SURVIVAL KINETICS OF LACTOBACILLUS SPECIES DURING EXPOSURE TO SIMULATED GASTRIC FLUID

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UNIVERSITI MALAYSIA SABAH
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I hereby declare that the material in this thesis is original except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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DR. CHAN ENG SENG

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ABSTRACT

Survival Kinetics of Lactobacillus Species during Exposure to Simulated Gastric Fluid

The overall aim of this research was to develop a standard quantitative method to evaluate the acid tolerance of probiotic cells. The approach was to expose the cells of different concentrations (10^3 – 10^9 CFU/ml) to a range of pH (i.e. pH 1.5 to 2.5) in order to simulate the varying acidity of stomach. A standard simulated gastric fluid of fixed volume and three model probiotic cells were used in this study. The cell survival kinetics was determined and was described with a mathematical correlation. It was found that the overall death constant (k_d) for three tested strains increased with decrease in the pH and cell concentration. The death constant could be expressed by a general mathematical correlation, $k_d = k_{AII}[pH^{-9.02} \cdot N_0^{-0.19}]$ where k_{AII} is the acid intolerance indicator and N_0 is the initial cell concentration. The data fitting analysis of this equation give 0.97701 values for coefficient of determination. The k_{AII} values of *Lactobacillus acidophilus* ATCC4356 was found to be lowest followed by *Lactobacillus casei* Shirota and *Lactobacillus casei* 01 was the highest. This indicates decreasing in acid intolerance of the cells. In conclusion, a standard and quantitative method has been developed to measure the acid tolerance of probiotic cells and to facilitate selection of strain and process technology.

ABSTRAK

Secara umumnya kajian ini bertujuan merangka satu piawaian yang kuantitatif bagi menilai keupayaan *probiotic* hidup dalam medium asid. Pendekatan kami adalah dengan mendedahkan kepekatan sel yang berlainan (10^3 – 10^9 CFU/ml) ke dalam medium asid yang berjulat (pH 1.5 hingga pH 2.5) bagi menyimulasi keasidan perut yang berubah. Dalam kajian ini piawaian perihal bendalir perut buatan dengan kandungan isipadu tetap dan tiga jenis baka sel *probiotic* telah digunakan. Kinetic hidupan sel dalam medium asid telah digambarkan dengan mengalikasikan persamaan matematik. Didapati bahawa pemalar kepupusan keseluruhan (k_d) bagi kesemua baka bertambah dengan setiap pengurangan pH dan kepekatan muatan sel. Pemalar kepupusan boleh dijelaskan dengan persamaan matematik berikut, $k_d = k_{AII}[pH^{-9.02} \cdot N_0^{-0.19}]$ dimana k_{AII} adalah petunjuk ketidaktahanan asid dan N_0 adalah kepekatan muatan sel. Stastistik data analisis menunjukkan R^2 bersamaan 0.97701. *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* Shirota dan *Lactobacillus casei* 01 menunjukkan peningkatan nilai k_{AII} dan ini menunjukkan bahawa penurunan keupayaan sel berhidup dalam medium asid di kalangan sel-sel ini. Sebagai kesimpulan, satu keadah paiwaian yang kuantitatif telah dirangka bagi menilai ketahanan asid dikalangan baka *probiotic* dan juga membantu dalam perihal pemilihan baka *probiotic* dan seterusnya cabaran teknologi pemprosesan.

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LIST OF ACRONYMS

ATCC	American Type Culture Collection
BHI	Brain-heart infusion
CFU	Colony forming per unit
DNA	Deoxyribonucleic acid
FOSHU	Food for Specific Health Use
LAB	Lactic acid bacteria
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MRD	Maximum recovery diluent
MRS	De Man, Gogosa, Sharpe
NFDM	Nonfat dry milk
OD	Optical density
PBS	Phosphate buffer saline
SGF	Simulated gastric fluid
SLB	Sodium lactate broth
USP	United States Pharmacopeia

LIST OF SYMBOLS

a_{pH}	pH coefficient
a_{N0}	Initial cell concentration coefficient
c	Number of colony forming
D	Dilution factor
k	Death rate of cell
k_{AII}	Acid intolerance indicator
k_d	Overall death constant
k_{pH}	Specific death constant for pH
k_{N_0}	Specific death constant for initial cell concentration
N	Viable cell count
N_0	Initial cell concentration
N_t	Viability count at time t
τ_d	Doubling time
μ_{net}	Net specific growth rates

CHAPTER 1

INTRODUCTION

1.1 Research Background

Perception on the role of diet virtual influences the health status of men and animal has been long understood. Lexis such as 'what we eat represents what we are', 'eat healthily' and 'healthy diet' are widespread phrases which have been utilized. Whereas, many discoveries have addressed that intestinal microflora are the key to host health, as they undertake a number of important biochemical, physiological and immunological activities. The concept of microorganism mixtures may modulate the intestinal environment, thus improved host health was first postulated by Elie Metchnikoff back in 1901 (Shortt, 1999). Traditionally, these microorganisms have been added to yogurt and other fermented foods include dairy, vegetable, fruit, meat and cereal products. These benefits and health potential diets are still available today and the microorganisms which involved in the production of healthy foods are known as 'probiotics' these days.

Since then, there has been tremendous growth in research pertaining to the use of probiotics as biotherapeutic agents. The principles of therapy and mechanisms of action for probiotics have been reported by many review papers. Among the documented therapeutic effects are treatments of diarrhea, improvement of lactose metabolism, prevention of hypercholesterolemia, and suppression colorectal cancer (Salminen *et al.*, 1993; Fooks *et al.*, 1999; McCracken and Gaskins, 1999; Sanders, 1999; Kailasapathy and Chin, 2000; Elmer, 2001; Wollowski *et al.*, 2001; Tuohy *et al.*, 2003; Prakash and Jones, 2005). Whereas, there are a few mechanisms action of probiotics in the intestinal tract have been observed; these include productions of antimicrobial bacteriocins (Jack *et al.*, 1995; Avonts *et al.*, 2004) and organic acids (Shortt, 1998; Fooks *et al.*, 1999; Makras and De Vuyst, 2006), competition for nutrients and adhesion sites on the intestinal wall (Fooks *et al.*, 1999), reduction in toxin-producing microorganism (Fooks *et al.*,

1999; Rafter, 2003) and modulation of the immune response (Mattila-Sandholm *et al*., 1999).

In addition, numerous publications address certain criteria that have been developed to evaluate the potential of microorganisms to function as therapeutic cells. In order for these probiotics to effectively confer benefit on the host, they must reach the distal ileum and colon in large quantities to facilitate adhesion and colonization (Kos *et al*., 2000; Salminen *et al*., 1993). However, the low pH and anti-microbial agents [e.g. pepsin (Zhu *et al*., 2006)] of gastric fluids in stomach provides a barrier against entry of bacteria into the intestinal tract. Therefore, resistance to the gastric fluid is one of the main criteria for selection of beneficial and therapeutic cell (Salminen *et al*., 1993; Shortt, 1999; Kailasaphty and Chin, 2000). The knowledge on the acid tolerance of the cells may also determine the need to apply process technology (e.g. encapsulation) to improve cell survival during transit through the gastro-intestinal tract. The acid tolerance of many beneficial and therapeutic bacteria has been studied in the past. Examples of such bacteria are *Lactobacillus* species, *Bifidobacterium* species, *Bacillus* spesies, *Sporolactobacillus* spesies, propionibacteria strains and *Streptococcus thermophilus*.

1.2 Research Problem

The common method to evaluate acid tolerance of cells is to expose the cells to a simulated gastric fluid (SGF) for certain amount of time. Generally, it was found that the acid tolerances of these bacteria are pH and strain dependent (Conway *et al*., 1987; Heidebach *et al*., 2009b; Huang and Adams, 2004). However, the previous finding remained qualitative and subjective because the evaluation and procedure were not standardized among studies. For instance, in many studies, acid tolerance test was based on a specific pH and there is no agreement to which pH should be used (Gbassi *et al*., 2009; Heidebach *et al*., 2009a; Mandal *et al*., 2006; Minelli *et al*., 200). Consequently, the cells were tested within a specific pH ranged from pH 1 to 4.

Additionally, there was no consistency over the initial cell concentration to be used and the initial cell concentration can range from 10^5 CFU/ml to 10^{10} CFU/ml

(Charteris *et al.*, 1998; Mathara *et al.*, 2008; Thantsha *et al.*, 2009). Most of the previous studies fail to notice that the probability of cell death during exposure to low pH can be related to initial cell present. Some of the study indicated that the cell survivability in high acidity medium were not affected by the initial cell concentration (Lee and Heo, 2000; Charalampopoulos *et al.*, 2003), while some study showed otherwise (Chandramouli *et al.*, 2004).

Furthermore, the composition of gastric fluid was not standardized. The materials varied from pepsin, saline, glucose, yeast extract, cysteine, disodium hydrogen phosphate, potassium dihydrogen phosphate to non-fat skim milk. It was found that a number of sugar compounds such as glucose, maltose and fructose may aid survival of probiotic at low pH (Charalampopoulos *et al.*, 2003; Corcoran *et al.*, 2005). Besides the skim-milk composition within simulated gastric fluid (Sultana *et al.*, 2000; Chandramouli *et al.*, 2004), immobilized materials (Chan and Zhang, 2005; Heidebach *et al.*, 2009a, b) are believed to have buffering effect which may relieve probiotic survival in acid medium.

Moreover, there was no satisfactory quantitative method to measure the acid tolerance of probiotic cells. In most cases, their acid tolerance was determined by comparing the viable count at the end of incubation in an acidic medium or by the fraction of cell survival (Cui *et al.*, 2000; Hyronimus *et al.*, 2000; Lian *et al.*, 2003; Huang and Adams, 2004; Kim *et al.*, 2007; Annan *et al.*, 2008; Mathara *et al.*, 2008; Zanoni *et al.*, 2008; Heidebach *et al.*, 2009).

As can be seen, the lack of standardized assay parameters (i.e. pH; initial cell concentration; simulated gastric fluid composition; simulated gastric fluid volume; physical state of cells) and quantitative analysis in determining the acid tolerance of probiotic cells has led to rationale to carry out this work. In view of the growing number of newly discovered probiotic strains and the greater regulatory control on probiotics, there is a pressing need to develop a systematic and standard method to quantitatively measure the acid tolerance of probiotic cells.

1.3 Research Objectives And Scope

The overall objective of this research is to develop a standard quantitative method to evaluate the acid tolerance of probiotic cells by standardizing the procedure, material and analysis. Three main parameters were considered in this study. They were the strain of probiotic cells, the pH of simulated gastric fluid and the initial cell concentration. Three model strains of probiotic cells were studied: *Lactobacillus casei* 01, *Lactobacillus casei* Shirota and *Lactobacillus acidophilus* ATCC 4356. The initial cell concentration was ranged from 10^3 to 10^9 CFU/ml since the cell concentration of most probiotic products falls within this range.

Our approach was to mimic the gastric fluid and gastric condition. The gastric fluid used was in accordance to the United States Pharmacopeia (USP) dissolution medium, where the solution was established by suspending 3.2 g/L of pepsin and 2.0 g/L of sodium chloride into HCl acidified deionized water (Wong *et al.*, 1997). Instead of exposing the cells to just one specific pH, the cells were incubated over a range of pH, from pH 1.5 to 2.5. The lower pH limit was chosen since it is the common pH value found in human stomach during fasting and the ease of generating kinetic data. On the other hand, the higher pH limit was selected because it was found that most probiotic cells could tolerate acidic medium of pH and above.

Subsequently the relationships of the cell survival over the pH range and cell concentration range were evaluated and described with mathematical functions. The acid tolerance of the cells was quantified by an indicator. The specific objectives are shown as follows:

i. To study the effect of pH on the survival kinetics of three model strains
ii. To study the effect of initial cell concentration on the survival kinetics of the cells
iii. To determine the effect of pH and initial cell concentration on death rate
iv. To develop an empirical mathematical model to describe the acid tolerance of cells
v. To describe the validity and limitation of the developed approach

1.4 Significance Of Study

The significances of this study are to develop:

i. A standardized acid tolerance indicator for probiotic cells
ii. A systematic method for quantitative measurement to compare the acid tolerance of different strains and to solve the discrepancy of data among various studies
iii. A standard strain evaluation and selection method to guarantee the efficacy of probiotic products

1.5 Thesis Layout

The details of the work are reported in the following five chapters. Brief contents of each chapter are discussed as follow.

Chapter 1 is devoted to the introduction of the thesis topic. A brief description on historical development of probiotic is discussed in research background. The assay parameters and analysis method on previous related studies in acid tolerance of probiotic are briefly discussed in research problem. The research objectives and scope of research are also presented in this chapter. Thesis layout of this research is also discussed in Chapter 1.

Chapter 2 focuses on a review of probiotic definition, history and development. The beneficial and therapeutic effects of probiotics are listed in this section. The mechanisms action of probiotic is also included. Brief discussions about probiotics products and criteria for selection of probiotics are presented. This chapter emphasized on quantification of cell acid tolerance and survival kinetic. The approach on analysis acid tolerance of cell is outlined. This chapter is enclosed by conclusion.

Chapter 3 discussed the materials and methods used in this research. Cultivation of probiotic and acid tolerance assay were outlined. Data analysis which emphasized on determination of kinetics parameters and overall death constant are presented.

Chapter 4 focuses on the survival kinetics of cells and the development of a mathematical model to describe the kinetics. The effects of pH and initial cell concentration on survival of three model strains were determined. A mathematical model was then developed. An acid intolerance factor to indicate the acid tolerance of cells is proposed. Statistical analysis were undertaken to validate the proposed model. Limitation, validation and application of proposed model are also outlined in this chapter.

Chapter 5 concludes the thesis. A summary emphasizing the contributions made towards this research is discussed.

CHAPTER 2

LITERATURE REVIEW

2.1 Definition Of Probiotics

Oral intake health potential of food containing live microorganisms was recognized in ancient times. However, the concept of probiotics was first scientifically reported by Elie Methchnikoff in his book of 'The Prolongation of Life' published in 1907. He postulated that intake of yogurt containing Bulgarian bacillus (subsequently named *Lactobacillus delbrueckii* subsp. *Bulgaricus*) and *Streptococcus thermophilus* suppress the putrefactive-type fermentation. Consumption of yogurt played an important role in maintaining health (Shortt, 1999). These have lead to the growing interest in the use of live microorganisms as biotherapeutic agents in man's health.

In early 20th century, Henry Tissier isolated bifidobacterium from breast-fed infants and reported clinical benefits from modulating the intestinal flora in infants suffering from diarrhea (Shortt, 1998; Shortt, 1999; Schrezenmeir and de Vrese, 2001). During early 1930s, Minoru Shirota developed fermented milks using an intestinal strain, *Lactobacillus acidophilus* Shirota (subsequently named *Lactobacillus casei* Shirota). By the 1950s, yogurts were often used in treating patients suffering from antibiotics' side effect (Shortt, 1999). The advances in microbiological methodologies and remarkable clinical evidence surrounding the beneficial bacteria at the late of the century have given new dimension and perspective to the subject.

In 1965, the term 'probiotic' was first proposed by Lilly and Stillwell to describe the beneficial bacteria. It is initially derived from the Greek language which means 'for life' and was used originally to describe substances secreted by one microorganism which stimulates the growth of another. Apparently, it was an antonym to *antibiotics*. Since then, probiotics have been defined in several ways based on observations made when these microorganisms are studied. Later in 1971, Sperti used it to describe tissue extracts that stimulate microbial growth. However