nm}	Optical Density at wavelength of 600 nm
CFU/mL	Colony Forming Unit per mL
Amp	Ampicillin
Kan	Kanamycin
Chl	Chloramphenicol
Gen	Gentamicin
Inc.	Incorporation
w/v	weight per volume
TAE	Tris-acetate-EDTA
EDTA	Ethylenediaminetetraacetic acid
GC	Guanine-Cytosine
Tm	Melting temperature
$MgCl_2$	Magnesium chloride
dNTPs	Deoxynucleotide
Taq	*Thermus aquaticus*
1^{st}	First
RT	Room temperature
5’	Five prime end of a DNA fragment
3’	Three prime end of a DNA fragment
$CaCl_2$	Calcium chloride
SGM17	M17 media supplemented with sucrose and glucose
Pfu	*Pyrococcus furiosus*
× 10	10 times magnification

siRNA	Small interfering ribonucleic acid
FI	Fluorescent intensity
TM	Trademark symbol
©	Copyright symbol
3D	Three dimensional
P	Probability value
±SEM	Standard error of mean confidence interval
kb	kilo base pairs
SV40	Simian virus 40
Rep	Replication gene
Ori	Origin of replication
UV	Ultraviolet
~	Approximately
bp	Base pairs
DMSO	Dimethyl sulfoxide
NaOH	Sodium hydroxide

CHAPTER 1

INTRODUCTION

Since the past decades, lactic acid bacteria (LAB) has received attention from scientist as a tool for drug delivery and targeting agents (Kochut & Dersch, 2013). The LAB family includes the genera *Lactobacillus, Lactococcus, Streptococcus* and many more (Stiles & Holzapfel, 1997). Generally, LAB is an organism of choice because it is a Gram-positive microorganism that lacks of lipopolysaccharide (LPS), an endotoxin that is present in Gram-negative bacteria. In addition, LAB could survive the strong acidic stomach when it is delivered orally. They undergo fermentation and produce lactic acid as its end-product (Sharma & Devi, 2014). These bacteria have been studied in detail and are well-known for their applications in the food industry. For instance, *Lactococcus* strains are mainly being used in the production of dairy product like cheese (Konings, 2000). Since its certification of GRAS (Generally Recognised as Safe) organism by the US Food and Drug Administration (FDA), the potential of LAB in several applications have been greatly enhanced. LAB have been reported to be utilised as cell factories for expression of membrane proteins (Douillard, O'Connell-Motherway, Cambillau, & van Sinderen, 2011), suppressing spoilage and growth of pathogenic bacteria (Jalilsood, Baradaran, Song, Foo, Mustafa, Saad, Yusoff, & Rahim, 2015), production of biocatalyst (Hugenholtz, Kleerebezem, Starrenburg, Delcour, de Vos, & Hols, 2000), and delivery of therapeutic substances (Braat, Rottiers, Hommes, Huyghebaert, Remaut, Remon, Van Deventer, Neirynck, Peppelenbosch & Steidler, 2006; Rottiers, De Smedt & Steidler, 2009). The GRAS certification of LAB also opens a new era in immunotherapy. Replacing an attenuated pathogenic bacteria with LAB as a delivery vector for therapeutic vaccine portrays a much safer alternatives for cancer treatments (García-Fruitós, 2012). It is crucial to avoid the risk associated with using the attenuated pathogen as live vaccine as there is possible reversion of the attenuated strains into its virulent phenotype (Tao, Pavlova, Ji, Jin, & Spear, 2011).

A food grade bacterium such as LAB could be utilised as a delivery vector for the DNA vaccine as it can be safely consumed. It also protects the naked DNA from degradation by nucleases and hence improves the efficacy of the delivery system (Bermúdez-Humarán, Kharrat, Chatel & Langella, 2011; Kawabata, Takakura, & Hashida, 1995; Lechardeur, Sohn, Haardt, Joshi, Monck, Graham, Beatty, Squire, O'Brodovich & Lukacs, 1999). In previous review, commercially available *L. lactis* strains have been commonly listed as one of the potential candidates for the delivery vehicle of therapeutic DNA vaccine into the mammalian cells. It was shown in the study that incubation of the cell line with purified plasmid DNA resulted in no protein expression. However, protein expression of the transgene was detected in the cells when the plasmid

is incorporated within the bacterium suggesting that the plasmid being successfully delivered (Guimarães, Innocentin, Lefèvre, Azevedo, Wal, Langella & Chatel, 2006). Some of these studies also incorporated invasive genes isolated from the pathogenic bacterial strains into the LAB strains to make it more invasive (Innocentin, Guimarães, Miyoshi, Azevedo, Langella, Chatel & Lefèvre, 2009) whereas some study has used enzymatic treatments to enhance the delivery of plasmid into the mammalian cancer cells (Tao *et al.*, 2011). Previously in the laboratory, a naturally derived local cow's milk isolate of *L. lactis* M4 was assessed for its ability to maintain low molecular weight plasmid and express heterologous protein. It was shown that *L. lactis* M4 with a small genome size can be developed as a suitable expression host and able to retain transformed plasmid stably for more than 100 generation time. Hence in this study, we would like to further investigate the capacity of this strain to interact with the colorectal cancer cell line, SW620 and to deliver dual-expression plasmid via bactofection (bacteria mediated DNA delivery).

Cancer is one of the leading causes of death in the world accounting for almost 8.2 million of cancer-related deaths. Colorectal cancer alone has resulted in 694,000 deaths (American Cancer Society, 2015b). According to Pourhoseingholi (2014), the number of colorectal cancer cases worldwide was estimated to increase from 1.2 to 2.2 million in 20 years time. In the Asian community, less number of people supported and was aware on the importance of screening on colorectal cancer at the early stages (Pourhoseingholi, 2014). This problem is closely associated with the low income communities as well as due to lack of facilities and educational program being provided for colorectal cancer screening (Pourhoseingholi, 2014). Based on the report by National Cancer Institute (NCI), there are a total of five standard treatments available for cancer patients which are surgery, radiofrequency ablation, cryosurgery, chemotherapy, and radiation therapy. However, these treatments comes with detrimental side-effects (American Cancer Society, 2013). Therefore, an alternative treatment such as gene therapy is anticipated. Recently, the first certified therapeutic cancer vaccine known as Sipuleucel- T (Provenge®) was used to treat prostate cancer by eliciting an immune response against the antigens found on the cancer cells. This cancer vaccine consists of dendritic cells from the patient's blood that was fused to the prostate antigen and granulocyte macrophage colony-stimulating factor (Schlom, 2012) before administered to the patient via intravenously (Kantoff, Higano, Shore, Berger, Small, Penson, Redfern, Ferrari, Dreicer, Sims, Xu, Frohlich & Schellhammer, 2010). This progression has opened up a whole new horizon for scientist to develop new therapeutic DNA vaccine for treatment of colorectal cancer.

The main aim for this study is to determine whether *L. lactis* M4 can deliver plasmid DNA into cancer cell. In order to track the delivery, the plasmid will have two different expression systems that enable fluorescent protein expression in prokaryotic and eukaryotic cells. Hence, the specific objectives of this study are;

1. To investigate the interaction properties on adhesion, invasion, intracellular survival and invasion inhibition between *L. lactis* M4 and SW620 cells.
2. To construct a dual-expression plasmid, pHSR harbouring prokaryotic and eukaryotic expression cassettes.
3. To develop a model for bactofection mechanism of SW620 cells using *L. lactis* M4.

REFERENCES

A. Frese, S., W. Hutkins, R., & Walter, J. (2012). Comparison of the Colonization Ability of Autochthonous and Allochthonous Strains of Lactobacilli in the Human Gastrointestinal Tract. *Advances in Microbiology*, *2*(3), 399–409.

Ahmed, D., Eide, P. W., Eilertsen, I. A., Danielsen, S. A., Eknæs, M., Hektoen, M., Lind, G. E., & Lothe, R. A. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis*, *2*(9), e71.

Amdekar, S., Dwivedi, D., Roy, P., Kushwah, S., & Singh, V. (2010). Probiotics: multifarious oral vaccine against infectious traumas. *FEMS Immunology and Medical Microbiology*, *58*(3), 299–306.

American Cancer Society. (2013). Cancer Treatment. *Cancer Treatment & Survivorship Facts & Figures*, 44.

American Cancer Society. (2015a). Colorectal Cancer What is cancer ? What is colorectal cancer ? Retrieved from http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-treating-radiation-therapy

American Cancer Society. (2015b). Global Cancer Facts & Figures 3rd Edition. *American Cancer Society*, (800), 1–64. http://doi.org/10.1002/ijc.27711

Anuradha, K., Foo, H. L., Mariana, N. S., Loh, T. C., Yusoff, K., Hassan, M. D., Sasan, H., & Raha, a R. (2010). Live recombinant *Lactococcus lactis* vaccine expressing aerolysin genes D1 and D4 for protection against *Aeromonas hydrophila in tilapia* (Oreochromis niloticus). *Journal of Applied Microbiology*, *109*(5), 1632–42.

Bahey-El-Din, M., & Gahan, C. G. (2011). *Lactococcus lactis*-based vaccines: Current status and future perspectives. *Human Vaccines*, *7*(1), 106–109.

Balestrino, D., Anne Hamon, M., Dortet, L., Nahori, M. A., Pizarro-Cerda, J., Alignani, D., Dussurget, O., Cossart, P., & Toledo-Arana, A. (2010). Single-cell techniques using chromosomally tagged fluorescent bacteria to study *Listeria monocytogenes* infection processes. *Applied and Environmental Microbiology*, *76*(11), 3625–3636.

Becker, P. D., Noerder, M., & Guzmán, C. A. (2008). Genetic immunization: bacteria as DNA vaccine delivery vehicles. *Human Vaccines*, *4*(3), 189–202.

Bera, S., Thillai, K., Sriraman, K., & Jayaraman, G. (2015). Process strategies for enhancing recombinant streptokinase production in *Lactococcus lactis* cultures using ^{P}170 expression system. *Biochemical Engineering Journal*, *93*, 94–101.

Bermúdez-Humarán, L. G. (2009). *Lactococcus lactis* as a live vector for mucosal delivery of therapeutic proteins. *Human Vaccines*, *5*(4), 264–267.

Bermúdez-Humarán, L. G., Kharrat, P., Chatel, J.-M. M., Langella, P., Bermudez-Humaran, L. G., Kharrat, P., Chatel, J.-M. M., & Langella, P. (2011). Lactococci and lactobacilli as mucosal delivery vectors for therapeutic proteins and DNA vaccines. *Microbial Cell Factories*, *10*(Suppl 1), S4.

Berzofsky, J. A., Wood, L. V, & Terabe, M. (2013). Cancer vaccines: 21st century approaches to harnessing an ancient modality to fight cancer. *Expert Review of Vaccines*, *12*(10), 1115–1118.

Blanchette, C. D., Woo, Y.-H., Thomas, C., Shen, N., Sulchek, T. A, & Hiddessen, A. L. (2009). Decoupling internalization, acidification and phagosomal-endosomal/lysosomal fusion during phagocytosis of *InlA* coated beads in epithelial cells. *PloS One*, *4*(6), e6056.

Bolhassani, A., & Zahedifard, F. (2012). Therapeutic live vaccines as a potential anticancer strategy. *International Journal of Cancer*, *131*(8), 1733–1743.

Bolotin, A., Mauger, S., Malarme, K., Ehrlich, S. D., & Sorokin, A. (1999). Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. *Antonie van Leeuwenhoek*, *76*(1), 27–76.

Botta, C., Langerholc, T., Cencič, A., & Cocolin, L. (2014). *In vitro* selection and characterization of new probiotic candidates from table olive microbiota. *PloS One*, *9*(4), e94457.

Braat, H., Rottiers, P., Hommes, D. W., Huyghebaert, N., Remaut, E., Remon, J.-P. P., Van Deventer, S.J.H., Neirynck, S., Peppelenbosch, M. P., & Steidler, L. (2006a). A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clinical Gastroenterology and Hepatology : The Official Clinical Practice Journal of the American Gastroenterological Association*, *4*(6), 754–9.

Cano-Garrido, O., Seras-Franzoso, J., & Garcia-Fruitós, E. (2015). Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. *Microbial Cell Factories*, *14*(1), 137.

Casella, J. F., Flanagan, M. D., & Lin, S. (1981). Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. *Nature*, *293*(5830), 302–305.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science (New York, N.Y.)*, *263*(5148), 802–805.

Chatel, J.-M., Pothelune, L., Ah-Leung, S., Corthier, G., Wal, J.-M., & Langella, P. (2008). *In vivo* transfer of plasmid from food-grade transiting lactococci to murine epithelial cells. *Gene Therapy*, *15*(16), 1184–90.

Chauhan, A. S., Badle, S. S., Ramachandran, K. B., & Jayaraman, G. (2014). The ^{P}170 expression system enhances hyaluronan molecular weight and production in metabolically-engineered *Lactococcus lactis*. *Biochemical Engineering Journal*, *90*, 73–78.

Collado, M. C., Isolauri, E., Salminen, S., & Sanz, Y. (2009). The impact of probiotic on gut health. *Current Drug Metabolism*, *10*(1), 68–78.

Cortes-Perez, N. G., Poquet, I., Oliveira, M., Gratadoux, J. J., Madsen, S. M., Miyoshi, A., Corthier, G., Azevedo, V., Langella, P., & Bermudez-Humaran, L. G. (2006). Construction and characterization of a *Lactococcus lactis* strain deficient in intracellular ClpP and extracellular HtrA proteases. *Microbiology (Reading, England)*, *152*(Pt 9), 2611–2618.

Cronstein, B. N., Molad, Y., Reibman, J., Balakhane, E., Levin, R. I., & Weissmann, G. (1995). Colchicine alters the quantitative and qualitative display of selectins on endothelial cells and neutrophils. *Journal of Clinical Investigation*, *96*(2), 994–1002.

De Ruyter, P. G. G. A., Kuipers, O. P., Beerthuyzen, M. M., Van Alen-Boerrigter, I., & De Vos, W. M. (1996). Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. *Journal of Bacteriology*, *178*(12), 3434–3439.

de Vos, W. M. (1999). Gene expression systems for lactic acid bacteria. *Current Opinion in Microbiology*, *2*(3), 289–95.

de Vos, W. M., & Gasson, M. J. . (1989). Structure and Expression of the *Lactococcus lactis* Gene for. *Journal of General Microbiology*, *135*(1989), 1833–1846.

de Vos, W. M., & Vaughan, E. E. (1994). Genetics of lactose utilization in lactic acid bacteria. *FEMS Microbiology Reviews*, *15*(2–3), 217 LP-237.

Delgado, S., Leite, A. M. O., Ruas-Madiedo, P., & Mayo, B. (2014). Probiotic and technological properties of *Lactobacillus* spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis. *Frontiers in Microbiology*.

Donohue, D. C. (2006). Safety of probiotics. *Asia Pac J Clin Nutr*, *15*(4), 563–569.

Douillard, F. P., O'Connell-Motherway, M., Cambillau, C., & van Sinderen, D. (2011). Expanding the molecular toolbox for *Lactococcus lactis*: construction of an inducible thioredoxin gene fusion expression system. *Microbial Cell Factories*, *10*, 66.

Duan, F., Duitama, J., Al Seesi, S., Ayres, C. M., Corcelli, S. A., Pawashe, A. P., Blanchard, T., McMahon, D., Sidney, J., Sette, A., Baker, B. M., Mandoiu, I.I., & Srivastava, P. K. (2014). Genomic and bioinformatic profiling of mutational neoepitopes reveals new rules to predict anticancer immunogenicity. *The Journal of Experimental Medicine*, *211*(11), 2231–2248.

Dunham, S. P. (2002). The application of nucleic acid vaccines in veterinary medicine. *Research in Veterinary Science*, *73*(1), 9–16.

Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzgerald, G., Daly, C., Kiely, B., Quigley, E., O'Sullivan, G., Shanahan, F., & Collins, J. K. (1999). Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie van Leeuwenhoek*, *76*(1–4), 279–292.

Dunne, W. M. (2002). Bacterial Adhesion: Seen Any Good Biofilms Lately? *Society*, *15*(2), 155–166.

Durrbach, a, Louvard, D., & Coudrier, E. (1996). Actin filaments facilitate two steps of endocytosis. *Journal of Cell Science*, *109 (Pt 2*, 457–465.

Elliker, P. R., Anderson, A. W., & Hannesson, G. (1956). An Agar Culture Medium for Lactic Acid Streptococci and Lactobacilli. *Journal of Dairy Science*, *39*(11), 1611–1612.

Galdiero, M., De Martino, L., Pagnini, U., Pisciotta, M. G., & Galdiero, E. (2001). Interactions between bovine endothelial cells and *Pasteurella multocida*: Association and invasion. *Research in Microbiology*, *152*(1), 57–65.

García-Fruitós, E. (2012). Lactic Acid Bacteria: a promising alternative for recombinant protein production. *Microbial Cell Factories*, *11*(1), 157.

Glenting, J., & Wessels, S. (2005). Ensuring safety of DNA vaccines. *Microbial Cell Factories*, *4*, 26.

Grangette, C., Mu, H., Hols, P., Goudercourt, D., Delcour, J., Turneer, M., & Mercenier, A. (2004). Enhanced Mucosal Delivery of Antigen with Cell Wall Mutants of Lactic Acid Bacteria. *Infection and Immunity*, *72*(5), 2731–2737.

Grillot-Courvalin, C., Goussard, S., & Courvalin, P. (2011). Bacterial Vectors for Deliviering Gene and Anticancer Therapies. *Microbe*, *6*(3), 115–121.

Guimarães, V. D., Gabriel, J. E., Lefèvre, F., Cabanes, D., Gruss, A., Cossart, P., Azevedo, V., & Langella, P. (2005). Internalin-expressing *Lactococcus lactis* is able to invade small intestine of guinea pigs and deliver DNA into mammalian epithelial cells. *Microbes and Infection / Institut Pasteur*, *7*(5–6), 836–44.

Guimarães, V. D., Innocentin, S., Lefèvre, F., Azevedo, V., Wal, J.-M., Langella, P., & Chatel, J.-M. (2006). Use of native lactococci as vehicles for delivery of DNA into mammalian epithelial cells. *Applied and Environmental Microbiology*, *72*(11), 7091–7.

Guimarães, V., Innocentin, S., Chatel, J.-M., Lefèvre, F., Langella, P., Azevedo, V., & Miyoshi, A. (2009). A new plasmid vector for DNA delivery using lactococci. *Genetic Vaccines and Therapy*, *7*, 4.

Haas, J., Park, E. C., & Seed, B. (1996). Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Current Biology : CB*, *6*(3), 315–324.

Holo, H., & Nes, I. F. (1995). Transformation of *Lactococcus* by Electroporation. In J. A. Nickoloff (Ed.), *Electroporation Protocols for Microorganisms* (Vol. 47, pp. 195–199). Totowa, NJ: Humana Press.

Hornef, M. W., Wick, M. J., Rhen, M., & Normark, S. (2002). Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat Immunol*, *3*(11), 1033–1040.

Hugenholtz, J., Kleerebezem, M., Starrenburg, M., Delcour, J., de Vos, W., & Hols, P. (2000). *Lactococcus lactis* as a Cell Factory for High-Level Diacetyl Production. *Applied and Environmental Microbiology*, *66*(9), 4112–4114.

Ikeda, R., Iwashita, K., Sumizawa, T., Beppu, S., Tabata, S., Tajitsu, Y., Shimamoto, Y., Yoshida, K., Furukawa, T., Che, X., Yamaguchi, T., Ushiyama, M., Miyawaki, A., Takeda, Y., Yamamoto, M., Zhao, H., Shibayama, Y., Yamada, K., & Akiyama, S. (2008). Hyperosmotic stress up-regulates the expression of major vault protein in SW620 human colon cancer cells. *Experimental Cell Research*, *314*(16), 3017–

3026.

Innocentin, S., Guimarães, V., Miyoshi, A., Azevedo, V., Langella, P., Chatel, J. M., & Lefèvre, F. (2009). *Lactococcus lactis* expressing either *Staphylococcus aureus* fibronectin-binding protein A or *Listeria monocytogenes* internalin A can efficiently internalize and deliver DNA in human epithelial cells. *Applied and Environmental Microbiology*, *75*(14), 4870–4878.

Inouye, S., & Tsuji, F. I. (1994). LznY Aequorea green fluorescent protein Expression of the gene and fluorescence characteristics of the recombinant protein, *341*, 277–280.

Jalilsood, T., Baradaran, A., Song, A. A.-L., Foo, H. L., Mustafa, S., Saad, W. Z., Yusoff, K., & Rahim, R. A. (2015). Inhibition of pathogenic and spoilage bacteria by a novel biofilm-forming *Lactobacillus* isolate: a potential host for the expression of heterologous proteins. *Microbial Cell Factories*, *14*(1), 96.

Jay, J. M. (2000). *Modern Food Microbiology*.

Jordan, M. a, Thrower, D., & Wilson, L. (1992). Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. *Journal of Cell Science*, *102 (Pt 3*, 401–416.

Jørgensen, C. M., Vrang, A., & Madsen, S. M. (2014). Recombinant protein expression in *Lactococcus lactis* using the ^{P}170 expression system. *FEMS Microbiology Letters*, *351*(2), 170–178.

Kamal, N. M., Zamri-Saad, M., Masarudin, M. J., & Othman, S. (2017). Interaction between *Pasteurella multocida* B:2 and its derivatives with bovine aortic endothelial cell (BAEC). *BMC Veterinary Research*, *13*(1), 186.

Kantoff, P. W., Higano, C. S., Shore, N. D., Berger, E. R., Small, E. J., Penson, D. F., Redfern, C., Ferrari, A., Dreicer, R., Sims, R., Xu, Y., Frohlich, M., & Schellhammer, P. F. (2010). Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *The New England Journal of Medicine*, *363*(5), 411–422.

Kawabata, K., Takakura, Y., & Hashida, M. (1995). The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharmaceutical Research*, *12*(6), 825–830.

Kim, Y., Lee, D., Kim, D., Cho, J., Yang, J., Chung, M., Kim, K., & Ha, N. (2008). Inhibition of proliferation in colon cancer cell lines and harmful enzyme activity of colon bacteria by *Bifidobacterium adolescentis* SPM0212. *Archives of Pharmacal Research*, *31*(4), 468.

Kimoto, H., Kurisaki, J., Tsuji, N. M., Ohmomo, S., & Okamoto, T. (1999). Lactococci as probiotic strains: adhesion to human enterocyte-like Caco-2 cells and tolerance to low pH and bile. *Letters in Applied Microbiology*, *29*(5), 313–316.

Klaenhammer, T., Altermann, E., Arigoni, F., Bolotin, A., Breidt, F., Broadbent, J., Cano, R., Chaillou, S., Deutscher, J., Gasson, M., van de Guchte, M., Guzzo, J., Hartke, A., Hawkins, T., Hols, P., Hutkins, R., Kleerebezem, M., Kok, J., Kuipers, O., Lubbers, M., Maguin, E., McKay, L., Mills, D., Nauta, A., Overbeek, R., Pel, H., Pridmore, D., Saier, M., van Sinderen, D., Sorokin, A., Steele, J., O'Sullivan, D., de Vos, W., Weimer, B., Zagorec, M., & Siezen, R. (2002). Discovering lactic acid bacteria by genomics. *Antonie van Leeuwenhoek*, *82*(1–4), 29–58.

Kochut, A., & Dersch, P. (2013). Bacterial invasion factors: Tools for crossing biological barriers and drug delivery? *European Journal of Pharmaceutics and Biopharmaceutics*, *84*(2), 242–250.

Kokkinosa, A., Fasseas, C., Eliopoulos, E., & Kalantzopoulos, G. (1998). Cell size of various lactic acid bacteria as determined by scanning electron microscope and image analysis . *Lait*, *78*(5), 491–500.

Kolida, S., & Gibson, G. R. (2011). Synbiotics in Health and Disease. *Annu. Rev. Food Sci. Technol*, *2*, 373–93.

Konings, W. (2000). Lactic acid bacteria: the bugs of the new millennium. *Current Opinion in Microbiology*, *3*(3), 276–282.

Kreiter, S., Vormehr, M., van de Roemer, N., Diken, M., Löwer, M., Diekmann, J., Boegel, S., Schrors, B., Vascotto, F., Castle, J.C., Tadmor, A.D., Schoenberger, S.P., Huber, C., Tureci, O., & Sahin, U. (2015). Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature*, *520*(7549), 692–696.

Kuijpers, T. W., Raleigh, M., Kavanagh, T., Janssen, H., Calafat, J., Roos, D., & Harlan, J. M. (1994). Cytokine-activated endothelial cells internalize E-selectin into a lysosomal compartment of vesiculotubular shape. A tubulin-driven process. *The Journal of Immunology* , *152*(10), 5060–5069.

Lechardeur, D., Sohn, K. J., Haardt, M., Joshi, P. B., Monck, M., Graham, R. W., Beatty, B., Squire, J., O'Brodovich, H., & Lukacs, G. L. (1999). Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Therapy*, *6*(4), 482–497.

Leggett, B. A., Devereaux, B., Biden, K., Searle, J., Young, J., & Jass, J. (2001). Hyperplastic polyposis: association with colorectal cancer. *The American Journal of Surgical Pathology*, *25*(2), 177–184.

Leibovitz, A., Stinson, J. C., McCombs III, W. B., Mccoy, C. E., Mazur, K. C., & Mabry, N. D. (1976). Classification of human colorectal adenocarcinoma cell lines. *Cancer Research*, *36*(12), 4562–4569.

Li, X.-T., He, M.-L., Zhou, Z.-Y., Jiang, Y., & Cheng, L. (2015). The antitumor activity of PNA modified vinblastine cationic liposomes on Lewis lung tumor cells: *In vitro* and *in vivo* evaluation. *International Journal of Pharmaceutics*, *487*(1–2), 223–233.

Liong, M.-T. (2008). Safety of probiotics: translocation and infection. *Nutrition Reviews*, *66*(4), 192–202.

Lundberg, K. S., Shoemaker, D. D., Adams, M. W. W., Short, J. M., Sorge, J. A., & Mathur, E. J. (1991). High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. *Gene*, *108*(1), 1–6.

Madsen, S. M., Arnau, J., Vrang, A., Givskov, M., & Israelsen, H. (1999). Molecular characterization of the pH-inducible and growth phase-dependent promoter ^{P}170 of *Lactococcus lactis*. *Molecular Microbiology*, *32*(1), 75–87.

Madsen, S. M., Hindre, T., Le Pennec, J. P., Israelsen, H., & Dufour, A. (2005). Two acid-inducible promoters from *Lactococcus lactis* require the cis-acting ACiD-box and the transcription regulator RcfB. *Molecular Microbiology*, *56*(3), 735–746.

Madsen, S., & Vrang, A. (2006). A regulatory acceptable alternative to E. coli: high yield recombinant protein production using the *Lactococcus lactis* ^{P}170 expression system combined with “Reverse electro enhanced dialysis” (REED) for lactate control. *Microbial Cell Factories*, *2*(October 2016), 1–2.

Makarova, K. S., Slesarev, a, Wolf, Y. I., Sorokin, a, Mirkin, B., Koonin, E. V, Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J., Díaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., & Mills, D. a. (2006). Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences* , *103*(42), 15611–15616.

Mercenier, A., Muller-Alouf, H., & Grangette, C. (2000). Lactic acid bacteria as live vaccines. *Current Issues in Molecular Biology*, *2*(1), 17–25.

Mierau, I. (2007). *Lactococcus lactis* in the 21 st Century (pp. 7–9).

Mierau, I., & Kleerebezem, M. (2005). 10 Years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Applied Microbiology and Biotechnology*, *68*(6), 705–717.

Moeini, Hassan; Rahim, Raha; Omar, Abdul; Shafee, Norazizah; Yusoff, K. (2011). *Lactobacillus acidophilus* as a live vehicle for oral immunization against chicken anemia virus. *Applied Microbiology & Biotechnology*, *90*(1), 77.

Morse, M. A., Niedzwiecki, D., Marshall, J. L., Garrett, C., Chang, D. Z., Aklilu, M., Crocenzi, T.S., Cole, D.J., Dessureault, S., Hobeika, A.C., Osada, T., Onaitis, M., Clary, B.M., Hsu, D., Devi, G.R., Bulusu, A., Annechiarico, R.P., Chadaram, V., Clay, T.M., & Lyerly, H. K. (2013). A randomized phase II study of immunization with dendritic cells modified with poxvectors encoding CEA and MUC1 compared with the same poxvectors plus GM-CSF for resected metastatic colorectal cancer. *Annals of Surgery*, *258*(6), 879–86.

Mosolits, S., Nilsson, B., & Mellstedt, H. (2005). Towards therapeutic vaccines for colorectal carcinoma: a review of clinical trials. *Expert Review of Vaccines*, *4*(3), 329–350.

Mukherjee, S., Ghosh, R. N., & Maxfield, F. R. (1997). Endocytosis. *Physiological Reviews*, *77*(3), 759–803.

Noreen, N., Hooi, W. Y., Baradaran, A., Rosfarizan, M., Sieo, C. C., Rosli, M. I., Yusoff, K., & Raha, A. R. (2011). *Lactococcus lactis* M4, a potential host for the expression of heterologous proteins. *Microbial Cell Factories*, *10*(1), 28.

Ofek, I., Hasty, D. L., Doyle, R. J., & Ofek, I. (2003). *Bacterial adhesion to animal cells and tissues*. Washington, D.C.: ASM Press.

Othman, S. (2011). Interactions of an attenuated AroA - derivative of *Pasteurella multocida* B : 2 with mammalian cells and its potential for DNA vaccine delivery Doctor of Philosophy, (June).

Othman, S., Parton, R., & Coote, J. (2012). Interaction between mammalian cells and Pasteurella multocida B:2. Adherence, invasion and intracellular survival. *Microbial Pathogenesis*, *52*(6), 353–8.

Othman, S., Roe, A. J., Parton, R., & Coote, J. G. (2013). Use of a dual reporter plasmid to demonstrate Bactofection with an attenuated AroA(-) derivative of *Pasteurella multocida* B:2. *PloS One*, *8*(8), e71524.

Pálffy, R., Gardlík, R., Hodosy, J., Behuliak, M., Resko, P., Radvánský, J., & Celec, P. (2006). Bacteria in gene therapy: bactofection versus

alternative gene therapy. *Gene Therapy*, *13*(2), 101–5.

Pilgrim, S., Stritzker, J., Schoen, C., Kolb-Mäurer, a, Geginat, G., Loessner, M. J., Gentschev, I. & Goebel, W. (2003). Bactofection of mammalian cells by *Listeria monocytogenes*: improvement and mechanism of DNA delivery. *Gene Therapy*, *10*(24), 2036–45.

Pontes, D. S., de Azevedo, M. S. P., Chatel, J.-M., Langella, P., Azevedo, V., & Miyoshi, A. (2011). *Lactococcus lactis* as a live vector: Heterologous protein production and DNA delivery systems. *Protein Expression and Purification*, *79*(2), 165–175.

Potter, J. D. (1999). Colorectal cancer: molecules and populations. *Journal of the National Cancer Institute*, *91*(11), 916–32.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., & Cormier, M. J. (1992). Primary structure of the Aequorea victoria green-fluorescent protein. *Gene*, *111*(2), 229–233.

Rahman, A., Gleinser, M., Lanhers, M. C., Riedel, C. U., Foligné, B., Hanse, M., Yen, F., Klouj, A., Afzal, M., Back, A., Mangavel, C., Cailliez-Grimal, C., Revol-Junelles, A., & Borges, F. (2014). Adaptation of the lactic acid bacterium *Carnobacterium maltaromaticum* LMA 28 to the mammalian gastrointestinal tract: From survival in mice to interaction with human cells. *International Dairy Journal*, *34*(1), 93–99.

Rottiers, P., De Smedt, T., & Steidler, L. (2009). Modulation of Gut-Associated Lymphoid Tissue Functions with Genetically Modified *Lactococcus lactis*. *International Reviews of Immunology*, *28*(6), 465–486.

Sabidi, S. . (2012). *Amplification and cloning of promoters ^{P}23 and ^{P}170 from Lactococcus lactis MG1363*. Universiti Putra Malaysia.

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W. M., Fondén, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S., & Mattila-Sandholm, T. (1998). Demonstration of safety of probiotics -- a review. *International Journal of Food Microbiology*, *44*(1–2), 93–106.

Salyers, A. a, Gupta, A., & Wang, Y. (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in Microbiology*, *12*(9), 412–6.

Schlom, J. (2012). Therapeutic cancer vaccines: current status and moving forward. *Journal of the National Cancer Institute*, *104*(8), 599–613.

Schottenfeld, D., & Fraumeni, J. F. (2006). *Cancer epidemiology and prevention*. Oxford University Press.

Seegers, J. F. M. L. (2002). Lactobacilli as live vaccine delivery vectors: progress and prospects. *Trends in Biotechnology*, *20*(12), 508–15.

Settanni, L., & Moschetti, G. (2010, September). Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. *Food Microbiology*.

Sharma, M., & Devi, M. (2014). Probiotics: a comprehensive approach toward health foods. *Critical Reviews in Food Science and Nutrition*, *54*(4), 537–52.

Shurety, W., Bright, N. A., & Luzio, J. P. (1996). The effects of cytochalasin D and phorbol myristate acetate on the apical endocytosis of ricin in polarised Caco-2 cells. *Journal of Cell Science*, *109 (Pt 1)*, 2927–2935.

Sing, A., Roggenkamp, A., Geiger, A. M., & Heesemann, J. (2002). *Yersinia enterocolitica* evasion of the host innate immune response by V antigen-induced IL-10 production of macrophages is abrogated in IL-10-deficient mice. *Journal of Immunology (Baltimore, Md : 1950)*, *168*(3), 1315–1321.

Sizemore, D. R., Branstrom, A. A., & Sadoff, J. C. (1997). Attenuated bacteria as a DNA delivery vehicle for DNA-mediated immunization. *Vaccine*, *15*(8), 804–807.

Spector, I., Shochet, N. R., Blasberger, D., & Kashman, Y. (1989). Latrunculins--novel marine macrolides that disrupt microfilament organization and affect cell growth: I. Comparison with cytochalasin D. *Cell Motility and the Cytoskeleton*, *13*(3), 127–144.

Staff, C., Mozaffari, F., Haller, B. K., Wahren, B., & Liljefors, M. (2011). A Phase I safety study of plasmid DNA immunization targeting carcinoembryonic antigen in colorectal cancer patients. *Vaccine*, *29*(39), 6817–6822.

Steidler, L. (2001). *Lactococcus lactis*, a tool for the delivery of therapeutic proteins treatment of IBD. *TheScientificWorldJournal*, *1*, 216–7.

Stiles, M. E., & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology*, *36*(1), 1–29.

Sung, J. J. Y., Lau, J. Y. W., Goh, K. L., Leung, W. K., & Asia Pacific Working Group on Colorectal Cancer, T. (2005). Increasing incidence of colorectal cancer in Asia: implications for screening. *The Lancet. Oncology*, *6*(11), 871–6.

Tao, L., Pavlova, S. I., Ji, X., Jin, L., & Spear, G. (2011). A novel plasmid for delivering genes into mammalian cells with noninvasive food and

commensal lactic acid bacteria. *Plasmid*, *65*(1), 8–14.

Tariq, K., & Ghias, K. (2016). Colorectal cancer carcinogenesis: a review of mechanisms. *Cancer Biology & Medicine*, *13*(1), 120–35.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-tieulent, J., & Jemal, A. (2015). Global Cancer Statistics, 2012. *CA: A Cancer Journal of Clinicians.*, *65*(2), 87–108.

Trémillon, N., Issaly, N., Mozo, J., Duvignau, T., Ginisty, H., Devic, E., & Poquet, I. (2010). Production and purification of staphylococcal nuclease in *Lactococcus lactis* using a new expression-secretion system and a pH-regulated mini-reactor. *Microbial Cell Factories*, *9*, 37.

Tsuruo, T., Iida, H., Tsukagoshi, S., & Sakurai, Y. (1981). Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Research*, *41*(5), 1967–1972.

Veettil, S. K., Lim, K. G., Chaiyakunapruk, N., Ching, S. M., & Abu Hassan, M. R. (2016). Colorectal cancer in Malaysia: Its burden and implications for a multiethnic country. *Asian Journal of Surgery*.

Vromman, F., Laverriere, M., Perrinet, S., Dufour, A., & Subtil, A. (2014). Quantitative monitoring of the *Chlamydia trachomatis* developmental cycle using GFP-expressing bacteria, microscopy and flow cytometry. *PLoS ONE*, *9*(6).

Wakatsuki, T., Schwab, B., Thompson, N. C., & Elson, E. L. (2001). Effects of cytochalasin D and latrunculin B on mechanical properties of cells. *Journal of Cell Science*, *114*(Pt 5), 1025–1036.

Wan, Y., Xin, Y., Zhang, C., Wu, D., Ding, D., Tang, L., Owusu, L., Bai, J., & Li, W. (2014). Fermentation supernatants of *Lactobacillus delbrueckii* inhibit growth of human colon cancer cells and induce apoptosis through a caspase 3-dependent pathway. *Oncology Letters*, *7*(5), 1738–1742.

Wang, H. H., Manuzon, M., Lehman, M., Wan, K., Luo, H., Wittum, T. E., Yousef, A., & Bakaletz, L. O. (2006). Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. *FEMS Microbiology Letters*, *254*(2), 226 LP-231.

Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C., Ventura, M., Goesmann, A., Gasson, M., Kuipers, O., Van Sinderen, D., & Kok, J. (2007). Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. cremoris MG1363. *Journal of Bacteriology*, *189*(8), 3256–3270.

Wells, J. M., Wilson, P. W., Norton, P. M., Gasson, M. J., & Le Page, R. W. (1993). *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. *Molecular Microbiology*, *8*(6), 1155–1162.

Wollowski, I., Rechkemmer, G., & Pool-Zobel, B. L. (2001). Protective role of probiotics and prebiotics in colon cancer. *The American Journal of Clinical Nutrition*, *73*(2), 451s–455s.

Wood, B. J. B., & Warner, P. J. (Eds.). (2003). *Genetics of Lactic Acid Bacteria*. Boston, MA: Springer US.

Yap, T. W. C., Rabu, A., Abu Bakar, F. D., Abdul Rahim, R., Mahadi, N. M., Illias, R. M., & Abdul Murad, A. M. (2014). Growth phase-dependent proteomes of the Malaysian isolated *Lactococcus lactis* dairy strain M4 using label-free qualitative shotgun proteomics analysis. *The Scientific World Journal*, *2014*.

Yeng, H. W., Shamsudin, M. N., & Rahim, R. A. (2009). Construction of an expression vector for *Lactococcus lactis* based on an indigenous cryptic plasmid. *African Journal of Biotechnology*, *8*(21), 5621–5626.