



UNIVERSITI PUTRA MALAYSIA

**PROBIOTIC CHARACTERISTICS OF *Bifidobacterium* spp.
BY IN VITRO ASSESSMENT**

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By

KALAI VAANI d/o VENGRASALAM

**Thesis Submitted in Fulfilment of the Requirements for
the Degree of Master of Science in the Faculty
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TABLE OF CONTENTS

| | Page |
|---|-------------|
| ACKNOWLEDGEMENTS | ii |
| LIST OF TABLES | ix |
| LIST OF FIGURES | xi |
| LIST OF PLATES | xii |
| LIST OF ABBREVIATIONS | xiii |
| ABSTRACT | xiv |
| ABSTRAK | xvii |
| CHAPTER | |
| 1 INTRODUCTION..... | 1 |
| 2 LITERATURE REVIEW..... | 5 |
| The Human Gastrointestinal Microflora..... | 5 |
| The Type and Counts of Gastrointestinal Bacteria Normally Found in Healthy Adults..... | 5 |
| Changes in the Intestinal Microflora With Age..... | 8 |
| Probiotics..... | 12 |
| Definition and The Micro-organisms Used as Probiotic..... | 12 |
| Improvement of Intestinal Microflora..... | 14 |
| Alleviation of Lactose Intolerance..... | 18 |
| Reduction of Serum Cholesterol..... | 22 |

| | |
|---|----|
| Antitumour and Anticarcinogenic Activity..... | 24 |
| Characteristics of an Effective Probiotic..... | 28 |
| Survival at the Upper Gastrointestinal Tract..... | 29 |
| Attachment and Colonisation to the Epithelial Cells in Small Intestine or Colon..... | 35 |
| Antagonistic Action Against Undesirable Organisms..... | 40 |
| Viability During Processing and Storage..... | 49 |
| Bifidobacteria..... | 50 |
| Glucose Metabolism..... | 52 |
| Bifidogenic Factor..... | 54 |
| 3 CHARACTERISATION OF <i>Bifidobacterium</i> STRAINS..... | 59 |
| Introduction..... | 59 |
| Materials and Methods..... | 60 |
| Bacterial Strains..... | 60 |
| Culture Medium and Storage Condition..... | 62 |
| Recovery of Cultures..... | 63 |
| Gram Stain..... | 63 |
| Catalase Test..... | 64 |
| Growth in Aerobic Condition..... | 64 |
| Carbohydrate Fermentation Profile..... | 64 |
| Antimicrobial Susceptibility Testing..... | 65 |
| End Product Analysis by Using HPLC..... | 68 |
| Results and Discussion..... | 70 |

| | | |
|---|--|-----|
| | Gram Stain..... | 70 |
| | Catalase Test..... | 71 |
| | Growth in Aerobic Condition..... | 73 |
| | Carbohydrate Fermentation Profile..... | 73 |
| | Antimicrobial Susceptibility Testing..... | 82 |
| | Fermentation End Product Analysis..... | 99 |
| 4 | ACID AND BILE TOLERANCE OF BIFIDOBACTERIA..... | 104 |
| | Introduction..... | 104 |
| | Materials and Methods..... | 105 |
| | Survival In Vitro During Exposure to Low pH..... | 105 |
| | Growth of Bifidobacteria in the Presence of Bile..... | 106 |
| | Results and Discussion..... | 107 |
| | Survival In Vitro During Exposure to Low pH..... | 107 |
| | Growth of Bifidobacteria in the Presence of Bile..... | 116 |
| 5 | ANTIBACTERIAL ACTIVITY OF BIFIDOBACTERIA TOWARDS OTHER ORGANISMS..... | 125 |
| | Introduction..... | 125 |
| | Materials and Methods..... | 125 |
| | Bacterial Strains..... | 125 |
| | Culture Media and Growth Conditions..... | 126 |

| | |
|--|-----|
| Assay for Inhibitory Activity..... | 126 |
| Inhibitory Activity in Buffered and Sugar Limited Medium..... | 127 |
| Antibacterial Activity of Bifidobacteria in Skim Milk When Grown Together With <i>E. coli</i> | 128 |
| Results and Discussion..... | 130 |
| Inhibition of Other Organisms by Bifidobacteria..... | 130 |
| Antibacterial Activity of Bifidobacteria in Skim Milk When Grown Together with <i>E. coli</i> | 138 |
| 6 ACID PRODUCTION IN MILK AND SURVIVAL OF <i>Bifidobacterium</i> spp. IN ACIDIFIED MILK..... | 146 |
| Introduction..... | 146 |
| Materials and Methods..... | 147 |
| Bacterial Strains and Growth Conditions..... | 147 |
| Acidification of Milk..... | 147 |
| Viability During Storage in Acidified Milk..... | 148 |
| Results and Discussion..... | 149 |
| Acidification of Milk..... | 149 |
| Survival of Bifidobacteria in Acidified Milk Stored at Low Temperature..... | 154 |
| 7 GENERAL DISCUSSION AND CONCLUSION..... | 160 |
| General Discussion..... | 160 |
| Conclusion..... | 167 |
| BIBLIOGRAPHY..... | 168 |
| BIOGRAPHICAL SKETCH..... | 183 |



LIST OF TABLES

| Table | | Page |
|-------|--|------|
| 1 | The Species and Numbers of Bacteria Normally Found in the Microflora in Different Parts of the Adult Digestive Tract | 7 |
| 2 | List of <i>Bifidobacterium</i> Strains Obtained From ATCC and Used in This Study..... | 61 |
| 3 | Fermentation Profile of <i>Bifidobacterium</i> spp Obtained from ATCC, Tested Using API CH 50 Kit..... | 75 |
| 4 | Susceptibility of Bifidobacteria Obtained from ATCC to Cephalosporins, Sulfonamides and Tetracyclines, Tested Using Agar Diffusion Disc Assay..... | 83 |
| 5 | Susceptibility of Bifidobacteria Obtained from ATCC to Polypeptides, Macrolides and Nitrofurantoin Tested Using Agar Diffusion Disc Assay..... | 86 |
| 6 | Susceptibility of Bifidobacteria Obtained from ATCC to Penicillins and Phenicol, Tested Using Agar Diffusion Disc Assay..... | 89 |
| 7 | Susceptibility of Bifidobacteria Obtained from ATCC to Aminoglycosides, Tested Using Agar Diffusion Disc Assay..... | 91 |
| 8 | Susceptibility of Bifidobacteria Obtained from ATCC to Beta-lactams and Other Antibiotics, Tested Using Agar Diffusion Disc Assay..... | 94 |
| 9 | The Total Number of Antibiotics, Bifidobacteria Showed Resistance Against..... | 96 |



| | | |
|----|--|-----|
| 10 | End Product Analysis of <i>Bifidobacterium</i> spp. Ratio of Acetic Acid to Lactic Acid Produced by <i>Bifidobacterium</i> spp. Obtained by HPLC Analysis..... | 100 |
| 11 | Survival of Bifidobacterial Strains Obtained from ATCC in TPY Broth Adjusted to pH 2.5 at Various Exposure Time as Determined by HPLC Analysis..... | 109 |
| 12 | Classification of <i>Bifidobacterium</i> spp. Obtained from ATCC According to Their Survival in TPY Broth Adjusted to pH 2.5..... | 115 |
| 13 | Comparison of Growth Rate of Cultures of Bifidobacteria Obtained from ATCC in TPY Broth With and Without 0.3% Oxgall..... | 118 |
| 14 | Classification of <i>Bifidobacterium</i> spp. Obtained from ATCC According to Their Tolerance to Bile..... | 120 |
| 15 | Inhibitory Activity of <i>Bifidobacterium</i> spp. Obtained from ATCC Against Various Bacteria Tested by a Double Layer Assay..... | 131 |
| 16 | Classification of <i>Bifidobacterium</i> spp. According to Their Antimicrobial Activity Against Other Organisms..... | 136 |
| 17 | Alteration of the pH Value of Sterilised Milk by Different Strains of Bifidobacteria During 24 hr at 37° C..... | 150 |
| 18 | Classification of Bifidobacteria Based on Their Intensity of Acidification in Milk..... | 152 |
| 19 | Survival of Bifidobacteria Obtained from ATCC During Storage at 4° C in Sterilised Milk Acidified With Lactic Acid to pH 4.5-4.7..... | 155 |
| 20 | Classification of Bifidobacteria Obtained from ATCC Based on Their Survival in Storage at 4° C in Acidified Milk (pH 4.5-4.7) | 156 |



LIST OF FIGURES

| Figure | | Page |
|--------|--|------|
| 1 | Chromatograms of a Mix Standard Solution and Extract of <i>B. longum</i> (ATCC 15708)..... | 102 |
| 2 | Growth of <i>B. longum</i> (ATCC 15707), <i>B. bifidum</i> (ATCC 35914) and <i>B. breve</i> (ATCC 15701) in TPY Broth With and Without 0.3% Oxgall (bile)..... | 122 |
| 3 | Changes in Counts of <i>E. coli</i> in Skim Milk During Fermentation by Bifidobacteria and Lactic Acid Bacteria..... | 140 |
| 4 | Changes in pH of Skim Milk During Fermentation by Bifidobacteria and Lactic Acid Bacteria..... | 141 |



LIST OF PLATES

| Plate | | Page |
|-------|--|------|
| 1 | Photomicrograph of <i>B. breve</i> (ATCC 15700), Isolated from Intestine of Infants..... | 72 |
| 2 | Photomicrograph of <i>B. adolescentis</i> (ATCC 15703), Isolated from Intestine of Adult..... | 72 |
| 3 | Antibacterial Activity of Bifidobacteria Towards <i>L.monocytogenes</i> (ScottA)..... | 134 |
| 4 | Antibacterial Activity of Bifidobacteria Towards <i>S. enteritidis</i> | 134 |



LIST OF ABBREVIATIONS

| | |
|------|--|
| NCCL | National Committee for Clinical Laboratory |
| ATCC | American Type Culture Collection |
| nm | nanometre |
| µg | microgram |
| U | unit |
| °C | degree centigrade |
| ml | millilitre |
| hr | hour |
| rpm | round per minute |
| g | gram |
| mg | milligram |
| mm | millimetre |
| µl | microlitre |
| N | normality |
| min | minute(s) |
| mM | millimolar |
| µm | micrometre |
| > | more than |
| < | less than |
| % | percent |
| cfu | colony forming unit |



Abstract of the Thesis Presented to the Senate of
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for the Degree of Master of Science

**PROBIOTIC CHARACTERISTICS OF *Bifidobacterium* spp. BY IN
VITRO ASSESSMENT**

BY

KALAI VAANI D/O VENGRASALAM

Chairman : Dr. Mohd. Yazid Manap

Faculty : Food Science and Biotechnology

Probiotics are organisms which are natural gut isolates and used as dietary adjuncts that is believed to benefit man and animals. An organism to be selected as a probiotic should possess certain important characteristics. This project was designed to screen bifidobacterial strains to be used as dairy starter cultures. Few in vitro methodologies were selected for the above purpose. Eighteen strains of bifidobacteria were obtained from ATCC. Firstly characterization of the strains were carried out. All strains were gram positive rods of variable sizes with bifurcated Y and V forms, catalase negative and did not grow aerobically. Almost all the strains fermented glucose, fructose, galactose and lactose except few strains from honey bees did not ferment galactose and lactose. The fermentation characteristics of other sugars were comparable to previous reports on bifidobacteria. Most of the bifidobacteria strains tested were resistant to aminoglycosides, nalidixic acid, polymyxin B, colistin and sulfonamides. Tetracyclines, moxalactam, furazolidone,



nitrofurazone, cloxacillin and fusidic acid showed variable effectiveness. Bacitracin, erythromycin, oleandomycin, chloramphenicol, imipenam, nitrofurazone, penicillin, ampicillin, amoxicillin, carbenicillin and novobiocin were very inhibitory. Almost all the strains produced more or equal amount of acetic acid than lactic acid in the fermentation end product. The strain designated *B. adolescentis* (ATCC 11146) did not show the characteristics of bifidobacteria. The survival of bifidobacteria in low pH showed wide strain variation. *B. bifidum* (ATCC 35914), *B. breve* (ATCC 15698), and *B. infantis* (ATCC 27920) possess excellent acid tolerance followed by *B. adolescentis* (11146, 15705, and 15706). Growth in bile also showed strain variation. *B. longum* (ATCC 15707) had the best bile tolerance followed by *B. longum* (ATCC 15708). The others that showed good bile tolerance were *B. adolescentis* (ATCC 11146), *B. asteroides* (ATCC 25909 and 25910), and *B. breve* (ATCC 15700) followed by *B. indicum* (ATCC 25913) and *B. bifidum* (ATCC 35914). The double layered assay used to study the antibacterial activity of bifidobacteria against other organisms was found to be suitable to screen bifidobacteria strain for bacteriocin production. *Bifidobacterium* spp. inhibited *Pseudomonas aeruginosa*, *Bacillus megaterium*, *B. licheniformis*, *B. subtilis*, *B. sphaericus*, *E. coli*, *L. monocytogenes* and *S. enteritidis* in vitro. The inhibition was confirmed as due to the production of organic acids. The tested strains did not produce other inhibitory substances. The strains that possess the best inhibition activity include *B. adolescentis* (ATCC 15705), *B.*

breve (ATCC 15700), *B. infantis* (ATCC 27920 and 25962), *B. angulatum* (ATCC27535), *B. catenulatum* (ATCC 27539), *B. longum* (ATCC 15707 and 15708) and *B. pseudocatenulatum* (ATCC 27919). Extended incubation time was necessary before inhibitory activity against *E. coli* was detected when *B. breve* was grown together with *E. coli* in skim milk. *B. bifidum* (ATCC 35914), *B. breve* (ATCC 15698, 15700 and 15701), *B. infantis* (ATCC 27920) and *B. longum* (ATCC 15707) acidify skim milk at a faster rate with a 3% inoculation and formed smooth curd in less than 24 hours. The results of survival in acidified milk (pH 4.5-4,7) during cold storage (4°C) was comparable to the acid tolerance study. The strains that survived pH 2.5 for 90 minutes also survived storage in acidified milk for 5 weeks. The bifidobacteria strains selected for further studies based on the tests carried out include *B. bifidum* (ATCC 35914), *B. breve* (ATCC 15698), *B. breve* (ATCC 15701), *B. infantis* (ATCC 27920) and *B. longum* (ATCC 15707).

**Abstrak Tesis yang dikemukakan Kepada Senat
Universiti Pertanian Malaysia Sebagai Memenuhi Syarat
Keperluan Untuk Ijazah Master Sains**

**PENENTUAN SIFAT-SIFAT PROBIOTIK *Bifidobacterium* spp
SECARA PENILAIAN IN VITRO**

Oleh

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Probiotik merupakan organisma-organisma yang dipencil dari usus manusia dan haiwan, yang digunakan sebagai bahan tambahan dalam makanan. Tujuan kajian ini adalah untuk memilih strain bifidobakteria yang sesuai untuk digunakan sebagai kultur pemula dalam makanan tenusu. Beberapa kaedah in vitro telah dipilih untuk tujuan berkenaan. Sebanyak lapan belas strain bifidobakteria telah diperolehi dari ATCC. Pada mulanya sifat-sifat spesifik organisma berkenaan ditentukan untuk mengenal pasti dan menentukan identiti sebagai bifidobakteria. Kesemua organisma menunjukkan sifat sebagai rod gram positif yang terdiri dari berbagai saiz dengan bentuk unik Y dan V, katalase negatif dan tidak tumbuh dalam keadaan aerobik. Hampir kesemua strain fermentasikan glukosa, fruktosa, galaktosa dan laktosa kecuali beberapa strain dari lebah madu tidak fermentasikan galaktosa dan laktosa. Sifat fermentasi lain-lain gula adalah seperti yang dikemukakan dalam laporan-

laporan sebelum ini. Kebanyakan strain bifidobakteria boleh tumbuh dengan kehadiran aminoglikosida, asid nalidiksik, polimisin B, colistin dan sulfonamida. Antibiotik seperti tetrasiklina, moksalaktam, furazolidone, nitrofurazone, kloksasilin dan asid fusidik menunjukkan perencatan sederhana. Basitrasin, eritromisin, oleandomisin, kloramfenikol, imipenam, nitrofurazone, penisilin, ampisilin, amoksisilin, karbenisilin dan novobiosin merencat tumbesaran bifidobakteria dengan berkesan. Hampir kesemua strain menghasilkan lebih atau sama kuantiti asid asetik daripada asid laktik sebagai hasil fermentasi. Strain yang ditentukan sebagai *B. adolescentis* (ATCC 11146) tidak menunjukkan sifat bifidobakteria. Kebolehan sifat toleransi bifidobakteria terhadap keadaan asid menunjukkan berbagai variasi di antara strain. *B. bifidum* (ATCC 35914), *B. breve* (ATCC 15698), dan *B. infantis* (ATCC 27920) mempunyai rintangan yang baik terhadap asid diikuti oleh *B. adolescentis* (ATCC 11146, 15705, dan 15706). Pertumbuhan dalam cecair hempedu juga menunjukkan berbagai variasi antara strain. *B. longum* (ATCC 15707) menunjukkan pertumbuhan yang paling baik di ikuti oleh *B. longum* (ATCC 15708). Organisma lain yang menunjukkan rintangan yang baik terhadap cecair hempedu adalah *B. adolescentis* (ATCC 11146), *B. asteroides* (ATCC 25909 dan 25910), dan *B. breve* (ATCC 15700) diikuti oleh *B. indicum* (ATCC 25913) dan *B. bifidum* (ATCC 35914). ‘Cerakin dua lapisan’ yang digunakan untuk menentukan sifat antibakterial bifidobakteria terhadap organisma lain didapati berkesan untuk mengenali bifidobakteria yang

menghasilkan bakteriosin. *Bifidobacterium* spp. merencat pertumbuhan *Pseudomonas aeruginosa*, *Bacillus megaterium*, *B. licheniformis*, *B. subtilis*, *B. sphaericus*, *E. coli*, *L. monocytogenes* dan *S. enteritidis* dalam keadaan in vitro. Perencatan ditentukan sebagai perencatan asid dan bahan perencatan lain tidak dihasilkan oleh strain-strain yang dikaji. Strain-strain yang didapati mempunyai sifat perencatan terbaik terdiri dari *B. adolescentis* (ATCC 15705), *B. breve* (ATCC 15700), *B. infantis* (ATCC 27920 dan 25962), *B. angulatum* (ATCC 27535), *B. catenulatum* (ATCC 27539), *B. longum* (ATCC 15707 dan 15708) dan *B. pseudocatenulatum* (ATCC 27919). Masa inkubasi yang lama perlu sebelum bifidobakteria boleh merencat tumbesaran *E. coli* apabila keduanya diinokulat bersama dalam susu. *B. bifidum* (ATCC 35914), *B. breve* (ATCC 15698, 15700 dan 15701), *B. infantis* (ATCC 27920) dan *B. longum* (ATCC 15707) menunjukkan pengasidan yang baik di dalam susu dengan inokulasi sebanyak 3% dan menghasilkan kurd yang halus dalam masa kurang dari 24 jam. Keputusan ujian penstoran sejuk (4°C) dalam susu yang diasidifikasikan adalah setanding dengan ujian toleransi pada keadaan asid. Strain-strain yang menunjukkan toleransi yang baik pada pH 2.5 untuk masa selama 90 minit juga menunjukkan toleransi yang baik apabila ditambah pada susu yang diasidifikasikan untuk selama 5 minggu. Strain-strain bifidobakteria yang telah dipilih untuk kajian seterusnya terdiri daripada *B. bifidum* (ATCC 35914), *B. breve* (ATCC 15698), *B. breve* (ATCC 15701), *B. infantis* (ATCC 27920) dan *B. longum* (ATCC 15707).

CHAPTER 1

INTRODUCTION

Probiotics consist of viable mono or mixed culture of micro-organisms, ingested in the form of dried cells or in fermented food preparations that can benefit the host by maintaining the intestinal microbial balance. The word 'Probiotic' originated from Greek and it means 'pro-life'. The primary use of probiotics is to protect the integrity of the gut microflora. This can be achieved either by enhancing the growth of endogenous population of beneficial organisms or by increasing the numbers by routine continuous ingestion.

In healthy individuals, the composition of intestinal microflora is normally stable. However certain endogenous or exogenous factors can influence this equilibrium. Such factors include peristalsis disorder, cancer, surgical operations, radiation therapy, emotional stress, ageing and administration of antibiotics. These conditions, where the balance of the gut microflora is adversely affected, are all situations in which probiotics are of potential value.

Other claimed beneficial effects of probiotics include improvement of lactose tolerance, digestibility of milk products, anticarcinogenic properties,

reduction of serum cholesterol levels, increased calcium resorption, synthesis of vitamins and predigestion of proteins.

Lactic acid bacteria have been used for centuries in food preservation in many parts of the world. Nobel prize microbiologist, Elie Metchnikoff of the Pasteur Institute in his famous 'theory of longevity' postulated that, harmful effects produced by intestinal flora could be prevented by establishing the appropriate *Lactobacillus* flora in the gut. In recent years, the incorporation of intestinal species of organisms into fermented milk is gaining extensive interest.

Another genus, *Bifidobacterium spp.* has become popular since it's first isolation by Tissier in the period 1899 to 1900. They were thought to play an important role in human health since they were the predominant organism in the stools of breast fed infants. Bifidobacteria is an anaerobic, gram positive, non-sporing bacilli of varying morphology.

Numerous strains of lactobacilli and bifidobacteria have been isolated from diverse sources. And many probiotic preparations are available in the market that incorporate this two organisms. It has been widely accepted that, for an organism to qualify as a dietary adjunct, it should possess certain important traits. Besides being normal inhabitants of the gastrointestinal tract,



it should be able to survive passage through the upper digestive tract to reach the intestine. In other words, it should be able to tolerate the acidic condition in the stomach and the bile secreted into the duodenum. It should produce desired effect(s) in the intestinal tract. These may comprise antagonistic action against pathogenic organisms, assimilation of cholesterol and so on. And the organism of choice should be able to attach and colonise the intestine.

Besides the above mentioned properties, strains that possess certain level of tolerance towards commonly used antibiotics and a high rate of acid production in milk would have added advantage. The candidate organisms too, should maintain its viability in carrier food before consumption and most importantly it should be non pathogenic or non toxic.

Even though many food preparations are available in the market at present, facts regarding the effectiveness of the probiotic organisms are very scant. Therefore extensive research is being carried out to select the suitable organisms for probiotic preparations including the isolation of new strains. All the above mentioned properties are being taken into consideration in the effort to identify the correct organisms to be used as dietary supplements.

As it is not feasible to carry out such experiments in vivo without detail information, in vitro experiments are carried out beforehand to select the right

organisms. Although the result of in vitro experiments do not guarantee an organism's effectiveness in vivo, it is likely that carefully selected strains will have added advantages in performing their role as probiotics.

Therefore the main objective of this study, was to select suitable strains of bifidobacteria that possess these probiotic characteristics to be incorporated into fermented foods. We have included strains other than natural inhabitants of the human GIT as comparisons. Few in vitro methodologies have been selected for the screening purposes.

The specific objectives of this study were to determine:

- 1) the carbohydrate fermentation profile of *Bifidobacterium* spp. and their sensitivity to various antibiotics.
- 2) the survival of *Bifidobacterium* spp. at low pH and their growth rate in the presence of bile acids,
- 3) the antibacterial activity of *Bifidobacterium* spp. against undesirable bacteria and,
- 4) the rate of acid production in skim milk and survival during storage in fermented food.

CHAPTER 2

LITERATURE REVIEW

The Human Gastrointestinal Microflora

Man is exposed to bacterial contamination from his environment since birth. Some of these organisms, both from the birth canal and the environment may infect orally and colonise the digestive tract and become the normal intestinal flora. The colonisation of the intestine depends on the organism's ability to compete with other organisms as well as its interaction with the host physiology. Factors such as host physiological mechanisms, bacterial interactions and exogenous factors such as diet and antibiotics are all interrelated in determining the nature and distribution of bacteria in the gastrointestinal tract. Changes in physiological status of the host caused by ageing, changes in nutritional and lifestyle may modify the intestinal flora.

The Type and Counts of Gastrointestinal Bacteria Normally Found in Healthy Adults

The normal gut flora in human consists of an enormously complex ecosystem which live in a stable relationship with the host. This includes a diverse bacterial genera comprising Gram-negative and Gram-positive, anaerobic and facultatively anaerobic rods, and cocci.