ANTAGONISTIC ACTIVITY OF *Lactobacillus plantarum* 0612 AGAINST SELECTED FOODBORNE ENTEROPATHOGENS

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ABSTRACT

Probiotic has been previously used for the treatment and prevention of intestinal disorders caused by enteropathogens. However, the antagonistic effect of probiotic on these pathogens in the gastrointestinal tract is not fully understood. The study aims to investigate the antagonistic ability of a probiotic strain, *Lactobacillus plantarum* 0612 against the adhesion of selected foodborne enteropathogens (*Escherichia coli* ATCC 11775, *Salmonella* Enteritidis ATCC 13076, *Listeria monocytogenes* ATCC 13932 and *Vibrio parahaemolyticus* ATCC 17802) to the colon epithelial Caco-2 cells by exclusion, competition and displacement conditions. *L. plantarum* 0612 has been subjected to the gastrointestinal transits simulation (GITS) sequentially prior to assess its competitive inhibition on selected human pathogenic strains using intestinal epithelial cells with different colonic pH conditions. The surface layer proteins that are involved in adhesion inhibition were separated using SDS-PAGE and further identified by MALDI-TOF/MS. Results showed that the adhesion of *E. coli* and *L. monocytogenes* on to Caco-2 cells was significantly inhibited by *L. plantarum* 0612 with a reduction of 4.35 log CFU/ml and 4.14 log CFU/ml, respectively in exclusion mechanism. However, *L. plantarum* 0612 exhibited a significantly stronger ($p<0.05$) competition activity against *V. parahaemolyticus* as compared to its exclusion and displacement activity. The exclusion and competition mechanisms seemed to be more effective against the colonization of *E. coli* in the presence of *L. plantarum* 0612. The GITS exposed *L. plantarum* 0612 showed significantly higher ($p<0.05$) efficacy of competitive inhibition against the selected foodborne enteropathogens in all colonic conditions as compared to the unexposed strain. The highest antagonistic activity was observed against the adhesion of *E. coli* and *V. parahaemolyticus* on the Caco-2 cells, with a log reduction of 5.10 log CFU/ml and 4.53 log CFU/ml respectively, in the colonic pH 5.0 after 8 hours of exposure. However, the GITS exposed *L. plantarum* 0612 significantly reduced ($p<0.05$) the adhesion of *L. monocytogenes* (4.20 log reduction) and *S. Enteriditis* (4.12 log reduction) respectively in the same colonic condition. It seems the colonic pH of the intestines could influence the antagonism of *L. plantarum* 0612 with the highest anti-adhesion against the bacterial pathogens was shown at pH 5.0 and 6.0, signifying the protective role of probiotic in the human proximal colon. Six cell surface-associated proteins (30s ribosomal protein, ATP synthase subunit beta, enolase, phosphoglycerate kinase, lactate dehydrogenase, and glycealdehyde-3-phosphate dehydrogenase) and 6 adhesive moonlighting proteins, (elongation-factor Tu, 60 kDa chaperonin GroEL, pyruvate kinase, triosephosphate isomerase, and 2, 3 bisphosphoglycerate-dependant phosphoglycerate mutase fructose-bisphosphate aldolase) have been identified using MALDI-TOF/TOF mass spectrometry. The expression of these adhesive surface proteins seemed to be influenced by the colonic pH conditions. Therefore, the existing of surface adhesive proteins in *L. plantarum* 0612 is expected to be responsible for its adhesion and antagonistic effects against the foodborne enteropathogens in human colonic epithelial cells. The excellent adherence and the antagonistic properties of *L. plantarum* 0612 against the enteropathogens are promising for the prevention and management of foodborne infections/diseases.

ABSTRAK

AKTIVITI ANTAGONISTIK Lactobacillus plantarum 0612 TERHADAP ENTEROPATHOGEN BAWAAN MAKANAN TERPILIH

Probiotik telah digunakan selama ini untuk tujuan rawatan dan pencegahan masalah gangguan usus yang disebabkan oleh enteropatogen. Namun, kesan antagonis probiotik terhadap patogen dalam saluran gastrousus masih belum difahami secara mendalam. Kajian ini bertujuan untuk mengetengahkan keupayaan dan kesan antagonistik melalui penyingkiran, persaingan dan penyesaran strain probiotik Lactobacillus plantarum 0612 terhadap pelekatan empat jenis enteropatogen bawaan makanan (Escherichia coli ATCC 11775, Salmonella enteritidis ATCC 13076, Listeria monocytogenes ATCC 13932 dan Vibrio parahaemolyticus ATCC 17802) di permukaan sel usus Caco-2. Inokulasi L. plantarum 0612 ke dalam simulasi transit gastrousus (GITS) berurutan dinilai dari segi perencatan persaingannya ke atas strain patogen bawaan makanan terpilih dengan menggunakan sel epitelium usus dalam keadaan pH kolon yang berbeza. Protein dari lapisan permukaan dinding sel yang terlibat dalam perencatan lekatan telah dianalisis dan dikenalpasti menggunakan SDS-PAGE dan MALDI-TOF/MS. Hasil kajian menunjukkan bahawa kesan rencatan melalui mekanisma penyingkiran E. coli dan L. monocytogenes pada permukaan sel Caco-2 oleh L. plantarum 0612 adalah sangat ketara dengan penurunan masing-masing sebanyak 4.35 log CFU/ml dan 4.14 log CFU/ml. Namun, toleransi GITS L. plantarum 0612 ($p<0.05$) terhadap aktiviti persaingan adalah jauh lebih kuat terhadap V. parahaemolyticus berbanding dengan aktiviti pengecualian dan penyesaran. Mekanisma pengecualian dan persaingan seolah-olahnya lebih berkesan terhadap perencatan E. coli dengan kehadiran L. plantarum 0612. L. plantarum 0612 terdedah GITS menunjuknan keberkesanannya yang ketara ($p<0.05$) terhadap perencatan kompetitif enteropathogens bawaan makanan yang dipilih dalam semua keadaan kolon berbanding dengan strain yang tidak terdedah. Aktiviti antagonistik tertinggi L. plantarum 0612 ditunjukkan terhadap pelekatan E. coli dan V . parahaemolyticus pada sel Caco-2, dengan pengurangan masing-masing sebanyak 5.10 log CFU / ml dan 4.53 log CFU / ml selepas pendedahan 8 jam kepada kolon pH 5.0. Walau bagaimanapun, L. plantarum 0612 terdedah GITS berkurangan ($p <0.05$) terhadap L. monocytogenes (pengurangan log 4.20) dan S. Enteriditis (pengurangan log 4.12) dalam keadaan kolon yang sama. Ini menunjukkan yang pH kolon usus boleh mempengaruhi kesan antagonistik L. plantarum 0612 dengan nilai anti-lekatan yang paling tinggi terhadap patogen bakteria telah ditunjukkan pada pH 5.0 dan 6.0. sekaligus menyokong keberkesanan perlindungan probiotik dalam usus proksimal manusia. Enam protein dari lapisan permukaan dinding sel yang berkaitan (30s protein ribosom, ATP synthase subunit beta, enolase, phosphoglycerate kinase, dehidrogenase laktat, dan glycealdehyde-3-fosfat dehidrogenase) dan 6 pelekat "moonlighting protein", (pemanjangan faktor-Tu, 60 kDa chaperonin GroEL, pyruvate kinase, isomerase triosephosphate, dan 2, 3 bisphosphoglycerate phosphoglycerate mutase) telah dikenal pasti menggunakan spektrometri jisim MALDI-TOF/TOF. Ekspresi protein pelekat dari permukaan dinding sel seolah-olah dipengaruhi oleh keadaan pH kolon. Oleh itu, protein pelekat yang sedia ada di permukaan dinding sel L. plantarum 0612 dijangka bertanggungjawab untuk memberi kesan lekat dan kesan antagonistik terhadap enteropathogen bawaan makanan pada sel-sel epitelium kolon manusia.

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LIST OF SYMBOLS AND ABBREVIATIONS

A

AAD	: Antibiotic-associated diarrhea
ANOVA	: Analysis of Variance
ATCC	: American Type Culture Collection
AUD	: Australian Dollar

C

CFU	: Colony forming unit
CO_2	: Carbon dioxide
COI	: Cost of illness
^oC	: Celsius
cm	: Centimetre
CDC	: Centre for Disease Control, United States of America
CDAD	: *Clostridium difficile*-associated diarrhea
CD	: Crohn's disease

D

Da	: Dalton
DALY	: Disability-adjusted life year

E

et al.	: Et alii (and others)
EC	: *Escherichia coli*
EFSA	: European Food Safety Authority
ECDC	: European Centre for Disease Prevention and Control
EU	: European Union
EF-TU	: Elongation factor Tu
ENO	: Enolase

F

FAO : Food and Agriculture Organization

FDA : Food and Drug Administration, United States of America

G

g : Gram

GAPDH : Glyceraldehyde-3-phosphate dehydrogenase

H

HCl : Hydrochloric acid

HACCP : Hazard Analysis and Critical Control Point

h : hour

I

IBS : Irritable bowel syndrome

IBD : Inflammable bowel diseases

K

kg : Kilogram

kDa : Kilodalton

L

L : Litre

LiCl : Lithium chloride

LM : *Listeria monocytogenes*

LC : Liquid chromatography

LAB : Lactic acid bacteria

LDL : Low-density lipoprotein

M

µL : Microlitre

µg : Microgram

mg	: Miligram
mL	: Mililitre
mm	: Millimetre
µg/ml	: Microgram per millileter
mg/ml	: Milligram per millimeter
mg/L	: Milligram per liter
mol/L	: Mol per liter
M	: Molar
mM	: Millimolar
µM	: Micromolar
m/z	: mass to charge ratio
min	: Minute
MALDI	: Matrix-assited laser desorption/ionization
MS	: Mass spectrometry
MOH	: Ministry of health

N

nm	: Nanometre

P

%	: Percentage
±	: Plus-minus
pH	: Power of hydrogen
PBS	: Phosphate buffer sulphate
PAGE	: Polyacryl amide gel electrophoresis
PMF	: Peptide mass fingerprinting

S

NaCl	: Sodium chloride
NaOH	: Sodium hydroxide
SE	: *Salmonella* Enteritidis
STEC	: Shiga-toxin producing *Escherichia coli*
SDS	: Sodium dodecyl sulphate

SD : Standard deviation

SLP : Surface layer protein

SLAP : Surface layer adhesive protein

T

TSA : Tryptone soy agar

TSB : Tryptone soy broth

MS/MS : Tandem mass spectrometry

TOF : Time-of-flight

TPI : Triosephosphate isomerase

U

US : United State of America

USD : United State Dollar

UV : Ultra-violet

UC : Ulcerative colitis

V

v/v : Volume per volume

VP : *Vibrio parahaemolyticus*

W

w/v : Weight per volume

WHO : World Health Organization

WGI : World Gastrointestinal Institute

WTO : World Trade Organization

WTP : Willingness to pay

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Foodborne disease becomes an accumulative public health problem that is responsible for an extensive morbidity and mortality globally. Various pathogens (bacteria, viruses, and parasites) are known to cause more than 250 foodborne diseases of which the majority of deaths are caused by diarrheal diseases (Fleury *et al.,* 2008; Linscott, 2011). Food safety has emerged as a key global issue with international trade and public health implications. Among the bacterial pathogens, *Escherichia coli*, *Salmonella spp.*, *Listeria monocytogenes*, *Vibrio spp.*, *Campylobacter jejuni*, *Yersinia enterocolitica* and *Staphylococcus aureus* are the most important foodborne pathogens and represent a major public health problem worldwide. In general, all of the foodborne pathogens must compete with gut microflora to colonize in the human gastrointestinal (GI) tract specifically in the large intestine, where huge amounts of resident microbiota are colonized (Lawley and Walker, 2013). Some of these bacterial pathogens evolved the abilities to resist non-specific host defences, such as stomach acidity, peristalsis, mucosal cell exfoliation, intestinal mucins, and bacteriocins (dos Reis and Horn, 2010), adhere to intestinal epithelial and eventually colonize the epithelia, cause infection/illnesses to the host. Foodborne pathogens such as *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes* caused numerous gastrointestinal illnesses and death (CDC, 2013). The ability of acid- and/or bile- resistant enteric bacteria through the gastric transit increases their possibility of colonization on the intestinal lining and caused infection/illnesses (Thomas, Ockhuizen, and Suzuki, 2014).

In human gastrointestinal tract (GIT), it is crucial to maintain the intestinal microflora density as it increases from 10^1–10^4 microbial cells in the stomach and duodenum, 10^4–10^8 cells in the jejunum and ileum, 10^{10}–10^{12} cells in the colon and faeces (Booijink *et al.,* 2007; Dethlefsen *et al.,* 2006). The transit time in the large

intestine is highly variable, with range of 6-32 hours typically reported (Cook *et al.,* 2012). The pH values of the large intestine have been reported to lie within 5.26-6.72 (ascending colon) and 5.20-7.02 (descending colon). The reduction in the normal microflora has adverse effects on human well-being and can be frequently associated with greater host susceptibility to enteric pathogenic infection. Thus, administration of probiotics has been recognized to overcome problems associated with microflora imbalance, improving both gastrointestinal and general health.

Probiotics are "live microorganisms that when administered in adequate amounts confer a beneficial health effect on the host" (Boué *et al.,* 2016). The emergence of concept "probiotics" had created niche development in the food industry. Most probiotic strains used in the food industry belong to the lactic acid bacteria (LAB), especially from *Lactobacillus* and *Bifidobacterium* genus. LAB are the focus of probiotic research internationally and their health benefits include prevention of diarrhea (Wanke and Szajewska, 2014), reduction of cholesterol level (Jones *et al.,* 2012), pediatric atopic dermatitis prevention (Panduru *et al.,* 2015), relief of irritable bowel syndrome (Yoon *et al.,* 2014) and efficacy in management of lactose intolerance (Almeida *et al.,* 2012). Besides, the competitive exclusion properties of probiotics as well as their ability to displace and inhibit pathogens are most important for therapeutic manipulation of the enteric microbiota (Molinaro *et al.,* 2012; Ortiz *et al.,* 2014). Therefore, application of such approaches can contribute to expand the beneficial properties of the selected probiotics on human health against pathogen infection. However, the beneficial effects of the probiotics are known to be genus, species and strain specific against selected enteric pathogens (Shi *et al.,* 2016). Therefore, it is crucial to consider a particular probiotic strain for its effective and potential therapeutic use.

Despite of all the benefits discussed upon ingestion of LAB, it is suggested that the microorganisms must be capable to survive through gastrointestinal tract with low pH environment and destructive bile salt (Vandenplas *et al.,* 2015). Besides, the potential probiotic must be able to adhere and colonize the intestinal cell wall, which is necessary to trigger any direct interactions between probiotic and host cells leading to the competitive exclusion of pathogens and modulation of host

cell responses (Van Baarlen *et al.,* 2013). Moreover, it must exhibit significant antimicrobial activity against pathogenic bacteria while remain safe for human consumption (Bull *et al.,* 2013). In addition, the probiotic selected must be able to survive and retain their functionality upon passing through the harsh industrial processing operations so that the dose supplemented is sufficient to proliferate in the gut, thus provide the beneficial properties to the host (Sánchez *et al.,* 2012). Some studies demonstrated the ability of probiotic bacteria to inhibit the colonization of *Escherichia coli* and *Clostridium difficile* from human intestinal epithelial cells (Tejero-Sarinena *et al.,* 2012; Schoster *et al.,* 2013). In fact, both *L. crispatus* (Chen *et al.,* 2007) and *L. helveticus* (Johnson-Henry *et al*., 2009; Wine *et al*., 2009) have been shown to competitively exclude enteropathogenic bacteria from Caco-2 epithelial cells. *L. paracasei* subp. *paracasei* M5-L, *L. rhamnous* J10-L and *L. casei* Q8-L were effective in inhibiting adhesion of *S. sonnei* to HT-29 cells (Zhang *et al.,* 2010). The competitive exclusion by probiotic is based on a bacteria-to-bacteria interaction mediated by the competition for the available nutrients and adhesion sites (Bermudez-Brito *et al*., 2012). Nevertheless, the degree of competition is strain dependent (Johnson *et al.,* 2016).

The knowledge related to probiotic tremendously arisen in recent years, yet the underlying functional mechanisms of probiotic are still not fully understood. It is widely accepted that adhesion and colonization of probiotics to the intestinal mucosa is considered as one of the most important selection criterion for persistent beneficial effects of probiotics (García-Cayuela *et al.,* 2014; Verdenell *et al.,* 2014). The initial adhesion stage of the probiotic bacteria to intestinal cell wall involves complex physiochemical interactions including hydrophobicity and charges (Ramos *et al.,* 2013; Yadav *et al.,* 2015). The surface proteins are expected to give appreciable effects on the properties of the cell wall of many *Lactobacillus* strains. Indigenous lactobacilli often showed the presence of specific, highly basic, hydrophobic cell surface proteins (Wasko *et al.,* 2014). Surface molecules are likely to play an important role in establishment of colonization and may be involved in the exclusion of intestinal pathogens (Antikainen *et al.,* 2007). Certain probiotics are found to utilize proteinaceous components, such as surface layer proteins (SLPs) to adhere on the intestinal cells (Meng *et al.,* 2014). The SLPs are two-dimensional,

highly porous crystalline arrays of subunits that presents the outermost structure of cell envelope in bacteria composed of glycoproteins or proteins, which represents up to 15% total protein of the bacterial cell (de sa Peixoto *et al.,* 2015). SLPs mediating bacterial adhesion to intestinal mucosa and epithelial cells have been demonstrated for many *Lactobacillus* species (Roselli *et al.,* 2016). Besides acting as cell adhesion mediators, SLPs are also believed to be capable of maintaining the cellular shape as well as playing an important role as immune modulators (Hynönen and Palva, 2013).

Recently, microbiologists have been exploring proteomics as a tool in research on adaptation of microorganisms to their environment. Several multifunctional proteins have been identified as associated with the cell surface and/or in the extracellular space. *L. plantarum* was found in many habitats and is a naturally occurring species in the gastro-intestinal tract of humans and animals. Cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as elongation factor Tu (EF-Tu), triosephosphate isomerase, and enolase were identified on the surface of *Lactobacillus plantarum* as molecules mediating adhesion to intestinal epithelial cells (Dhanani and Bagchi, 2013; Jensen *et al.,* 2014; Zhang *et al.,* 2015). Interestingly, surface proteins of lactobacillus are revealed to play an important role in inhibiting pathogens. Given that surface layer protein forms the outermost layer of gram-positive bacteria, this protein may potentially play a role in competitive exclusion of pathogens (Johnson-Henry *et al.,* 2007; Chen *et al.,* 2007). Surface layer protein was identified on the outer surface of *L. plantarum* 423, where it is supposed to play a role in adhesion to Caco-2 cells, as well as in the competitive exclusion of *Clostridium sporogenes* and *Enterococcus faecalis* (Ramiah *et al.,* 2008). After removal or disruption of the surface proteins from *Lactobacillus* species, the ability of adhesion and inhibition against pathogens decreased (do Carmo *et al.,* 2016; Bouchard *et al.,* 2013). Nevertheless, the use of new molecular omics-based technologies is tremendously increasing and it will apparently replace traditional screening methods. Omics technologies may also turn out to be very effective in the follow-up analysis of probiotic candidate strains resulting from *in vitro* and/or *in vivo* screening with current methodologies. Therefore, we hypothesize that GIT exposed *L. plantarum* 0612 could antagonize against the adhesion of selected