5-13-2006

Evaluation of the Antimicrobial Activity of a Bifidobacteria Mix against Escherichia Coli 0157:H7 under Aerobic Conditions

Chenbo Wang

EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF A BIFIDOBACTERIA MIX AGAINST *ESCHERICHIA COLI* O157:H7 UNDER AEROBIC CONDITIONS

By

Chenbo Wang

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Food Science and Technology
in the Department of Food Science, Nutrition and Health Promotion

Mississippi State, Mississippi

May 2006

EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF A BIFIDOBACTERIA MIX AGAINST *ESCHERICHIA COLI* O157:H7 UNDER AEROBIC CONDITIONS

By

Chenbo Wang

Approved:

Juan L. Silva Professor of Food Science, Nutrition and Health Promotion (Director of Thesis) (Committee Member)	Charles H. White Professor and Graduate Coordinator for Food Science and Technology (Committee member)
M. Wes Schilling Assistant Professor of Food Science, Nutrition and Health Promotion (Committee member)	Vance H. Watson Dean of the College of Agriculture and Life Sciences

Name: Chenbo Wang

Date of Degree: May 13, 2006

Institution: Mississippi State University

Major Field: Food Science and Technology

Major Professor: Dr. Juan L. Silva

Title of Study: EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF A BIFIDOBACTERIA MIX AGAINST *ESCHERICHIA COLI* O157:H7 UNDER AEROBIC CONDITIONS

Pages in Study: 94

Candidate for Degree of Master of Science

A bifidobacteria mix (nine strains) was evaluated for its effect on the growth and survival of *Escherichia coli* O157:H7 (ATCC 43890) in 11% NFDM and MRS broth under the optimum growth conditions for *E. coli* O157:H7 growth (37°C, aerobic). Preliminary experiments were conducted to obtain the growth curves of the 9 strains of bifidobacteria and *E. coli* O157:H7 and confirm the inhibitory effect of acidity on *E. coli* O157:H7 (pH of media adjusted to 3.8). Acid-adapted *E. coli* O157:H7 showed no difference in resistance toward bifidobacteria ($P>0.05$) when compared to the non-acid adapted one. *Escherichia coli* O157:H7 did not survive in the supernatant of the bifodbacteria mix collected after incubation (37°C) with aerobic shaking (8 h). However, the pathogen was able to grow after the pH of the supernatant was adjusted to 6.50 (pH of fresh MRS broth). Results suggest that a high content of bifidobacteria has a strong inhibitory effect on *E. coli* O157:H7, in part due

to the low pH. However, products from bifidobacteria may also exert inhibitory effects.

ACKNOWLEDGEMENTS

I would like to express my deep appreciation and gratitude to Dr. Juan L. Silva, for all his support and guidance, teaching and help during my study at Mississippi State University. I also thank Dr. Yoshen Chen for her teaching and guidance. Without her idea and advice this work would have been impossible.

I am thankful to Dr. Mark W. Schilling for assistance with statistical analysis of data. Gratitude is also expressed to my other committee members, Dr. Charles H. White and Dr. W. Benjy Mikel, for providing their time and insightful comments.

I am especially thankful to Wei-Lien Weng, for her friendship and support. I also appreciate very much our other laboratory members: Dr. Taejo Kim, Jelena Stojanovic, Youkai Lu, John McGillivray, Sovann Kin, Bang-Yuan Chen and Wei-Chun Chen for their help, support and encouragement.

Love and thanks to my family and Dr. Jianxia Du for their encouragement, patience and support throughout my graduate study.

TABLE OF CONTENTS

LIST OF TABLES

TABLE Page

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

Bifidobacteria, regarded as probiotics, have generated significant research and commercial interest in recent years. “They are desirable, health-promoting bacteria, with a saccharolytic and acidogenic physiology, and without involvement in putrefying or toxigenic reactions or pathogenicity” (Crittenden, 2004). Fermented or unfermented dairy foods, like milk, yoghurt, ice cream and cheese, are the most popular food vehicles that are used to deliver these cultures (Boylston, *et al.*, 2004). They may also be found in powdered infant formulas, fermented soybean and oat-based products, and in the fermentation of some animals’ (goat’s, sheep’s and camel’s) milk. Although applications have developed very quickly, there are always numerous challenges in maintaining a high level of viable and functional bifidobacteria cultures throughout the shelf life of products, due to their strict anaerobic and limited acid tolerance characteristics (Crittenden, 2004). Therefore, the research on the response of bifidobacteria under unfavorable conditions requires more attention. This may help to extend the application of bifidobacteria in the industry and provide cost saving during processing.

One of the most important health properties of bifidobacteria is their antimicrobial activity toward pathogens. Bifidobacteria have been reported to inhibit *Clostridium perfringens*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Bacteroides vulgatus* and *Escherichia coli* when there is a large amount of viable cells (Leahy *et al.*, 2005). Many authors suggest that the inhibitory effect of bifidobacteria on pathogens in foods is derived from the production of organic acids, hydrogen peroxide, bacteriocins and other antimicrobial compounds when a large number of viable and functional colonies are present (Leahy *et al.*, 2005).

Escherichia coli O157:H7 is one of the most common food-borne pathogens and is a public health concern all over the world. Infected people may develop severe diarrhea, abdominal cramps, fever and/or blood in the stool. Its severe consequences of infection (especially to young children and old people), low infectious dose, and strong ability to survive under poor conditions make it imperative to eliminate it in foods (CDC, 2006).

Researchers have confirmed the inhibitory effect of bifidobacteria on *E. coli* O157:H7. However, methods used in experiments emphasize the need for anaerobic conditions and a large number of viable bifidobacteria. Little information is available about the influence of bifidobacteria on *E. coli* O157:H7 under aerobic conditions.

Research in this area may deflate the limitation of bifidobacteria and lead to its application in medicines and other food products besides dairy.

Therefore, the objectives of this project were: to study the inhibitory effect of a bifidobacteria mix (nine strains) on *E. coli* O157:H7 under optimum growth conditions for *E. coli* (37°C, aerobic), and to analyze the effect of pH of media and supernatant created by bifidobacteria under aerobic conditions, on *E. coli* O157:H7.

CHAPTER II

LITERATURE REVIEW

Bifidobacteria: History

Bifidobacteria was first discovered by Henry Tissier (1900) in 1899 at the Pasteur Institute, Paris, France. It was isolated from the feces of breast-fed infants and has an irregular Y shape. At the same time, a bacterium Moro (1900) discovered in similar conditions was considered to belong to the genus *Lactobcillus*, because of its rod-like shape and obligate fermentative characteristics. With the discovery of fructose-6-phosphoketolase (F6PPK) (characteristic enzyme in bifidobacteria) and specific DNA segment, the genus *Bifidoabcium* was found to be different from that of *Lactobacillus*, *Corynebacterium*, and *Propionibacterium* (Sebald *et al*. and Werner *et al*., 1965). Therefore, in the 8th editon of Bergey's Manual of Determinative Bacteriology, *bifidobacterium* was regarded as an individual genus and constituted 11 species (Rogosa, 1974). Presently, the genus has reached more than 30 species (Appendix Table 1), isolated mainly from the gastrointestinal tract of various animals and humans. (Biavati *et al*., 2000)

Characteristics of Bifidobacteria

Morphology

Bifidobacteria are Gram-positive, non-sporeforming, non-motile, irregular rods. Some of them are curved, some are clubbed and most are branched. Bifidobacteria include cornynebacteria, mycobacteria and streptomycetes (Embley and Stackerandt, 1994). In unnatural habitat, strains of bifidobacteria may have different forms, like bifurcated Y and V forms, spatulate or club shapes, branching and pleomorphism. Different composition and different levels of certain constituents of the culture medium are also responsible for different shapes of bifidobacteria strains (Mayer *et al.*, 1950). The amount of N-acetylglucosamine, alanine, aspartic acid, glutamic acid, serine and Ca^{2+} ions in the growth medium influence the cell shape of bifidobacteria (Scardovi, 1984).

Ecology

All species of bifidobacteria are found in six different habitats: the human intestine, oral cavity, food, the animal gastrointestinal tract (GIT), the insect intestine, and sewage (Appendix Table 1). Bifidobacteria is a major part of the normal intestinal microflora in humans for all their life and about 3% of the total fecal microflora of adults (Sghir *et al.*, 2000). They appear in the feces of human infants a few days after

their birth and then continue to increase in number after that. Bifidobacteria enters the body of infants through their mouth. Several factors, like methods of feeding, constituents of milk and environment may influence the growth of bifidobacteria *in vivo*. Breast-fed babies were found to have much more bifidobacteria in the infant gut than formula-fed infants. This might be attributed to the bifidogenic factors present in human milk. (Harmsen *et al.*, 2000). The number of bifidobacteria in the colon of adults is 10^8 - 10^{11} CFU, but this number decreases with age. *Bifidobacterium adolescentis* and *B. longum* are major bifidobcteria species in the adult intestine and *B. infantis* and *B. breve* are predominant species in the intestinal tract of human infants (Mutai *et al.*, 1987; Gavini *et al.*, 2001). The number of viable *bifidobacteria* is regarded as a marker of the stability of the human intestinal microflora (Mutt and Tanaka, 1987).

Physiology

Bifidobacteria species isolated from humans grow at 36ºC-38ºC, whereas bifidobacteria from animals have a higher optimum growth temperature, 41-43ºC. Bifidobacteria cannot grow below 20 ºC. However, Simpson *et al.* (2004) discovered that *B. thermacidophilum* was able to grow at a maximal temperature of 49.5 ºC and *B. psychraerophilum* was shown to grow at as low as 4 ºC.

Bifidobacteria are acid-tolerant microorganisms. They also cannot survive in an environment at pH higher than 8.5 (Biavati *et al.*, 2000) The suitable pH is between 6.5 and 7.0. No growth is recorded at pH lower than 4.5; except for strains of *B. lactis* and *B. animalis*, which can survive at pH 3.5, and *B. thermoacidophilum*, which has a delayed growth at pH 4.0 (Matsumoto *et al.*, 2004).

Bifidobacteria are described as strictly anaerobic microorganisms (Scardovi 1984). However, the sensitivity toward oxygen can vary between species and between different strains within a species (Shimamura *et al.*, 1992; Ahn *et al.*, 2001). Hydrogen peroxide and superoxide are the products of fructose-6-phosphoketolase (F6PPK) under aerobic conditions. They are able to inactivate the enzyme and therefore cause the death of bifidobacteria.

There are three kinds of responses when conditions change from anaerobic to aerobic. First, the reduction of NAD-oxidase, NAD-peroxidase and superoxide dismutase helps *B. breve, B. infantis* and *B. longum* strains survive from oxygen toxicity. The weak catalase activity and the effect of NADH oxidase can remove or avoid the synthesis of hydrogen peroxide, which increases the tolerance of some strains to oxygen (Ventura *et al.*, 2004). Secondly, bifidobacteria strains are able to grow very slowly or just survive with the accumulation of hydrogen peroxide. Thirdly, some bifidobacteria strains require a low redox potential (accumulation of hydrogen

peroxide) for growth. The metabolism of oxygen dissimilation in bifidobacteria is shown in Figure 2.1 (Ballongue, 1993).

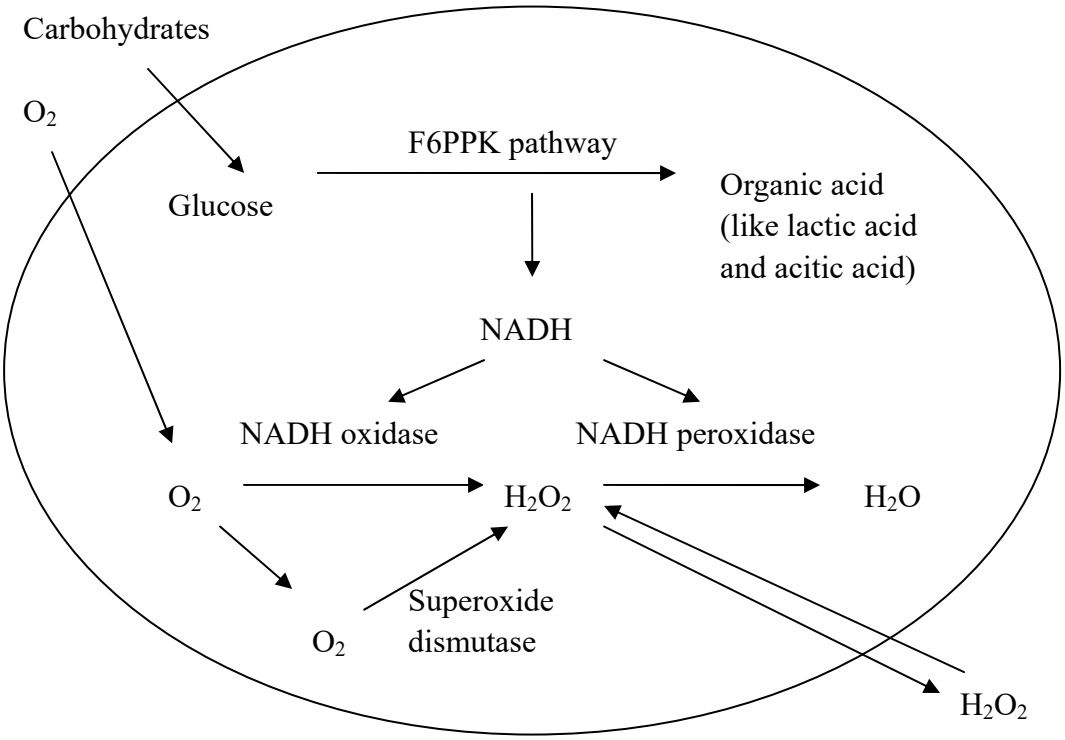
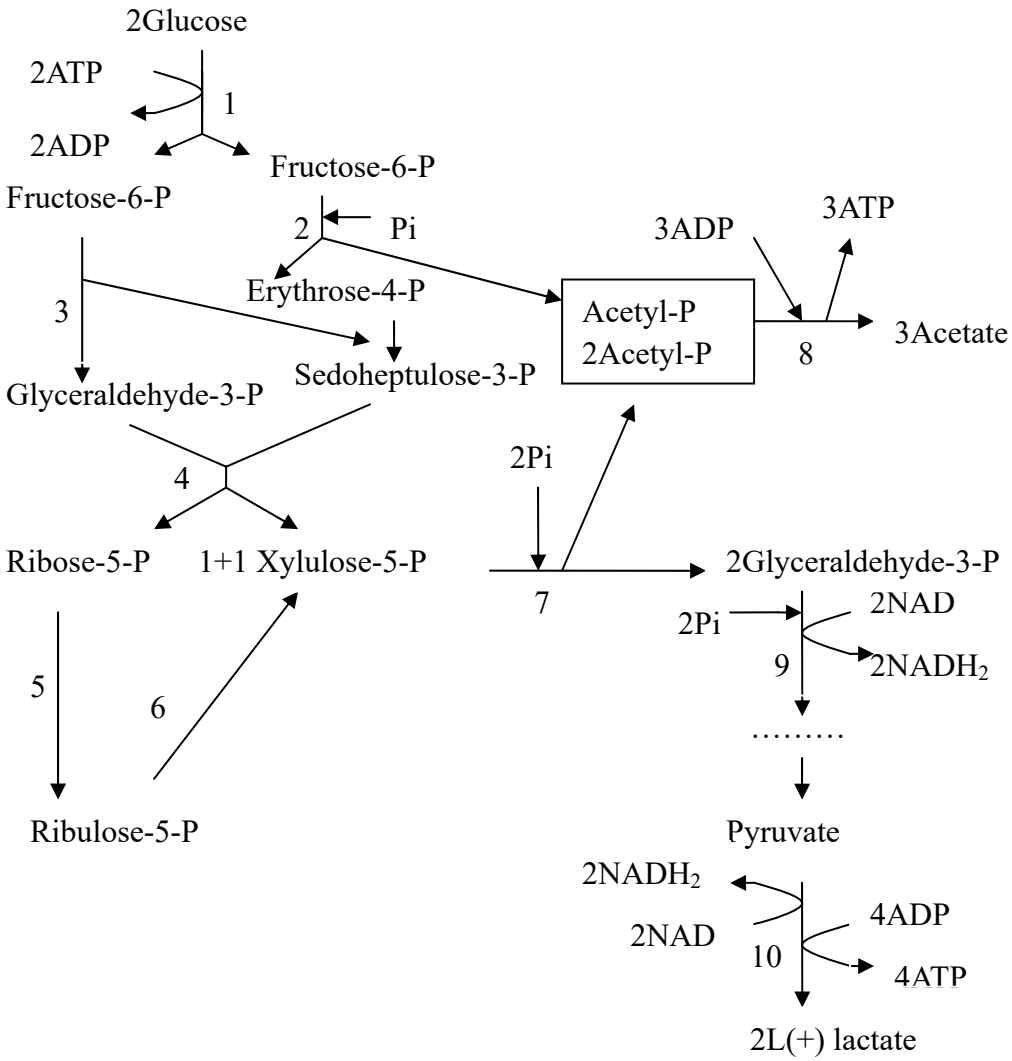


Figure 2.1 Oxygen dissimilation in *Bifidobacterium* (Ballongue, 1993)



1 = hexokinase and glucose-6- phosphate isomerase; 2 = fructose-6-phosphoketolase; 3 = transaldolase; 4 = transketolase; 5 = ribose-5-phosphate isomerase; 6 = ribulose-5-phosphate epimerase; 7 = xylulose-5-phosphate phosphocetolase; 8 = acetate kinase; 9 = homofermentative pathway enzymes; 10 = L(+) lactate dehydrogenase

Figure 2.2 Metabolic pathway of *Bifidobacterium* sp. (Tamine *et al.*, 1995)

Metabolism

Bifidobacteria create organic acids (Figure 2.2). Hexoses are degraded by F6PPK and this mechanism led to the discovery of this characteristic enzyme (Scardovi, 1986). Acetate and half of the lactate are created by the fermentation of glucose; the other half of the lactate is transferred from pyruvic acid through L(+) lactate dehydrogenase. (Tamine *et al.*, 1995)

Bifidobacteria strains isolated from human can also synthesize vitamins, thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), folic acid (B_9), cyanocobalamine (B_{12}) and nicotinic acid (PP) (Deguchi *et al.*, 1985).

As was mentioned, F6PPK is the key enzyme for bifidobacteria for the fermentation of sugars. There are three types of this enzyme, from three different ecological sources: mammalian, bee or human. (Scardovi *et al*., 1971) The F6PPK is also a specific enzyme to the genus which is absent in other anaerobic bacteria similarto bifidobacteria (Scardovi and Trovatelli, 1965). Therefore, the activity of F6PPK is often used to detect and identify bifidobacteria (formation of reddish-violet color in cellular extracts containing F6PPK). “The detection of F6PPK in cellular extracts, is considered to be the most reliable indication that Gram-positive rod-shaped bacteria belong to the genus Bifidobacterium” (Gavini *et al.*, 1996). The method has been modified several times. Orban and Patterson (2000) used the method

of incubation in hexadecyltrimethylaminonium bromide (CTAB) for five minutes instead of sonication to release cellular extract and to reduce cost and time. Other tests, like pulsed field gel electrophoresis (PFGE) of whole chromosomal DNA, random amplified polymorphic DNA (RAPD) assays, and genus–specific PCR primers, can be used to identify certain strains of bifidobacteria. However, most of these tests are expensive and time-consuming.

Health Benefits of Bifidobacteria

Bifidobacterium adolescentis, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis* and *B. longum* are probiotics (Holzapfel *et al.*, 1998). The health of humans and animals are improved when these viable cells are added to diets (Salminen et al., 1998). They help to synthesize the vitamins in yogurt and improve the absorption of minerals and protein (Ding *et al.*, 2005).

As for therapeutic benefits, bifidobacteria can be used to treat gastrointestinal tract diseases and lactose intolerance. In Egypt, *B. bifidum* combined with other probiotics reduced the incidence of travel diarrhea from 71 to 43% (Black et al., 1989). These bacteria can also improve host immune system, modulate abnormal inflammatory responses in allergies, bowel disease and also improve the function of immune system against pathogens and cancer cells (Crittenden, 2004). *Bifidobacterium longum* and *B. breve* were reported to prevent carcinogens from

affecting DNA. *Bifidobacterium longum* reduces the creation of tumors, and creates anticancer linoleic acid (Rosberg-Cody *et al.*, 2004). As for other diseases, bifidobacteria was also suggested to reduce serum cholesterol and alleviate constipation (Leahy *et al.*, 2005). Plummer *et al.* (2004) suggested that *B. bifidum* could also help to neutralize toxin and reduce the possibility of diarrhea caused by antibiotics.

Antagonistic Effect of Bifidobacteria on *Escherichia coli* O157:H7 - Mechanism

Antimicrobial activity of bifidobacteria *in vivo* is mainly based on adhesiveness properties, bacterial interference, production of antimicrobial substances and stimulation of mucosal immunity. Until now, research has emphasized that high levels of viable cells of bifidobacteria are necesary for antimicrobial activity. Therefore, all experiments has been conducted under anaerobic conditions, and not much information is available pertaining to the behavior of bifidobacteria under aerobic conditions.

Caco-2 and HT29-MTX cells are the constituents of intestinal epithelium. *Bifidobacterium adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. infantis*, *B. longum* and *B. pseudocatenulatum* can adhere to Caco-2 cells, and the mucus secreted by HT29-MTX cells, the places that are also adhered by pathogens. The competition for adherence between bifidobacteria and pathogens helps to reduce

the frequency of infection. *Bifidobacterium lactis* DR 10 interferes with the binding of *E. coli* O157:H7 to the intestinal cells, and therefore reduces the invasiveness of the pathogen (Servin, 2004). Chiang *et al.* (2000) also suggested that *B. lactis* induced intestinal mucin gene expression to modulate the barrier effect of the gut, that inhibiting *E. coli* adherence.

The main antimicrobial products of bifidobacteria are organic acids (lactic and acetic acid), hydrogen peroxide, and bacteriocins. Shelef (1994) cited that the inhibitory effect of organic acids were due to chelation of iron and inhibition of lactate dehydrogenase. Weak acids have stronger antimicrobial activity at low pH, making acetic acid more effective than other acids. Antimicrobial ability of organic acids depends on their undissociated form. Therefore, low pH is necessary. Lactic acid can reduce pH and permeate membranes of pathogens. Asahara *et al.* (2004) concluded that a high concentration of acetic acid and a low pH that can be created by bifidobacteria were able to inhibit Shiga-like toxin production of *E. coli* O157:H7. Hydrogen peroxide can oxidize cells of bacteria, sulfhydryl groups of cell proteins, and membrane lipids. Bacteriocins have antimicrobial properties and are thought to be natural preservatives. However, so far "there is not enough evidence to improve the effect of bacteriocins, collected from gram-positive bacteria, on gram-negative bacteria without the addition of any membrane-active compounds" (Ouwehand and

Vesterlund, 2004). Therefore, there is no evidence to make sure that bacteriocins can be utilized *in vitro*. More research needs to be accomplished. Besides the direct effect of bifidobacteria on the survival of pathogens, Li *et al.* (2005) suggested that bifidobacteria DNA can improve the activity of murine macrophages J774A.1 (immune system) *in vitro*.

***Escherichia coli* O157:H7**

Escherichia coli are one of the main bacteria that live in the intestines of humans and animals. They are Gram-negative, facultative, anaerobic rods. Most of them are harmless. However, there are six pathogenic groups: enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and diffusely adherent (DAEC). *Escherichia coli* O157:H7 belongs to the EHEC group and because of their ability to produce Shiga-like toxins (or verotoxins), they are also considered to be Shiga-like toxin producing *E. coli* (STEC) (or verotoxin *E. coli,* VTEC). (Levine, 1987)

The expression of somatic (O) antigen 157 and flagellar (H) antigen 7 defines *E. coli* O157:H7 and the bacteria were first isolated in the U.S. in 1975. In 1982, *E. coli* O157:H7 caused bloody diarrhea in two separate cases that occured in Oregon and Michigan. These outbreaks were associated with contaminated hamburgers (Konowalchuk *et al.*, 1977). The next year, a report on hemorrhagic

colitis (HC) confirmed *E. coli* O157:H7 as a pathogen (Riley *et al*., 1983). In 1985, Karmali *et al.* reported that STEC caused hemolytic uremic syndrome (HUS). Due to its wide range of distrubution vehicles and infection with limited colony number, *E. coli* O157:H7 has been regarded as one of the leading bacterial cause of human gastrointestinal disease. Large outbreaks have occurred in a number of countries and great attention has been received thereafter.

Escherichia coli O157:H7 is indole positive and oxidase negative. It can produce lactose-fermenting, nonmucoid colonies like other *E. coli*, whereas it fails to ferment sorbitol causing it to appear colorless, while other coliform bacteria produce pink colonies on MacConkey sorbitol agar. (Padhye and Doyle, 1992)

Although, MacConkey sorbitol agar is a good selective medium for *E. coli* O157:H7, it is useless on the recovery of injured or stressed colonies. Silk and Donnelly (1997) reported that Tryptic Soy Agar (TSA) or TSA overlayed with violet red bile agar can be used to recover injured and stressed *E. coli* O157:H7 colonies.

***Escherichia coli* O157:H7 Pathogenicity and Outbreaks**

Escherichia coli O157:H7 is a normal inoffensive bacterium that becomes pathogenic after being combined with foreign DNA through transfer (such as conjugation, bacteriophagic transfers or natural transformations (Boerlin, 1999;

Boerlin *et al*., 1999)). The main pathogenicity mechanisms of *E. coli* O157:H7 are described in Figure 2.3.

The first pathogenicity mechanism of *E. coli* O157:H7 is Locus for enterocyte effacement (LEE). This locus encodes a type III secretion system (TTSS), the translocated intimin receptor (Tir), intimin and other effector proteins (*E. coli*-secreted proteins (Esp)). These bacterial genes are able to attach pathogens to intestinal cells and destroy microvilli, known as the Attaching and Effacing (A/E) lesion. The TTSS is responsible for transporting virulent factors (Esp) directly to the cell cytoplasm (LeBlanc, 2003). Tir is regarded as an intimin receptor and does not phosphorylate in *E. coli* O157:H7 which is different with Enteropathogenic *E. coli* (EPEC) (Calderwood, 1996). Recent studies discovered that nucleolin and betal integrin were also present as receptors for intimin (function as a membrane adhesion (Frankel *et al.*, 1996)) on the luminal surface of intestinal epithelia during the infection of *E. coli* O157:H7 (Sinclair *et al.*, 2006).

The second pathogenicity mechanism of *E. coli* O157:H7 (Figure 2.3) is Shiga-like toxins (vero cytotoxins). Shiga toxin-producing *E. coli* may produce Stx1 or Stx2 or both of these two toxins (Chart, 2000). Stx2 was reported to be more toxic to human renal endothelial cells than Stx1 (Scotland *et al.*, 1987). Toxins have an AB structure, five B polypeptides linked with one A polypeptide (O'Brien and Holmes,

1987). After entering the cell through endocytosis, the B-pentamer plays an important role for toxins to bind to globotriaosylceramide (Gb3), which is in the cortex of the human kidney. (Sandvig and van Deurs, 1996; Schmidt *et al.*, 1996) Meanwhile, the A subunit is an N-glycosidase that inhibits the synthesis of proteins by cleaving a single adenine residue from the 28S (Sandvig *et al.*, 1992). In the intestine, the destruction of epithelial cells alters the secretion and absorption of electrolytes, which might cause diarrhea (Kandel *et al.*, 1989). Acetic acid and low pH was suggested for the inhibition of the Stx production of the pathogen (Asahara *et al.*, 2004)

The third pathogenicity mechanism of *E. coli* O157:H7 (Figure 2.3) plasmid. Its main virulent genes are *KatP*, *EspP*, *etpC-O*, *hlyA-D* and *toxB* (LeBlanc, 2003). Nataro and Kaper (1998) proposed that *KatP*, as a bifunctional catalase-peroxidase, could protect bacteria from oxidative stress derived from host cells. Since *EspP*, as plasmidic serine proteases, can dissolve Vero cells and destroy pepsin A and human coagulation factor V, it helps bacteria to access to host cells (Brunder *et al.*, 1997). A type II secretion system exists from *etpC* to *etpO* (LeBlanc, 2003). The four genes from *hlyA* to *hlyD* are responsible for the creation of EHEC-hemolysin (Brunder *et al.*, 1997).

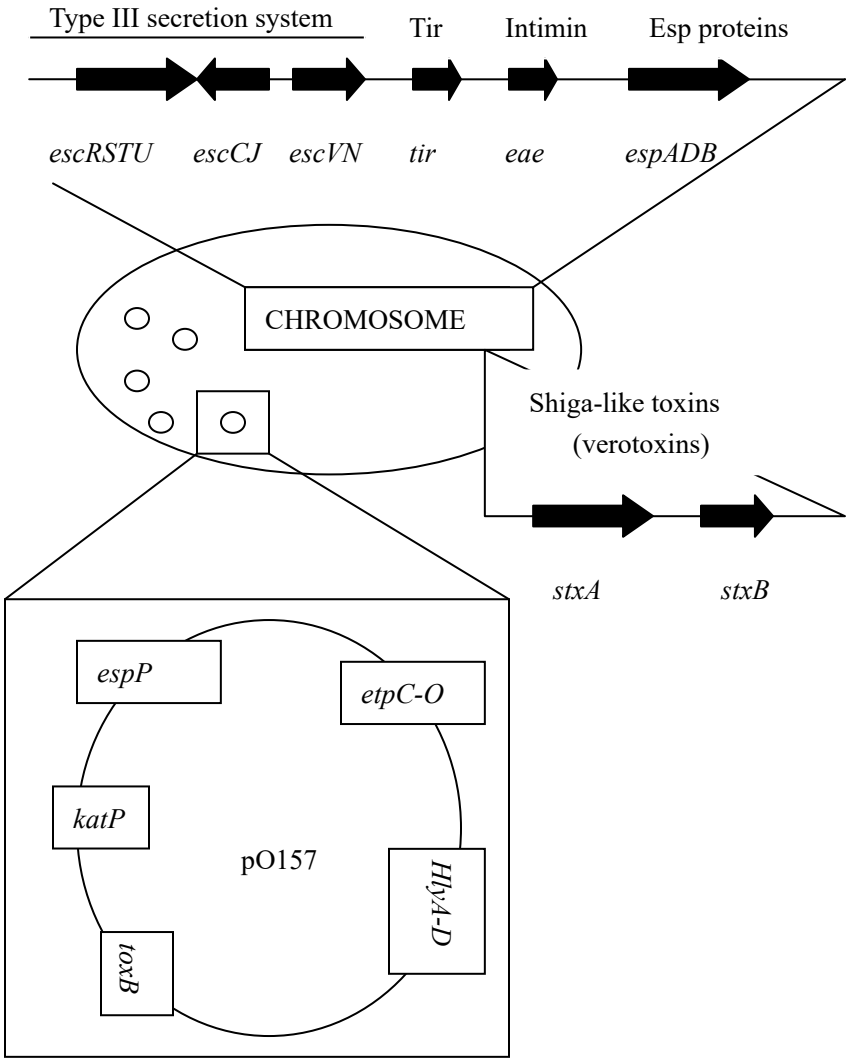


Figure 2.3 Schematic representation of the main pathogenicity mechanisms of *Escherichia coli* O157:H7 (LeBlanc, 2003)

The fourth pathogenicity mechanism of *E. coli* O157:H7 (Figure 2.3) is through Lipopolysaccharides (LPS). LPS is known as somatic (O) antigen and regarded as a marker for the detection of *E. coli* O157:H7 (De Boer and Heuvelink,

2000). Lipid A and O polysaccharides constitute LPS. The O polysaccharide was suggested to have an effect on the adherence of *E. coli* O157:H7 to the host cells. (Bilge *et al.*, 1996) Lipopolysaccharide and Stx2 have been found to induce the release of chemokines and other factors in the microvascular endothelial cells. These factors combine with low level of primary agonists to activate the renal thrombosis associated with hemolytic uremic syndrome (HUS). (Guessous *et al.*, 2005)

The ingestion of contaminated food (ground beef, sprouts, lettuce, salami, unpasteurized milk and juice) or water, or by person-to-person transmission, causes the *E. coli* O157:H7 infection. *Escherichia coli* O157:H7 mainly causes hemorrhagic colitis (HC), HUS, and/or thrombotic thrombocytopenic purpura (TTP). (Park *et al.*, 2001) The symptoms of these diseases are severe bloody diarrhea and abdominal cramps. Sometimes, a little fever might be present. Usually, the infection will end without specific treatment in 5-10 days and antibiotics and antimotility medications will induce the complications. Children under 5 years of age and elders will usually have HUS which is complicated from the *E. coli* O157:H7 infection, causing destruction of red blood cells and kidney failure (CDC, 2006). The TTP often occurs in adults. It lacks prodromal and therefore it is much more dangerous and less predictable in patients than HUS. Patients often develop a blood clot in the brain and die. (Proesmans, 1996)

Outbreaks of *E. coli* O157:H7 occur all over the world. With the improvement in surveillance for *E. coli* O157:H7 in the United States (U.S.), the number of infectious cases caused by contaminated hamburgers from restaurants has decreased (Park *et al.*, 2001). During 2004-2005, petting zoos was reported as vehicles for *E. coli* O157:H7 infections. Three outbreaks occurred in North Carolina, Florida, and Arizona. Children, who visited these zoos were infected with HUS (CDC, 2005). Unlike in the U.S., infections caused by *E. coli* O157:H7 in the United Kingdom (U.K.) have increased in recent years. Infections often happen in the summer and on children 0-4 years old (Douglas and Kurien, 1997). Ground beef products are typical foods to cause the infection in the U.S. and U.K. Among Asian countries, Japan has the most reports on *E. coli* O157:H7 infections. In South America, *E. coli* O157:H7 frequently causes diarrhea, HUS. In Canada, *E. coli* O157:H7 is one of most prevalent pathogens that causes infection, and it has been suggested that control of this pathogen at the farm level will reduce the risk of its infection (Park *et al.*, 2001). *Escherichia coli* O157:H7 has been more frequently regarded as an enteric pathogen in developed countries than in developing countries. This may be due to a lack of surveillance systems in developing countries (Phillip *et al.*, 2001).

Factors Affecting Survival and Growth of *Escherichia coli O157:H7*

E. coli O157:H7 infections occur through consuming a variety of foods, such as raw or undercooked meat, contaminated apple cider and juice, fruit, vegetables, and water. This suggests that *E. coli* O157:H7 may have strong tolerance to rigorous environments. (Tarr *et al*., 1997; Raghubeer and Matches, 1990)

Temperature

Many *E. coli* O157:H7 strains grow poorly above 44°C. The minimum growth temperature for *E. coli* O157:H7 is around 8-10°C (Buchanan and Bagi, 1994; Rajkowski and Marmer, 1995). However, cell growth depends on the constitution of media. They cannot grow well in Tryptic Soy Broth (TSB) at 44-45°C (Doyle and Schoeni,, 1984). All strains can grow in brain heart infusion (BHI) broth at 45°C, but six of them cannot grow in *E. coli* (EC) broth at the same temperature. (Palumbo *et al*., 1995)

Nevertheless, *E. coli O*157:H7 can grow fairly well in foods. There is no great change in colony number of *E. coli* O157:H7 in ground beef that is initially stored at -80°C and then held at -20°C for up to 9 months (Doyle and Schoeni, 1984). *Escherichia coli* O157:H7 grows very quickly in cantaloupe and watermelon cubes at 25°C (del Rosario and Beuchat, 1995). The greater survival of *E. coli* O157:H7 at 22°C than at -10 and 4°C in feces from corn- and barley-fed steers suggested that

temperature may help to seasonally transmit and spread the pathogen in feedlot cattle (Bach *et al.*, 2005). Modified atmospheres have no effect on the growth of *E. coli* O157:H7 at both 12 and 21°C, on shredded lettuce or sliced cucumbers (Abdul-Raouf *et al.*, 1993a).

Low pH

Escherichia coli O157:H7 survives in several acid foods (Buchanan and Doyle, 1997). It can grow quite well at pH 5.5-7.5. The minimum pH for growth is 4.0-4.5, below which populations will decline very quickly (Buchanan and Bagi, 1994; Buchanan and Klawitter, 1992). Acid tolerance depends on the growth phase of *E. coli* O157:H7 (Gorden and Small, 1993). Stationary-phase cells are more resistant to acid than log-phase cells and do not need exposure to low pH to induce acid resistance. Log-phase cells need to be exposed to pH 5.5-6.0 to induce acid resistance (Arnold and Kaspar, 1995). The type of acid and acid concentration influence the effect of pH on *E. coli* O157:H7. Some organic acids (acetic, lactic and citric acid) have an inhibitory effect on *E. coli* O157:H7. (Abdul-Raouf *et al.*, 1993) Recently, study on this area found that colonic acid (CA), created by *E. coli* O157:H7, increased the tolerance of *E. coli* O157:H7 to the extreme pH of microbiological culture media (Lee and Chen, 2004).

The acid tolerant state of *E. coli* can last longer if incubated at refrigeration temperature. *Escherichia coli* O157:H7 can survive in apple cider for only 2-3 days at 25°C, but 31 days at 8°C (Zhao *et al.*, 1993). At high populations, *E. coli* O157:H7 can survive in mayonnaise (pH 3.6-3.9) for 5-7 weeks at 5°C, but only 1-3 weeks at 20°C (Zhao and Doyle, 1994). *Escherichia coli* O157:H7 (10^3-10^7 CFU/ml) survived yogurt fermentation for 5 h at 42°C but 1 week at 4°C (Gorden and Small, 1993). Acid tolerance also helps *E. coli* O157:H7 resist other environmental stresses. It has been reported that the resistance of *E. coli* O157:H7 toward heating, radiation and antimicrobials increases due to the induction of acid tolerance (Rowbury, 1995) causing cells to more easily cause infections.

Water (Effect of NaCl)

Escherichia coli O157:H7 (initially 10^4 CFU/g) was reported to survive fermentation, drying and storage for 2 months at 4°C in fermented sausage which contained 69 ppm sodium chloride (Glass *et al.*, 1992). Since *E. coli* O157:H7 is in a viable but nonculturable (VBNC) state in water, it cannot be detected by traditional plating methods (Masaki *et al.*, 1992), thus difficult to detect in contaminated water. A risk assessment process should be performed when the effect of the pathogen in drinking water cannot be predicted by monitoring system (Schets *et al.*, 2005).

Escherichia coli O157:H7 cannot grow in TSB containing greater than 6.5% NaCl (Harrison, 1996). Harrison (1996) also indicated that *E. coli* O157:H7 could not be recovered from drying beef strips at 60°C for 10 h. Keene *et al.* (1997) reported that *E. coli* O157:H7 was killed slowly in marinated meat when dried at 51.7°C; and was only detectable by enrichment after drying for 6-8 h at 62.8°C. However, Hengge-Aronis *et al*. (1993) indicated that if *E. coli* O157:H7 was grown in relatively high levels of NaCl, thermotolerance and H_2O_2 resistance increased, and that the pathogen can survive for many weeks in a dry state, especially at refrigerated temperatures (Bagi and Buchanan, 1993).

Probiotics

Probiotics, such as lactic acid bacteria and especially bifidobacteria, are often applied as starter cultures in fermented foods. These fermented food products have been produced safely for many years without heat treatment. The growth of *E. coli* O157:H7 is inhibited in those foods (Barrantes *et al.*, 2004).

As for yogurt, although different cultures vary in their ability to inhibit the growth of *E. coli* O157:H7 in fermented systems (Dineen *et al.*, 1998), Barrantes *et al.*, (2004) confirmed that yogurt with additional probiotic cultures had inhibitory effects on *E. coli* O157:H7, when *E. coli* O157:H7 was cultured in yogurt with no additional probiotics. In addition, fermentation temperature and subsequent storage

temperature are also quite important to the growth and survival of *E. coli* O157:H7 in yogurt involving probiotics (Ogwaro *et al.*, 2002).

Probiotics were able to inhibit the growth of *E. coli* O157:H7 *in vivo* and *in vitro*. The creation of organic acids (lactates and acetates), hydrogen peroxide and bacteriocins, the competition, and adhesion receptors in the body, aid human beings ability in fighting against *E. coli* O157:H7 (Leahy *et al.*, 2005). Use of *Bdellovibrio* spp. reduce the *E. coli* O157:H7 counts on stainless steel surfaces after 7 h incubation within the temperature range from 19 to 37°C and can be used to protect food from contaminated processing equipment (Fratamico and Cooke, 1996).

CHAPTER III

MATERIALS AND METHODS

Bacterial Cultures: Bifidobacteria and *Escherichia coli* O157:H7

Bifidobacterium lactis/animalis Bb12; *B. bifidum* 15696; *B. longum* 15708; *B. bifidum* 791; *B. longum* VMKB S2B; *B. longum* VMKB 211; *B. bifidum* VMKB 221; *B. bifidum* VMKB 213 and *Bifidobacterium* spp. VSB1 were obtained from James L. Steele (University of Wisconsin, Madison, WI, USA), All of which were isolated from human intestines except *Bifidobacterium lactis/animalis* Bb12, which was isolated from milk (Appendix Table 1). *Escherichia coli* O157:H7 (ATCC 43890) was obtained from Dr. Juan L. Silva (Mississippi State University, MS, USA).

All cultures of bifidobacteria and *E. coli* O157:H7 were maintained in 50% glycerol and 11% nonfat dry milk (NFDM) stock and stored at -80°C. The 11% NFDM was prepared by combining agglomerated instant nonfat dry milk (Land O'Lakes, Inc., St. Paul, MN, USA) with double-distilled water to contain 11% total solids. This mixture was then autoclaved at 121°C for 15 min. Bifidobacterial strains were reactivated in de Man, Rogosa, Sharpe (MRS) broth (Becton Dickinson, Sparks, MD, USA) (De Man *et al*., 1960) and incubated under anaerobic conditions using an

anaerobe container system (BD GasPak ™ EZ, Becton Dickinson, Sparks, MD, USA) at 37°C. *Escherichia coli* O157:H7 was reactivated in Tryptic Soy Broth (TSB; Becton Dickinson) and incubated aerobically at 37°C with shaking at 100 rpm (C24 Incubator shaker, New Brunswick Scientific, Edison, NJ, USA). All cultures were subcultured at least two times prior to experimentation. (Gagnon *et al*., 2004)

Ten microliters of each of the nine strains of bifidobacteria were transferred to fresh MRS broth and incubated at 37°C for 16 h under anaerobic conditions to develop the growth curve. Their pH value (Corning Pinnacle 530 pH meter, Corning Incorporated, Corning, NY, USA) and turbidity at 600 nm (UV-1201 Spectrophotometer, Shimadzu Corporation, Columbia MD, USA) were measured.

Ten microliters of *E. coli* O157:H7 were transferred to fresh TSB and incubated at 37°C for 24 h with aerobic shaking at 100 rpm (C24 Incubator shaker) to obtain the growth curve. As for acid-adapted *E. coli* O157:H7, 10 µl of *E. coli* O157:H7 were transferred to TSB supplemented with 10 g/l glucose (final pH following incubation 4.8±0.007) and incubated at 37°C for 24 h with aerobic shaking at 100 rpm (C24 Incubator shaker) to obtain the growth curve. Media were acidified with 1 N hydrochloric acid (Dave and Shan, 1996). Their pH value and turbidity at 600 nm was measured. The growing colony number of acid-adapted and unadapted *E. coli* O157:H7 was also measured every hour during incubation. Serial dilutions in

sterile 0.1% peptone (Fisher, Pittsburgh, PA, USA) (0.1 g/100ml double-distilled H_2O) were prepared from the samples, and appropriate dilutions were spread plated in duplicate on Tryptic Soy Agar (TSA; Becton Dickinson, Sparks, MD, USA). Plates were incubated at 37°C for 24 h (Leenanon and Drake, 2001).

Bifidobacterial colonies were confirmed by Gram-staining and by the detection of intracellular fructose-6-phosphoketolase (F6PPK). Detection of F6PPK was accomplished by a modification of the microanalysis procedure described by Orban *et al.* (2000) and Vlková *et al.* (2002). All reagents for this analysis were obtained from Fisher (Pittsburghm, PA, USA) unless stated otherwise. Each of the nine strains of bifidobacteria was cultivated anaerobically in 10 ml MRS broth at 37°C for 14 h in an anaerobic box (full-growth), respectively. The cells of each strain were washed twice with phosphate buffer and centrifuged (Sorvall® Biofuge Fresco, Newtown, CT, USA) at 16,000×*g* (4°C, 3 min). The phosphate buffer was prepared as a mixture (1:1(v/v)) of KH_2PO_4 (0.05M) and cysteine-HCl (500 mg/liter) and the buffer was adjusted to pH 6.5 with fresh NaOH. The newly washed cells were resuspended in 1.0 ml phosphate buffer. These cells were incubated with 0.4 ml of hexadecyltrimethylaminonium bromide (CTAB) (450mg CTAB in 1 ml of double-distilled H_2O) for 5 min for cell disruption. Then, a reaction mixture containing 0.25 ml of a solution containing sodium fluoride (Sigma, St. Louis, MO,

USA) and sodium iodoacetate (6 mg NaF and 10 mg Na iodoacetate in 1ml double-distilled H_2O), 0.25 ml of sodium fructose-6-phosphate (Sigma) (80 mg/ml in water) was added into the mixture of each strain and vortexed (Fisher Scientific Mini Vortexer). After 30 min of incubation at 37°C, the reaction was terminated by the addition of 1.5 ml hydroxylamine HCl (13.9 g/100ml of double-distilled water, freshly neutralized with NaOH to pH 6.5) and vortexed. The mixture of each strain was kept for 10 min at room temperature (around 20°C) and then 1 ml of TCA (trichloroactic acid; 15% (w/v) in water), 1.0 ml of 4N HCl and 1.0 ml of color-developing ferric chloride ($FeCl \cdot 6H_2O$ (Sigma), 5% w/v in 0.1N HCl) were added. Tubes were vortexed and reddish-violet color formation was determined using a Spectronic® GENESTS™ Spectrophotometer (Spectronic Instruments, Inc., Rochester, NY, USA). The supernatant of the reaction mixture of each strain was collected by centrifugation at 16,000×*g* (Sorvall® Biofuge Fresco) (4°C for 3 min) and measured at 435 nm, respectively. The blank was prepared by mixing all reagents except bifidobacteria cells (negative when the reaction mix develops a light yellow color).

Effect of Low pH on the Growth of *Escherichia coli* O157:H7

Escherichia coli O157:H7 cells were prepared by culturing for 12 h at 37°C in TSB with aerobic shaking at 100 rpm (C24 Incubator shaker). One milliliter of

cells was washed twice by centrifugation at 1000×*g* (Sorvall® Biofuge Fresco) (4°C, 2 min) with 0.85% NaCl (0.85 g/100ml double-distilled water). Ten microliters of cells were suspended in acid-adjusted 11% NFDM (pH 3.81 before growth) and MRS broth (pH 3.94 before growth), and incubated at 37°C for 24 h with aerobic shaking at 100 rpm (C24 Incubator shaker). Media were acidified with 1 N hydrochloric acid (Dave and Shan, 1996). For the control, 10 µl of cells were suspended and then cultured in unadjusted 11% NFDM (pH 6.39 before growth) and MRS broth (pH 6.46 before growth) at 37°C for 24 h with aerobic shaking at 100 rpm (C24 Incubator shaker). Samples were collected during the incubation period at 0, 4 (log phase), 8 (stationary phase) and 24 h. The colony forming units (CFU/ml) of *E. coli* O157:H7 were measured using a spread plate method on TSA (Silk and Donnelly, 1997).

Inhibitory Effect of Bifidobacteria on *Escherichia coli* O157:H7

The cultures of nine bifidobacteria strains were centrifuged (Sorvall® RC-5B PLUS Superspeed Centrifuge, Sorvall® Centrifuges, Newtown, CT, USA) together at 4,300×*g* (4°C, 15 min) after each was grown separately at 37°C for 14 h in 10 ml MRS broth under anaerobic conditions. One milliliter of *E. coli* O157:H7 was centrifuged at 1,000×*g* (Sorvall® Biofuge Fresco) (4°C, 2 min) after being grown at 37°C for 12 h in 10 ml TSB with aerobic shaking at 100 rpm (C24 Incubator shaker). The supernatant was then discarded. All cell pellets of the bifidobacteria mix were

washed twice with 0.85% NaCl at 4,300×*g* (Sorvall® RC-5B PLUS Superspeed Centrifuge) (4°C, 15 min). The cells of *E. coli* O157:H7 were washed twice with 0.85% NaCl at 1,000×*g* (Sorvall® Biofuge Fresco) (4°C, 2 min) and then resuspended in 1 ml 0.85% NaCl. The bifidobacteria mix was suspended in 10 ml 11% NFDM with the addition of 10 µl *E. coli* O157:H7 from the 0.85% NaCl suspended solution, and incubated at 37°C for 24 h with aerobic shaking at 100 rpm (C24 Incubator shaker). To prepare the control, 10 µl of *E. coli* O157:H7 from the 0.85% NaCl suspended solution were incubated in 11% NFDM at 37°C for 24 h with aerobic shaking at 100 rpm (C24 Incubator shaker). The *E. coli* O157:H7 counts were measured during incubation at 0, 4, 8, 24 h, by the spread plate method on TSA (Silk and Donnelly, 1997).

In further research, the bifidobacteria mix and 10 µl of *E. coli* O157:H7 were incubated in MRS broth. Method was identical to the one utilized for the bifidobacteria mix and 10 µl of *E. coli* O157:H7 incubated in 11% NFDM (described above). The colony number of *E. coli* O157:H7 was measured at 0, 4, 8, 24 h during incubation, by the spread plate method, on TSA. The pH of media was also measured during incubation.

Inhibitory Effect of Bifidobacteria on Acid-adapted *Escherichia coli* O157:H7

One milliliter of *E. coli* O157:H7 was collected by centrifugation at 1,000×*g* (Sorvall® Biofuge Fresco) (4°C, 2 min) after being grown at 37°C for 12 h in 10 ml TSB with aerobic shaking at 100 rpm (C24 Incubator shaker). The supernatant was discarded. The cell pellets of *E. coli* O157:H7 were washed twice with 0.85% NaCl at 1,000×*g* (Sorvall® Biofuge Fresco) (4°C, 2 min) and then suspended in 1 ml TSB supplemented with 10 g/l glucose. Media were acidified with 1 N hydrochloric acid (final pH following incubation 4.8±0.007). After incubation at 37°C for 12 h with aerobic shaking at 100 rpm (C24 Incubator shaker), the cells of acid-adapted *E. coli* O157:H7 were prepared. (Leenanon and Drake, 2001)

The cultures of nine strains of bifidobacteria were centrifuged together at 4,300×*g* (Sorvall® RC-5B PLUS Superspeed Centrifuge) (4°C, 15 min) after being grown separately at 37°C for 14 h in 10 ml MRS broth under anaerobic conditions. All cell pellets of bifidobacteria mix were washed twice with 0.85% NaCl at 4,300×*g* (Sorvall® RC-5B PLUS Superspeed Centrifuge) (4°C, 15 min). The cells of acid-adapted *E.coli* O157:H7 were collected by centrifugation at 1,000×*g* (Sorvall® Biofuge Fresco) (4°C, 2 min), followed by washing twice with 0.85% NaCl at 1,000×*g* (Sorvall® Biofuge Fresco) (4°C, 2 min). Cells were then resuspended in 1 ml 0.85% NaCl. The bifidobacteria mix was suspended in 10 ml 11% NFDM with the

addition of 10 µl of acid-adapted *E. coli* O157:H7 from the 0.85% NaCl suspended solution, followed by incubation at 37°C for 24 h with aerobic shaking at 100 rpm (C24 Incubator shaker). Ten microliters of acid-adapted *E. coli* O157:H7 from 0.85% NaCl were incubated in 11% NFDM at 37°C for 24 h with aerobic shaking at 100 rpm (C24 Incubator shaker) was the control. The colony number of acid-adapted *E. coli* O157:H7 was measured at 0, 4 (log phase), 8 (stationary phase) and 24 h during incubation, by the spread plate method on TSA.

Effect of Supernatant of Bifidobacteria on *Escherichia coli* O157:H7

The cultures of nine strains of bifidobacteria were centrifuged together at 4,300×*g* (Sorvall® RC-5B PLUS Superspeed Centrifuge) (4°C, 15 min) after each species was grown separately at 37°C for 14 h in 10 ml MRS broth under anaerobic conditions. All cell pellets of bifidobacteria mix were washed twice with 0.85% NaCl at 4,300×*g* (Sorvall® RC-5B PLUS Superspeed Centrifuge) (4°C, 15 min). The bifidobacteria mix was suspended in fresh MRS broth, followed by incubation at 37°C with aerobic shaking at 100 rpm (C24 Incubator shaker) for 8 h. The mixture was centrifuged at 30,000×*g* (Sorvall® RC-5B PLUS Superspeed Centrifuge) (4°C, 20 min). The supernatant was collected and divided into two parts. The pH of one part was adjusted to 6.50 (pH of fresh MRS broth) with 6 N NaOH (Yamamoto *et al.*, 2003).

To prepare the *E. coli* O157:H7 culture, 1 ml of *E. coli* O157:H7 were centrifuged at 1,000×*g* (Sorvall® Biofuge Fresco) (4°C, 2 min) after being grown at 37°C for 12 h in 10 ml TSB with aerobic shaking at 100 rpm (C24 Incubator shaker). The culture was washed twice with 0.85% NaCl by centrifugation at 1,000×*g* (Sorvall® Biofuge Fresco) (4°C, 2 min).

Ten microliters of the *E. coli* O157:H7 cells were suspended into the two previous parts of the bifidobacteria supernatant that were prepared previously, and then incubated at 37°C with aerobic shaking at 100 rpm (C24 Incubator shaker) for 24 h. The *E. coli* O157:H7 counts were measured during incubation at 0, 4, 8 and 24 h by the spread plate method on TSA. The pH value of the bifidobacteria supernatant was also measured at each sampling period.

Statistical Analysis

Mean values and standard deviations (SD) were calculated from data obtained from three replications in each experiment. A randomized complete block design was utilized to examine differences ($P \leq 0.05$) among treatments, and a completely randomized design was utilized to examine differences ($P \leq 0.05$) among treatments for each incubation time, in each experiment. Fisher's protected least significance difference (LSD) test was then used to separate treatment means ($P \leq 0.05$) (SAS version 9.0; SAS, 2004) when significant differences occurred.

CHAPTER IV

RESULTS AND DISCUSSION

Reddish-violet color (Appendix Table 2) formation indicating the presence of Fructose-6-phosphoketolase (F6PPK) and observation of purple, rod-shaped colonies observed under the microscope after Gram-staining confirmed that the cultures and stocks produced and stored at -80°C were bifidobacteria.

The growth curves for the nine strains of bifidobacteria selected (Figures 4.1 and 4.2) (incubated in MRS broth at 37°C in anaerobic conditions) showed that full-growth was attained after 14h. The growth of *E. coli* O157:H7 (ATCC 43890), shown in Figure 4.3, (incubated in TSB at 37°C with aerobic shaking at 100 rpm) demonstrated that full-growth was attained after 12 h. This figure also indicated that the log and stationary phases of *E. coli* O157:H7 occur at 4 and 8 h, respectively. Acid-adapted *E. coli* O157:H7 (ATCC 43890) (Figure 4.4) (incubated in TSB at 37°C with aerobic shaking at 100 rpm) attained full-growth after 12 h. This growth curve also revealed that the log and stationary phases occur as 4 and 8 h, respectively. Accordingly, 4 h and 8 h were chosen as the times to take samples for the survival of acid-adjusted and unadjusted *E. coli* O157:H7 in the experiments.

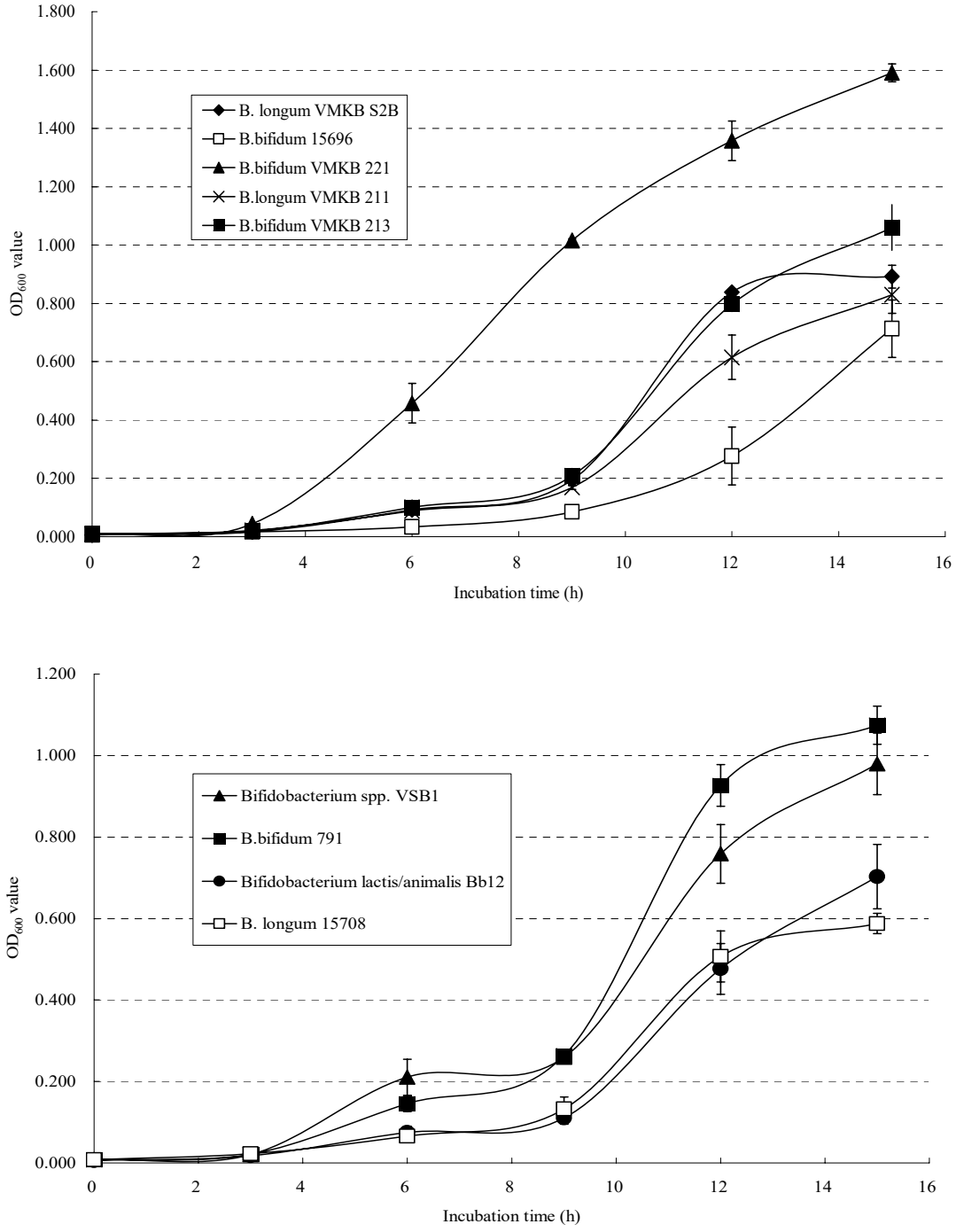


Figure 4.1 Growth curve (absorbance, OD_{600} value) of bifidobacteria in MRS broth for 16 h of incubation at 37°C under anaerobic conditions (n=3)

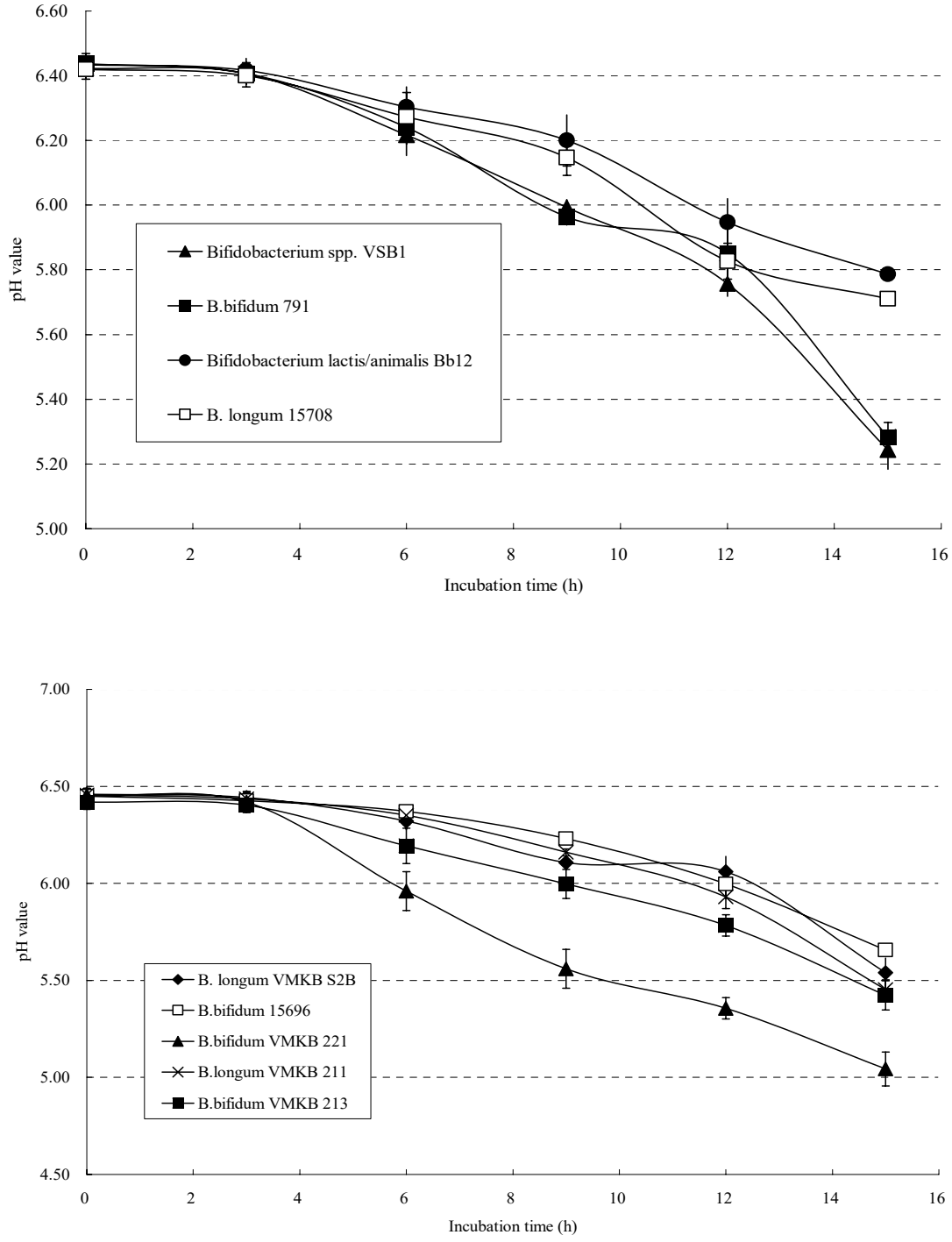
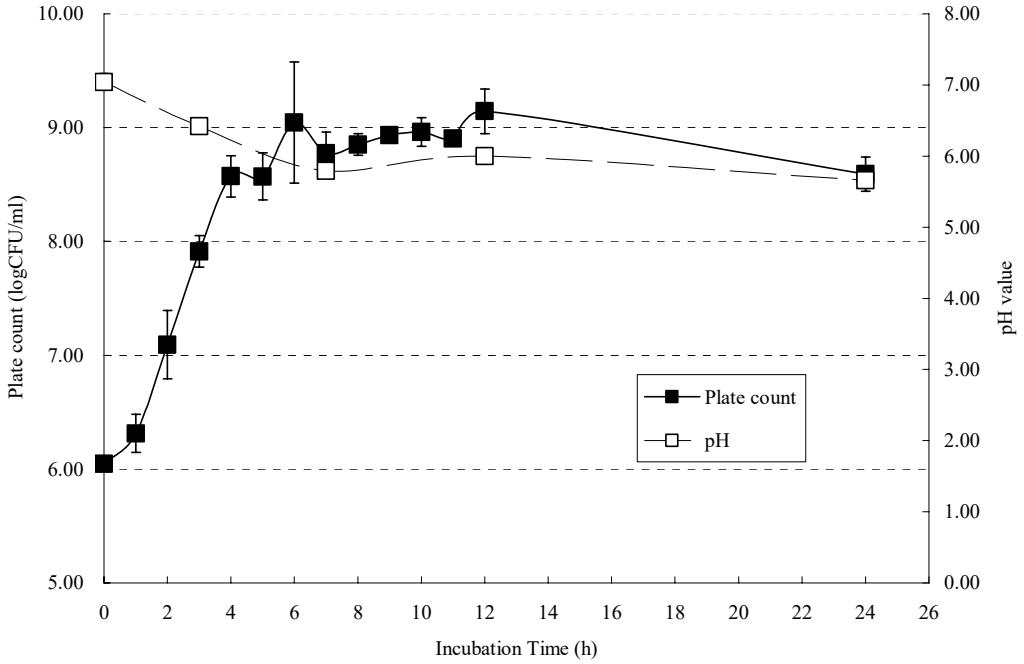


Figure 4.2 pH value of bifidobacteria in MRS broth for 16 h of incubation at 37°C under anaerobic conditions (n=3)



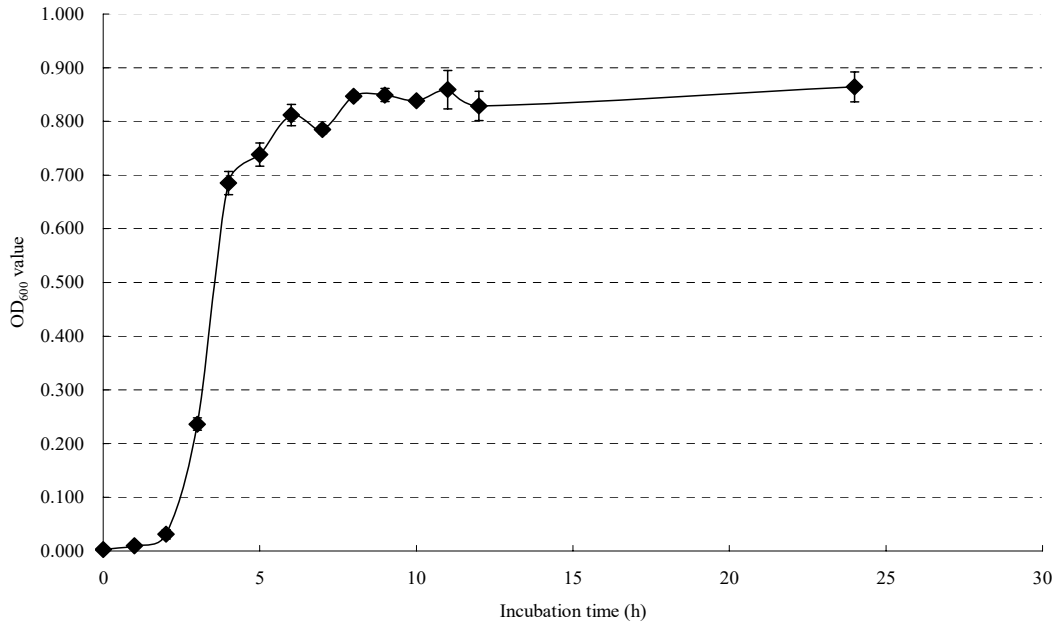


Figure 4.3 Growth curve of *E. coli* O157:H7 (ATCC 43890) and media pH in TSB for 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

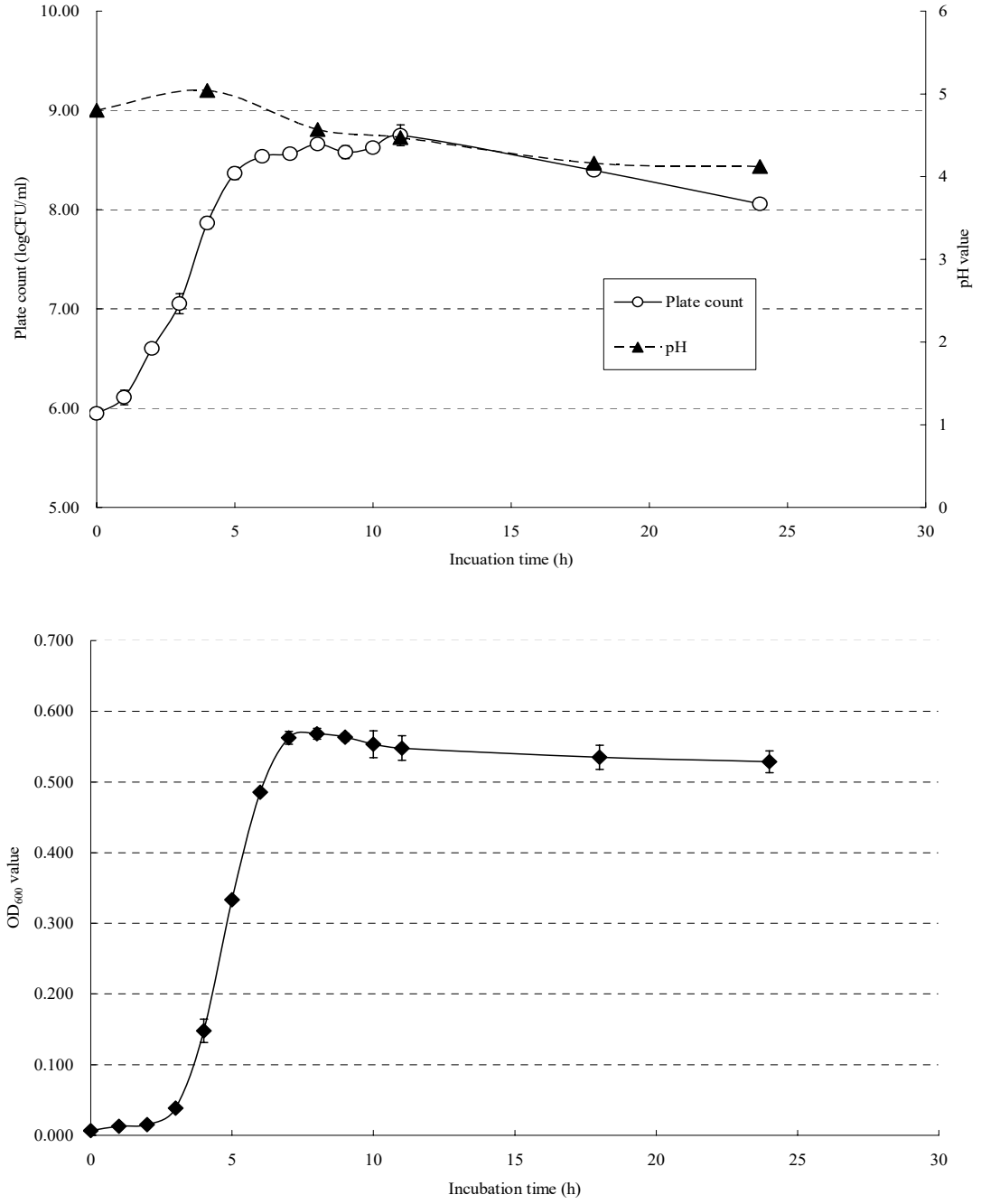


Figure 4.4 Growth curve of acid-adapted *E. coli* O157:H7 (ATCC 43890) and media pH in TSB supplemented with 10g/l glucose (final pH following incubation: 4.8±0.007) and incubated at 37°C for 24 hours with aerobic shaking at 100 rpm (n=3)

Preliminary Experiment: Effect of Low pH

In order to detect pH effect on *E. coli* O157:H7, 10 µl of *E. coli* O157:H7 were incubated in acid-adjusted and unadjusted media (11% NFDM and MRS broth) during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (Figure 4.5). Initial colony number of *E. coli* O157:H7 for 10 µl *E. coli* O157:H7 in acid-adjusted 11% NFDM (pH 3.81 before growth) and MRS broth (pH 3.94 before growth) was about 6.06 and 6.03 log CFU/ml, respectively (Appendix Table 7). For unadjusted 11% NFDM (pH 6.39 before growth) and MRS broth (pH 6.46 before growth), initial colony number of *E. coli* O157:H7 was the same ($P>0.05$), about 6.0 log CFU/ml. The *E. coli* O157:H7 counts increased over time ($P\leq0.05$) and reached around 8.87 log CFU/ml after 24 h incubation in unadjusted 11% NFDM. *E. coli* O157:H7 counts increased ($P\leq0.05$) by 2.79 log CFU/ml after 8 h incubation MRS broth, and finally reached 8.81 log CFU/ml after 24 h incubation. As for acid-adjusted media, *E. coli* O157:H7 counts decreased ($P>0.05$) shortly after 8 h incubation in acid-adjusted 11% NFDM and then decreased ($P\leq0.05$) greatly to around 1.20 log CFU/ml after 24h. On the other hand, *E. coli* O157:H7 counts from acid-adapted MRS broth decreased ($P\leq0.05$) greatly to non-detectable levels between 4 and 24 h. From these data above, it was concluded that the survival of *E. coli* O157:H7 was influenced by the pH of the two media.

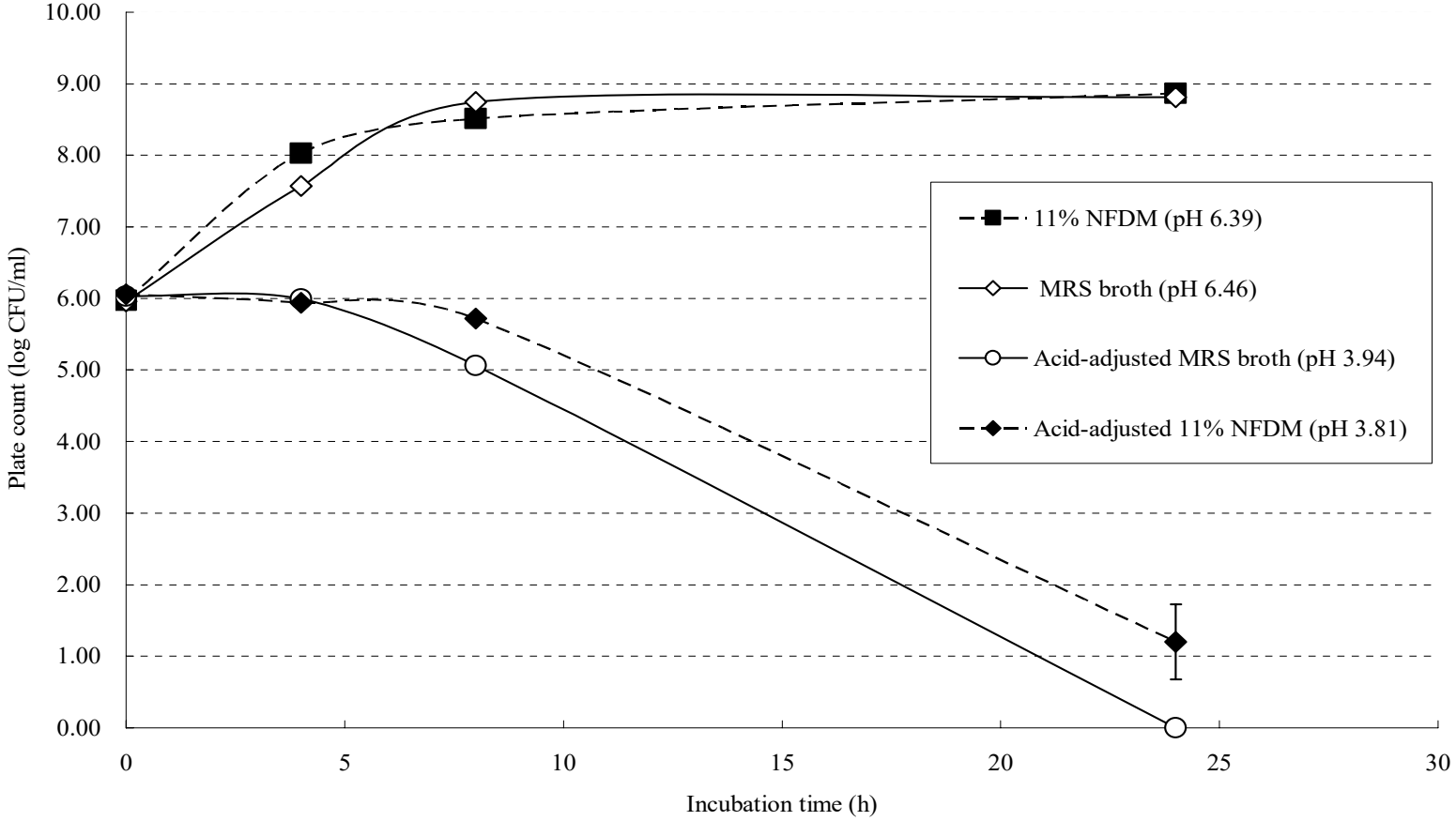


Figure 4.5 Survival of *E. coli* O157:H7 (ATCC 43890) in acid-adjusted or unadjusted media (11% NFDM and MRS broth) during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Escherichia coli O157:H7 is highly acid tolerant. Many researches have been accomplished on the effect of low pH on *E. coli* O157:H7. Buchanan and Bagi (1994) reported that the minimum pH for the growth of *E. coli* was 4.0-4.5. When the pH falls below the minimum, the numbers of *E. coli* O157:H7 will decline. This experiment confirmed the conclusion. However, *E. coli* O157:H7 is still able to survive for a long time (much more than one day) in acid food, like fermented sausages, apple cider, and apple juice (pH 3.6-4.0). Since several outbreaks contributed by a low level of *E. coli* O157:H7 in acid food had been reported (Park *et al.* 2001).

The Food and Drug Administration requires that a heating or pasteurization step must be added for acid food products, and that their pH be maintained at or below 4.6 by acid or acid ingredients (Doyle, 1991). Therefore, Breidt *et al.* (2004) indicated that if only pH is considered, acid-resistant pathogens, like *E. coli* O157:H7, might be a potential threat to acidified foods. In order to remove the pathogen thoroughly, more measures need to be applied. Acidified food products were reported to be safe for many years without other treatments due to organic acids present in those products (Breidt *et al.*, 2004). Type of acid and acid concentration influences the effect of pH on the growth of *E. coli* O157:H7. Shelef (1994) cited that the inhibitory effect of organic acids were due to "chelation of iron and inhibition of

lactate dehydrogenase". Abdul-Raouf *et al.* (1993) recorded that acetic > lactic ≥ citric was the sequence of acids which had relative inhibitory effect on *E. coli* O157:H7 in beef slurries. Considering those factors above, 1 N hydrochloric acid was applied in the experiment to reflect the effects of pH alone.

Inhibitory Effect of Bifidobacteria on *Escherichia coli* O157:H7

Inhibitory Effect of Bifidobacteria on *E. coli* O157:H7 in 11% NFDM

Survival of *E. coli* O157:H7 in 11% NFDM combined with nine strains of full-growth bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm is shown in Figure 4.6 (Appendix Table 8). In the control test, *E .coli* O157:H7 counts increased (P≤0.05) around 2.26 log CFU/ml after 8 h of incubation and reached 8.72 log CFU/ml (from 6.06 log CFU/ml at 0 h) after 24 h in the control. *Escherichia coli* O157:H7 counts decreased (P≤0.05) greatly in the bifidobacteria mix + 10µl *E. coli* O157:H7 between 8 h and 24 h to non-detectable numbers. This suggests that bifidobacteria may have an inhibitory effect on *E. coli* O157:H7 during incubation in 11% NFDM at 37°C with aerobic shaking at 100 rpm.

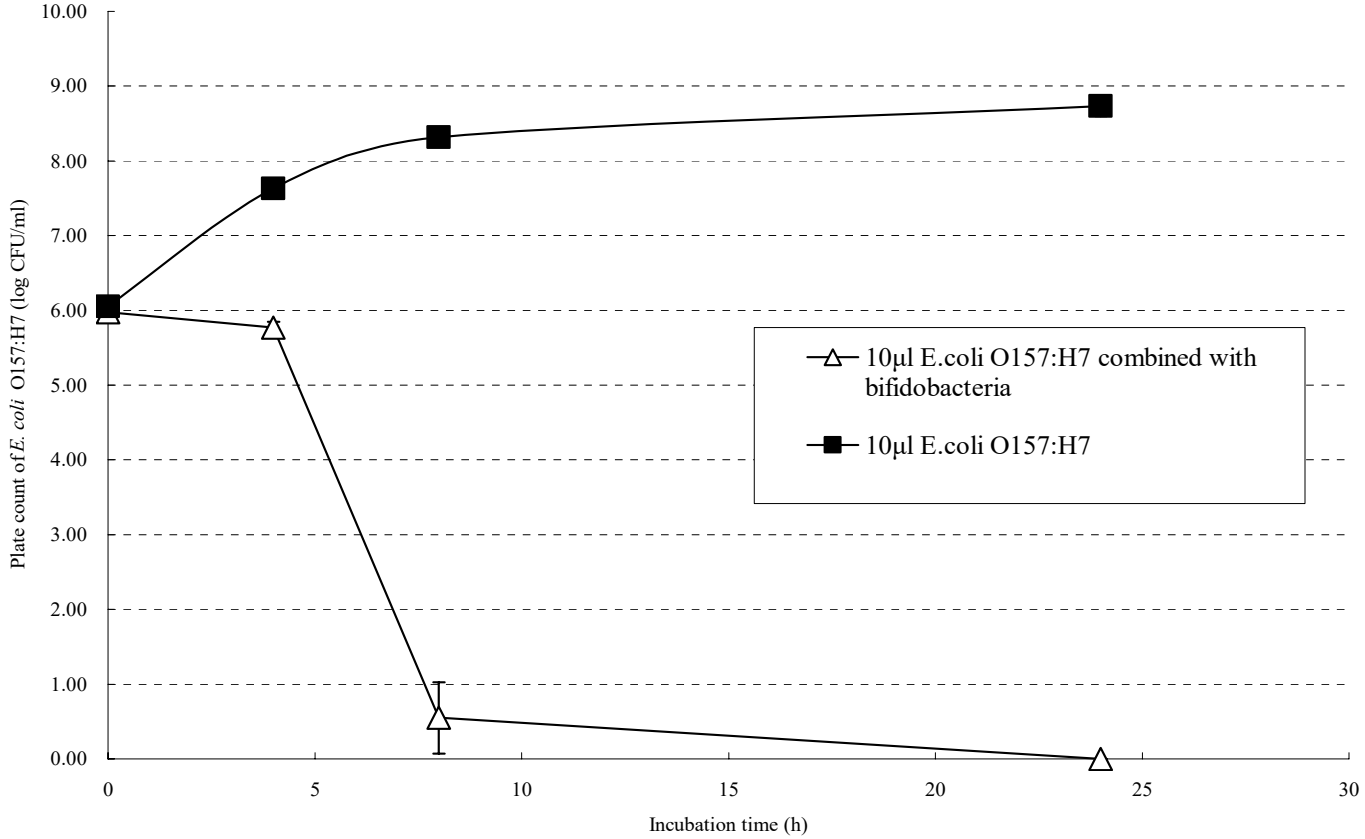


Figure 4.6 Survival of *E. coli* O157:H7 (ATCC 43890) in 11% NFDM combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Bifidobacteria are regarded as probiotics and thought to provide health benefits to humans. The injection of bifidobacteria with foods may help to lighten the symptom of gastrointestinal tract disease, inhibit some pathogens (like *Salmonella*, *Campylobacteria*, *Escherichia coli* etc.), and enhance the immune system and so on (Ding *et al.*, 2005). Although scientific data is very limited, bifidobacteria have been utilized in many kinds of dairy foods. Yogurt is milk fermented by probiotics and is one of the most popular food delivery systems for bifidobacteria cultures. Ding *et al.* (2005) have confirmed that cell-free extracts of milk fermented by 10 probiotic bacteria (five *Bifidobacterium* strains and five *Lactobacillus* strains) inhibited the growth of *Campylobacter jejuni*. However, numerous challenges, skills, knowledge and advanced technology are required during its manufacture. In order to develop high-quality Bifidus products, not only the taste and other sensory properties are important, but the viability of bifidobacteria is also critical. A 10^7 CFU/ml viable bifidobacteria has been demanded to be the minimum count for fresh dairy products in Japan (Ishibashi and Shimamura, 1993).

This experiment revealed that although the effect of bifidobacteria under aerobic conditions toward human health benefits is unknown, the bifidobacteria may still have an inhibitory effect on *E. coli* O157:H7, regardless of the viability.

Inhibitory Effect of Bifidobacteria on *E. coli* O157:H7 in MRS Broth

One medium suitable for the growth of bifidobacteria is MRS broth. In order to further study the inhibitory activity of bifidobacteria against *E. coli* O157:H7, the medium was changed from 11% NFDM to MRS broth (Figure 4.7). In the bifidobacteria mix + 10µl *E. coli* O157:H7, *E. coli* O157:H7 counts decreased ($P \leq 0.05$) to non-detectable levels after 8 h incubation, Simultaneously, *E. coli* O157:H7 counts increased ($P \leq 0.05$) about 2.8 log CFU/ml in the control. When comparing the survival of *E. coli* O157:H7 in MRS broth combined with bifidobacteria mix with the one in 11% NFDM (Figure 4.8), the results are quite similar ($P > 0.05$) (Appendix Table 10). *Escherichia coli* O157:H7 combined with bifidobacteria mix in either media reached non-detectable levels after 8 h, while *E. coli* O157:H7 counts reached non-detectable levels after 24 h incubation in acid-adjusted media (Figure 4.9). Those comparisons indicate that bifidobacteria have an inhibitory effect on *E. coli* O157:H7 during incubation in 11% NFDM and MRS broth at 37°C with aerobic shaking at 100 rpm. Also, this shows that these phenomenon were created not only by the low pH of media.

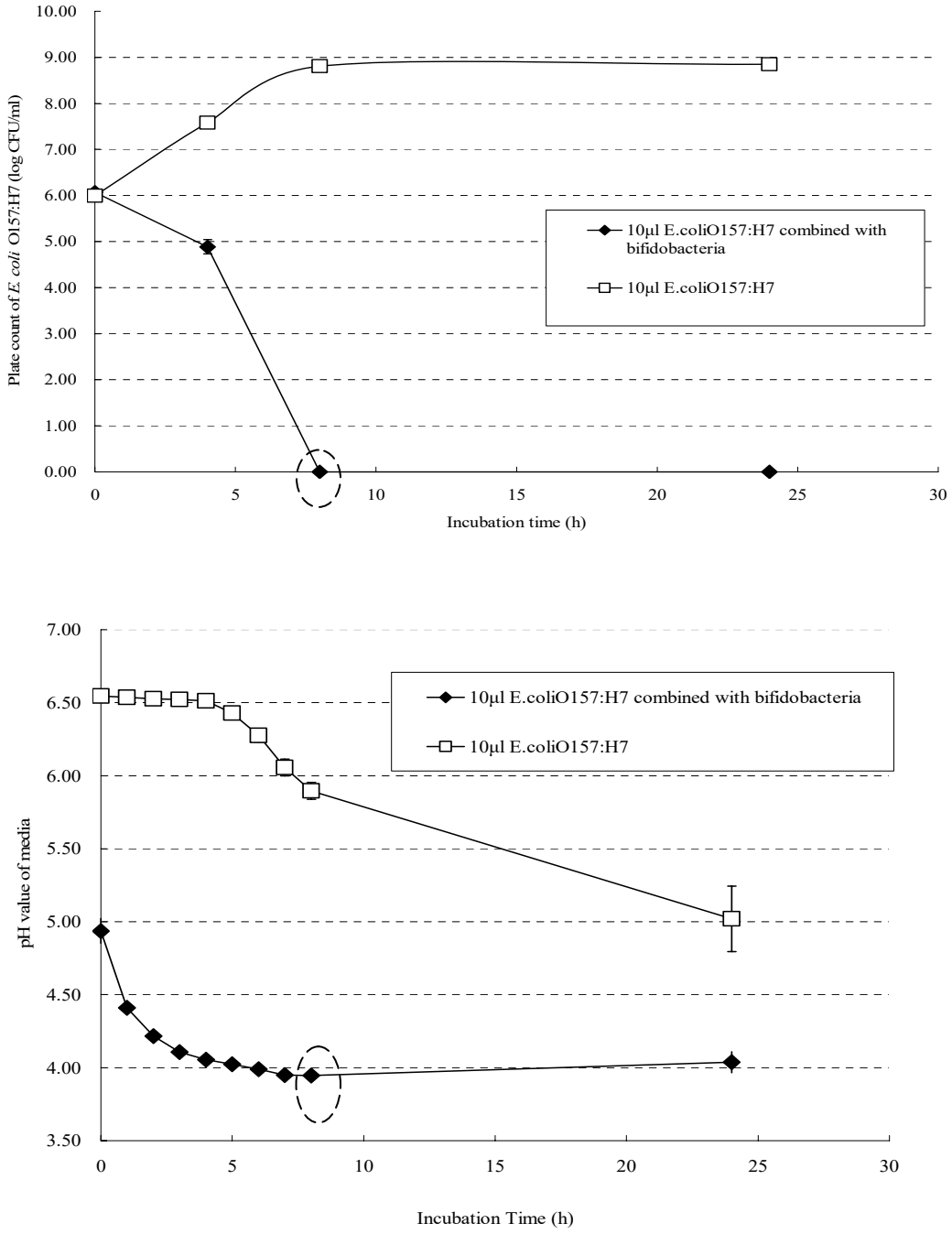


Figure 4.7 Survival of *E. coli* O157:H7 (ATCC 43890) and media pH in MRS broth combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

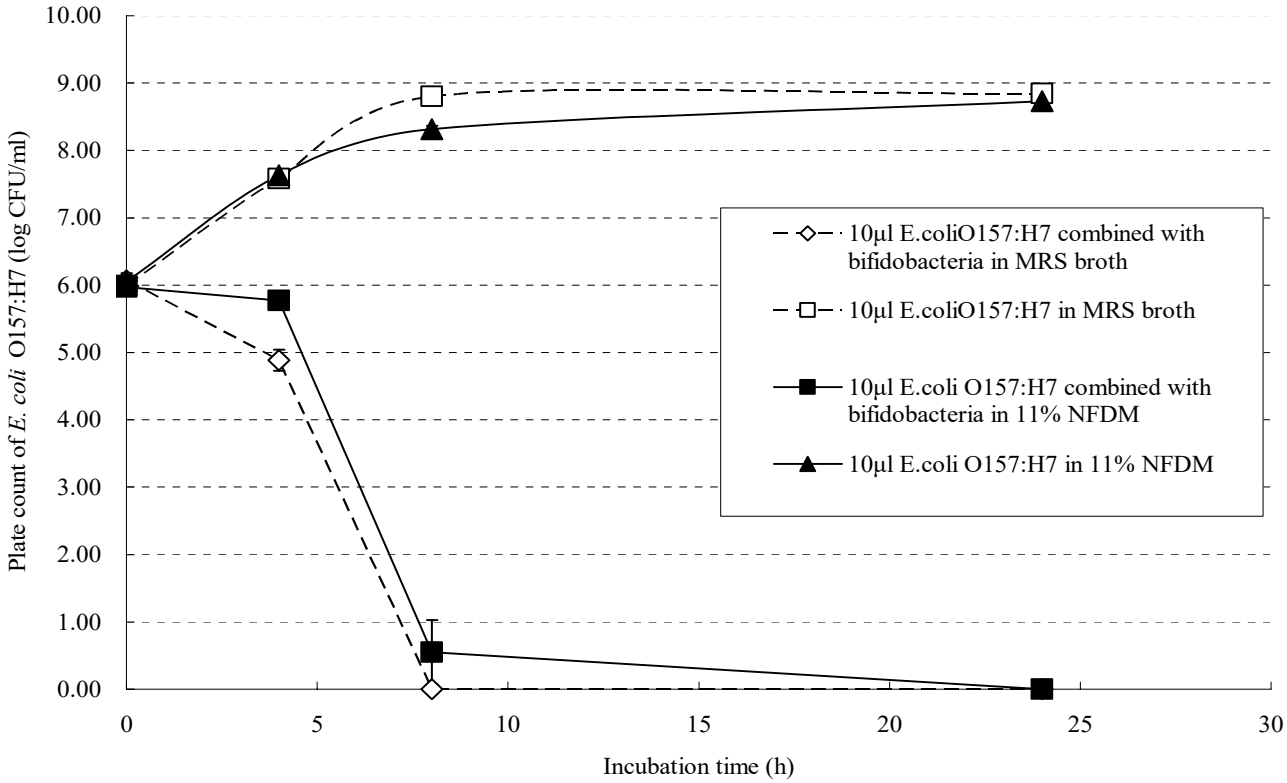


Figure 4.8 Survival of *E. coli* O157:H7 (ATCC 43890) in 11% NFDM or MRS broth combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

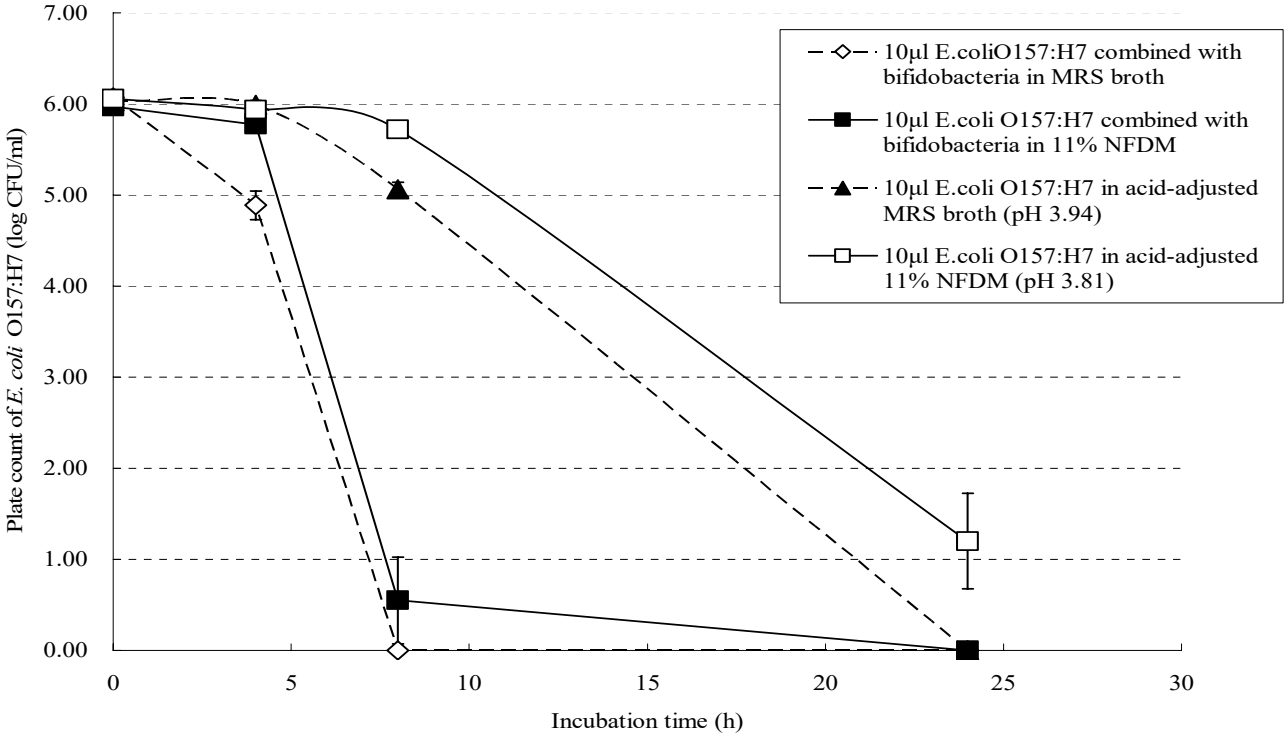


Figure 4.9 Survival of *E. coli* O157:H7 (ATCC 43890) when combined with bifidobacteria mix in the media (11% NFDM and MRS broth) and *E. coli* O157:H7 in acid-adjusted media during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Research on the inhibitory effect of bifidobacteria on the *E. coli* O157:H7 has been conducted *in vitro* and *vivo*. The effect of bifidobacteria on the survival of *E. coli* O157:H7 *in vitro* was mainly attributed to several factors: the production of lactic acid and acetic acid, hydrogen peroxide, bacteriocins and the competition for nutrients (Leahy *et al.*, 2005). According to Gagnon *et al.* (2004), 2µl of overnight bifidobacterial cultures spotted on 1.5% MRS agar plates, overlaid with *E. coli* O157:H7 were able to create zones of clear agar around themselves. As for *in vivo*, besides the forementioned factors, competition of adhesion receptors (Caco-2 cells), the creation of anti-toxin materials and improvement of the immune system also affected *E. coli* O157:H7 survival (Leathy *et al.*, 2005). Asahara *et al.* (2004) reported inhibitory effect of (10^9 CFU/ml) bifidobacteria on (10^4 CFU/ml) *E. coli* O157:H7 in drinking water. Decreased *E. coli* O157:H7 counts in our study (Appendix Table 10) were consistent with other research findings. However, their studies also emphasized that the inhibitory effect of bifidobacterial cultures depended on the concentration and viability of bifidobacteria cells. Exposure of bifidobacteria mixture under aerobic condition in our experiments showed that large amount of bifidobacteria had strong inhibitory effect on *E. coli* O157:H7, regardless if the viability.

Since *E. coli* O157:H7 was non-detectable after 8 h incubation (Figure 4.7), the amount of substances created by bifidobacteria to inhibit the pathogen must have

been enough at this incubation time. Thereby, the pH value of the mixture at 8 h is regarded as a standard to change the pH of those media (11% NFDM and MRS broth) to evaluate the effect of low pH on *E. coli* O157:H7 in the preliminary experiment (Figure 4.5). Eight hours were also chosen as the incubation time for the bifidobacteria mix under aerobic conditions to prepare their supernatant in order to estimate the effect of outcome on *E. coli* O157:H7.

Acid Adaption

Inhibitory Effect of Bifidobacteria on Acid-adapted *E. coli* O157:H7

Acid adaptation has been reported to prolong the survival time of *E. coli* O157:H7 in severe acidic conditions (Cheng *et al.*, 2002). The induction of acid tolerance also helps organisms to survive other stresses (Rowbury, 1995). Acid-adapted *E. coli* O157:H7 (Figure 4.4) achieved full growth in 12 h. Acid-adapted *E. coli* O157:H7 counts (Figure 4.10) increased ($P \leq 0.05$) around 3.10 log CFU/ml after 24 h incubation in the control, while acid-adapted *E. coli* O157:H7 counts were non-detectable after 8 h when combined with the bifidobacteria mix. The results indicate that bifidobacteria also have an inhibitory effect on the acid-adapted *E. coli* O157:H7.

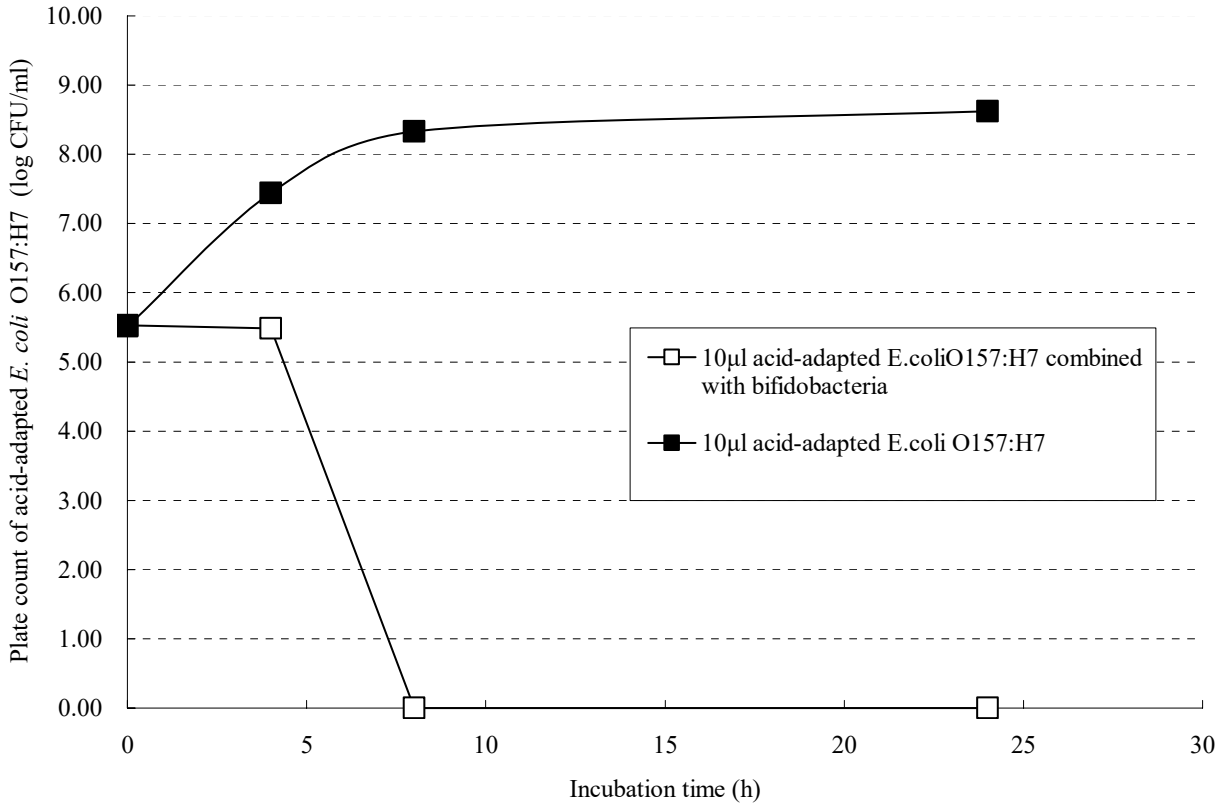


Figure 4.10 Survival of acid-adapted *E. coli* O157:H7 (ATCC 43890) in 11% NFDM combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

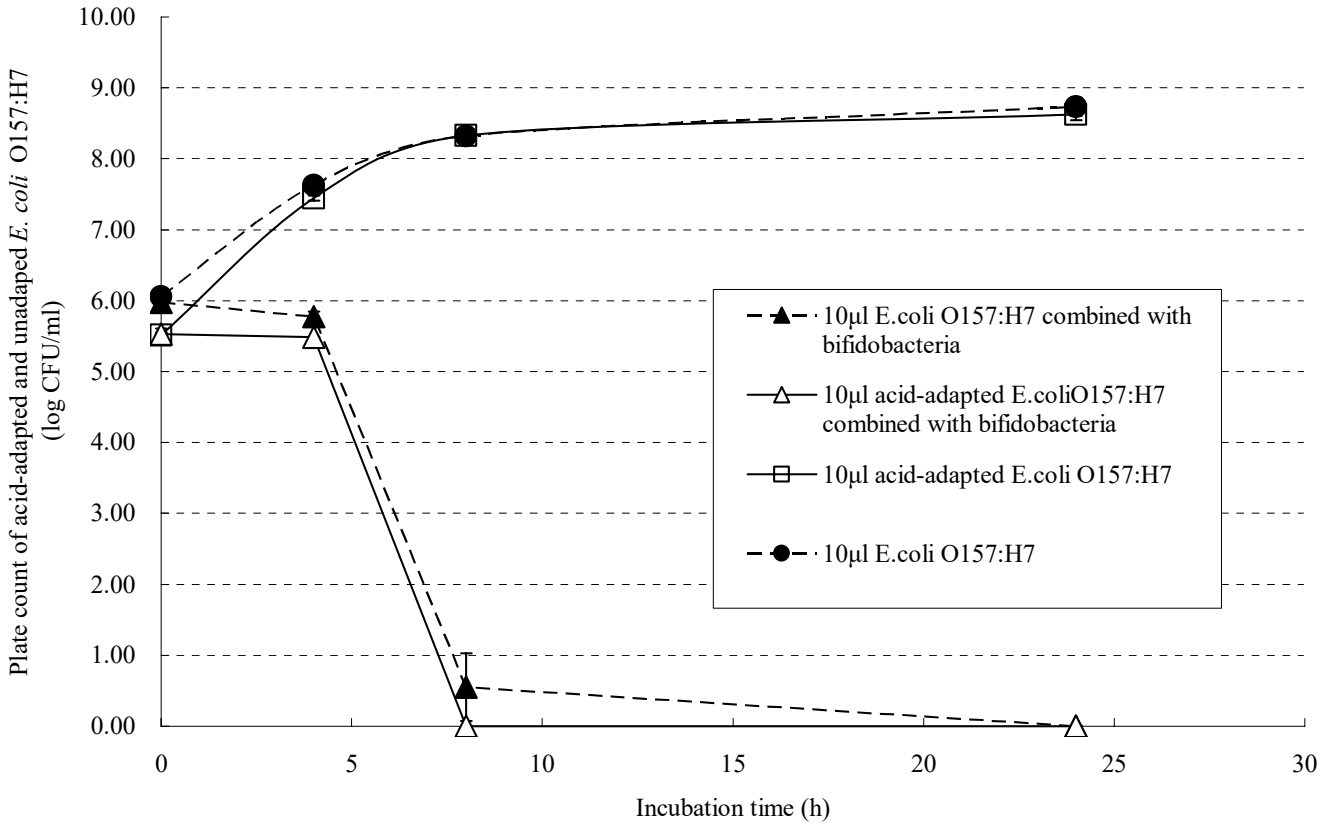


Figure 4.11 Survival of acid-adapted and unadapted *E. coli* O157:H7 (ATCC 43890) in 11% NFDM combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

There was no difference (P>0.05) between colony numbers of acid-adapted and unadapted *E. coli* O157:H7 in NFDM, when combined with the bifidobacteria mix (Figure 4.11). Leyer *et al*., (1995) reported that *E. coli* O157:H7, pre-adapted to pH 5, increased in survival rates in acidic foods, such as fermented salami and apple cider. Some other reports also indicate that acid adaptation prolong the survival of *E. coli* in different food systems (Goodson and Rowbury, 1989; Cheng and Chou, 1999). However, Cheng and Chou (2001) pointed out that acid adaptation reduced the survival of *E. coli* O157:H7 in low-fat yoghurt stored at 7°C. Our findings differ from these researcher since we found was no difference between acid-adapted and unadapted cell counts for *E. coli* O157:H7. The difference between our findings and others may be due to: (1) different storage temperature: the lower the storage temperature, the more resistant the *E. coli* O157:H7 towards milk fermented by bifidobacteria mix; (2) different cultures of bifidobacteria and *E. coli* O157:H7; (3) different composition of milk (i.e., different concentration of fat and other ingredients); and (4) different procedures for preparation of the acid-adapted *E. coli* O157:H7: For example, Tsai and Ingham (1997) reported another method to create (acid-adapted) cells of *E. coli* O157:H7: *E. coli* O157:H7 incubationed at 37°C for 18 h in TSB followed by washing and transfering to pH 5.0 (pH adjusted with 6 N HCl)

and incubated at 37°C for 4 h. The choice of the method, culture and media depends on the requirement and objective of the experiment.

Supernatant of Bifidobacteria Mix

Survival of *E. coli* O157:H7 in the supernatant of bifidobacteria mix

Another factor that is considered to be one of the main reasons that inhibitory effect on *E. coli* O157:H7are the substances that are created by bifidobacteria mix under aerobic conditions. After 4 h incubation, *E. coli* O157:H7 counts decreased ($P \leq 0.05$) by 6.06 log CFU/ml and reached undetectable levels (Figure 4.12). These data reveals that the supernatant without bifidobacteria mix might contain substances that are lethal to *E. coli* O157:H7. However, after changing the pH value of the supernatant to 6.50 (pH of fresh MRS broth), the *E. coli* O157:H7 counts increased ($P \leq 0.05$) by 2.59 log CFU/ml after 24 h incubation at 37°C (Figure 4.12). These results appear similar to the ones in fresh MRS broth (Figure 4.13).

This comparison showed that low pH may be the main factor that contributes to the inhibitory effect of bifidobacteria on *E. coli* O157:H7. This agrees with reports by Asahara *et al.* (2004), who concluded that the creation of acetic acid (thus low pH) inhibited the production of Shiga-like toxin. However, in comparison with the change of colony number of *E. coli* O157:H7 incubated in acid-adjusted MRS broth (pH 3.94

before growth) (Figure 4.14), the time to achieve non-detectable levels of *E. coli* O157:H7 incubated in the supernatant and death rate was much faster (4 h vs. 24 h), ($P \leq 0.05$) for nonacid-adjusted (≥1.5 log CFU/ml) than for adjusted (≥0.3 log CFU/ml) *E. coli* O157:H7. The pH value of supernatant (pH 4.10-4.20) was also higher than the one of acid-adjusted MRS broth (Appendix Table 16). Therefore, it is possible that there are antimicrobial substances created by bifidobacteria to accelerate the death of *E. coli* O157:H7 and that these substances may lose their antimicrobial characteristic without acid condition or combined with NaOH (the pH of the supernatant was adjusted by 6 N NaOH).

Gibson and Wang (1994) indicated that the antagonistic activity of bifidobacteria toward Gram-negative pathogens was acid due more to the secretion of large amounts of antimicrobial substances (like several organic acids, hydrogen peroxide, and bacteriocins as mentioned above) than acid production. Inhibitory effect of soluble substances in the supernatant of bifidobacteria cultures on *E. coli* O157:H7 has been studied both *in vivo* and *vitro*. Kim *et al.* (2001) reported that the supernatant of *B. longum* HY8001 might have inhibitory effects by binding to vero cytotoxins (VTs) that are produced by *E. coli* O157:H7, thus disturbing the attachment of VTs to globotriaosylceramide (Gb_3). The attachment of VTs to Gb_3 in the gut epithelium is the primary step for *E. coli* O157:H7 to cause and develop

disease. Cell-free extracts of milk fermented by bifidobacteria and lactobacilli at 37°C for 24 h inhibited the growth of *Campylobacter jejuni* (Ding *et al.*, 2005). These results are similar to our experiments, confirming the antimicrobial effect of substances created by bifidobacteria. However, since fermentation is an anaerobic process and caused by enzymes of living microorganisms, those substances are most likely different from the ones created under aerobic conditions in our experiments.

As for the ingredients in the substances created by bifidobacteria under aerobic conditions, there are some possibilities: the F6PPK found in *Bifidobacterium* species ferments glucose to produce acetate and lactate (Figure 2.2). The F6PPK pathway also transfers oxygen to create various compounds such as superoxide and hydrogen peroxide and cause oxygen toxicity under aerobic conditions (Figure 2.1) (Tamine *et al*. 1995). As has been mentioned, acetic and lactic acids have inhibitory effects on *E. coli* O157:H7. Hydrogen peroxide was reported to "have a strong oxidizing effect on bacterial cells, sulphydryl groups of cell proteins and membrane lipids" (Ouwehand and Vesterlund, 2004). Hydrogen peroxide is also known as a weak acid and can be neutralized by NaOH just like acetic and lactic acids. In conclusion, organic acids and hydrogen peroxide might be the main reasons that caused the death of *E. coli* O157:H7 in the supernatant isolated from nine strains of bifidobacteria.

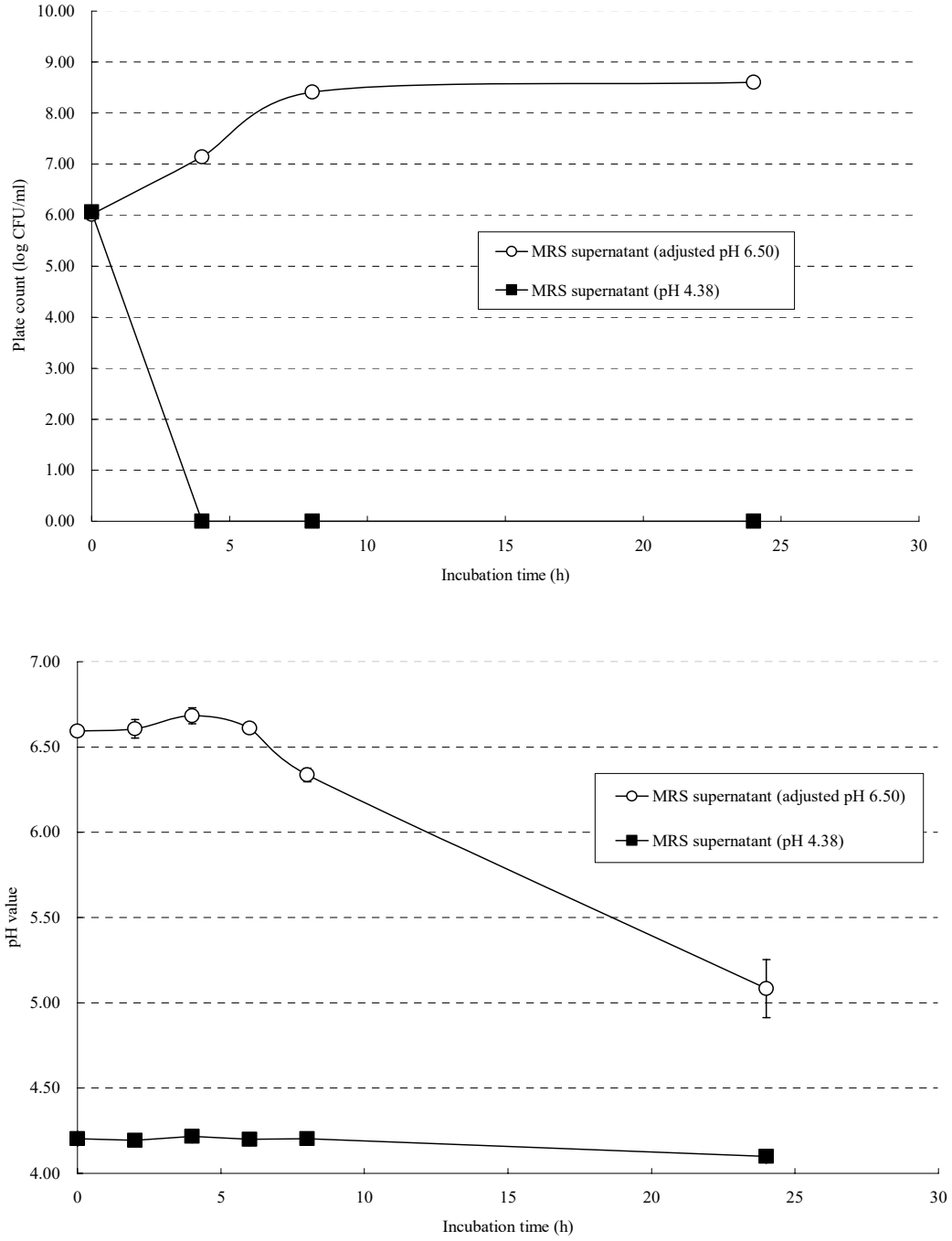


Figure 4.12 Survival of *E. coli* O157:H7 (ATCC 43890) and media pH in the supernatant of bifidobacteria during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

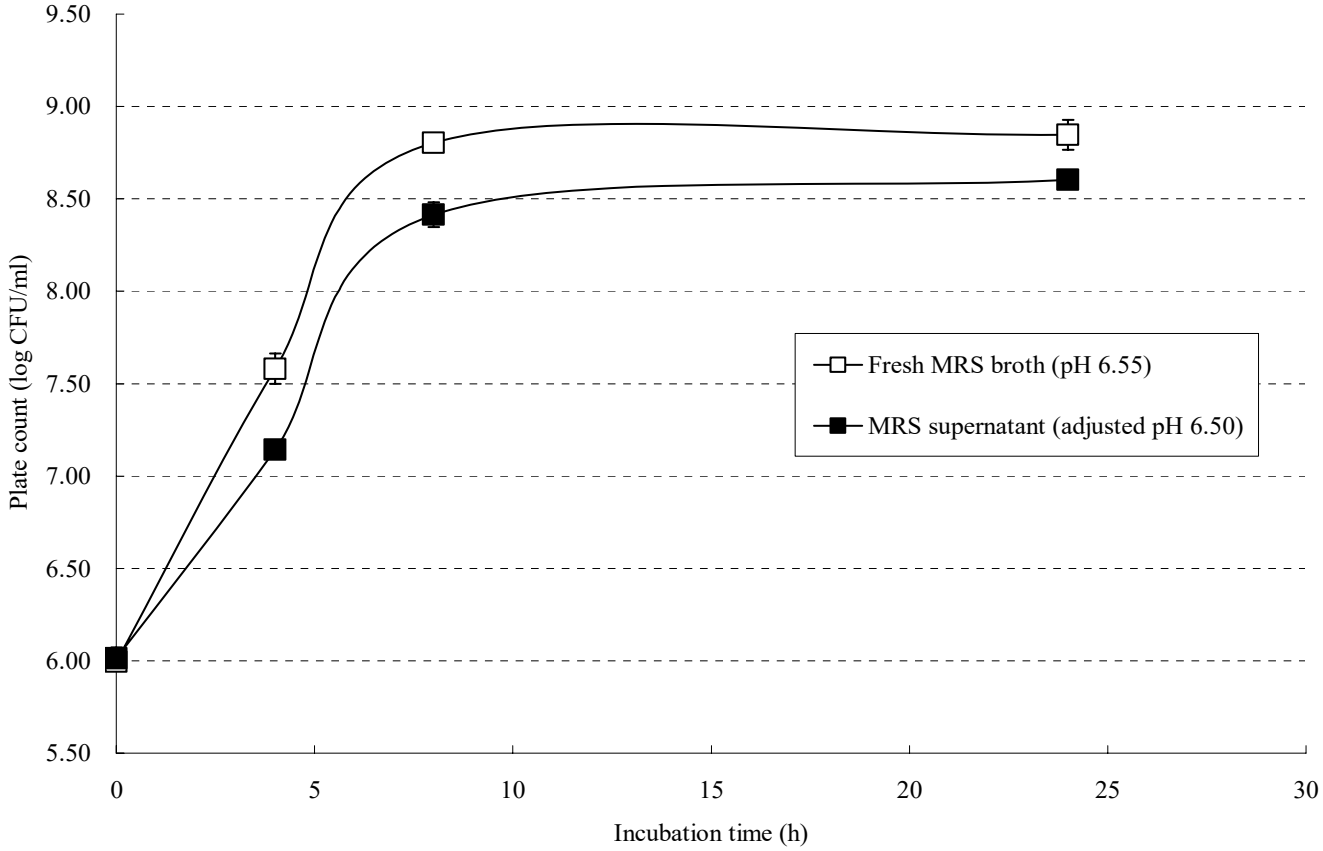


Figure 4.13 Survival of *E. coli* O157:H7 (ATCC 43890) in pH adjusted MRS supernatant or fresh MRS broth during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

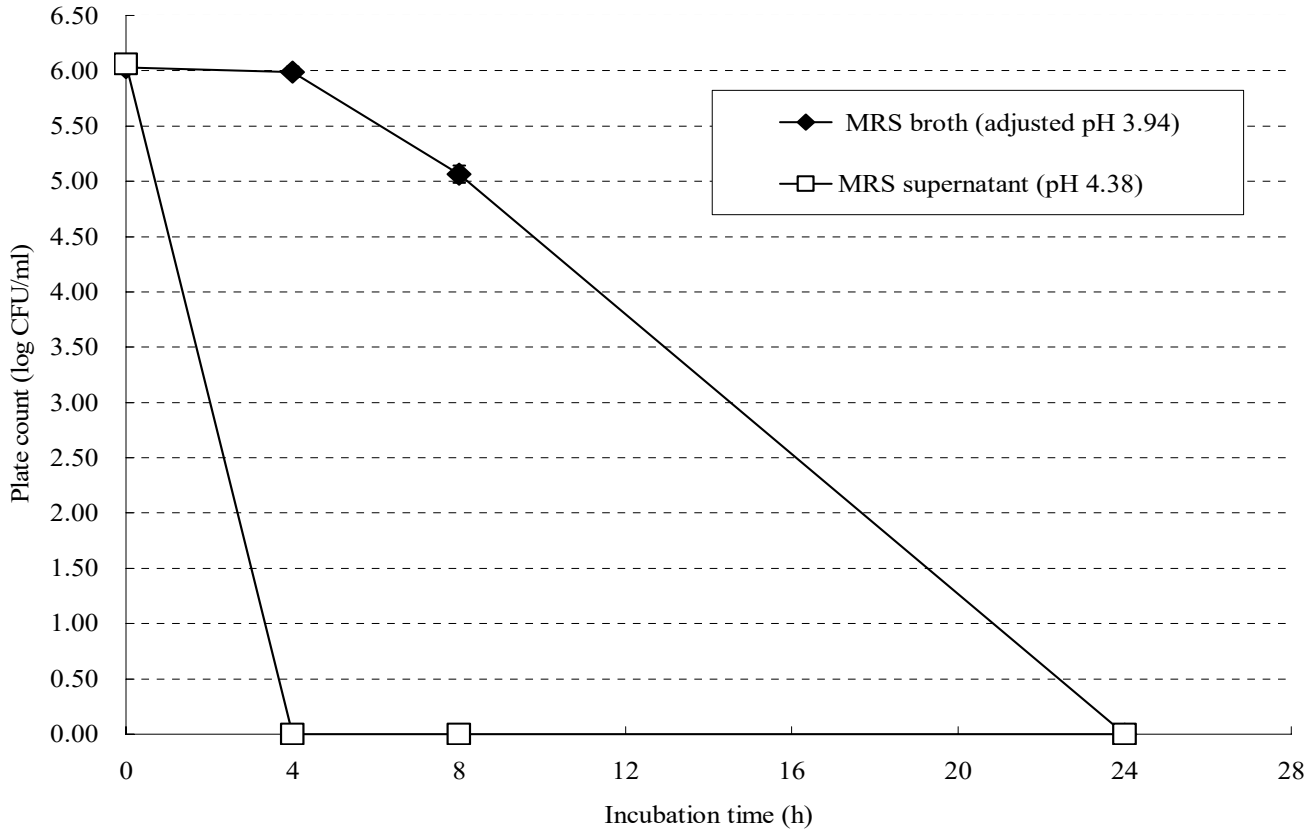


Figure 4.14 Survival of E. coli O157:H7 (ATCC 43890) in MRS supernatant or acid-adjusted (fresh) MRS broth during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

As for other materials produced by bifidobacteria, since only some of them have been extensively studied, further research in the area is needed. For example, bacteriocins found in almost every bacterium were close to antibiotics, however, so far "there is no enough evidence to improve the effect of bacteriocins collected from gram-positive bacteria on gram-negative bacteria, without the addition of any membrane-active compound" (Ouwehand and Vesterlund, 2004).

CHAPTER V

SUMMARY AND CONCLUSIONS

The antimicrobial activity of a bifidobacteria mixture against *Escherchia coli* O157:H7 under aerobic conditions was systematically evaluated.

The mixture of nine strains of bifidobacteria had a strong inhibitory effect on *E. coli* O157:H7 during incubation in 11% NFDM or MRS broth aerobically at 37°C.

Two main factors had been proposed to cause the effect of bifidobacteria on the survival of *E. coli* O157:H7: (1) low pH (pH around 3.8); (2) antimicrobial substances created by bifidobacteria during incubation under aerobic conditions.

Low pH had the ability to destroy *E. coli* O157:H7 after incubation in acid-adjusted 11% NFDM and MRS broth at 37°C. There were no differences between acid-adapted and unadapted *E. coli* O157:H7 in withstanding bifidobacteria when incubated in 11% NFDM.

The supernatant collected from bifidobacteria mix after incubation in MRS broth at 37°C with shaking was lethal to *E. coli* O157:H7. However, once the pH of the supernatant was adjusted to 6.5 (from 4.3), *E. coli* O157:H7 was able to grow.

This seemed to indicate that low pH was the main reason for the antagonistic effect of bifidobacteria. However, comparing the results from *E. coli* O157:H7 incubated in supernatant with the one incubated in acid-adjusted MRS broth (4 h vs. 24 h), a different conclusion was reached. The outcome revealed that other materials contained in the supernatant accelerate the death of *E. coli* O157:H7 and the material might be inactivated by NaOH or under low-acid conditions.

Additional research needs to be conducted in order to determine the exact ingredient in the supernatant that inhibit *E. coli* O157:H7 to confirm those results. The effect of cell extracts of bifidobacteria on the pathogen may be another meaningful subject to investigate. As for further application in industry, cost and safety must also be considered.

REFERENCES

Abdul-Raouf, U.M., Beuchat, L.R. and Ammar, M.S. 1993. Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. Appl. Environ. Microbiol. 59: 1999-2006.

Ahn, J.B., Hwang, H.J., and Park, J.H. 2001. Physiological responses of oxygen-tolerant anaerobic *Bifidobacterium longum* under oxygen. J. Microbiol. Biotechnol. 11: 443-451.

Arnold, K.W. and Kaspar, C.W. 1995. Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 61: 2037-2039.

Asahara, T., Shimizu, K., Nomoto, K., Hamabata, T., Ozawa, A. and Takeda, Y. 2004. Probiotic bifidobacteria protect mice from lethal infection with shiga toxin-producing *Escherichia coli* O157:H7. Infec. Immun. Apr. 2240-2247.

Bach, S.J., Stanford, K. and McAllister, T.A. 2005. Survival of *Escherichia coli* O157:H7 in feces from corn- and barley-fed steers. FEMS Microbiol. Lett. 252(1): 25-33.

Bagi, L.K. and Buchanan, R.L. 1993. Preservation of *Listeria monocytogenes* and other pathogenic foodborne bacteria on silica gel. Lett. Appl. Microbiol. 17: 37–39.

Ballongue, J. 1993. Bifidobacteria and probiotic action. In: S. Salminen and Wright A. eds. Lactic Acid Bacteria. Marcel Deker, Inc. New York. 357.

Barrantes, X., Railey, D., Arias, M.L. and Chaves, C. 2004. Evaluation of the effect of probiotic cultures added to commercial yogurt over a known population of *Listeria monocytogenes* and *Escherichia coli* O157:H7. Arch. Latinoam Nutr. 54(3): 293-297.

Biavati, B., Vescovo, M., Torriani, S. & Bottazzi, V. 2000. Bifidobacteria: history, ecology, physiology and applications. Ann. Microbiol. 50: 117–131.

Bilge, S.S., Vary, J.J.C., Dowell, S.F. and Tarr, P.I. 1996. Role of the *Escherichia coli* O157:H7 O side chain in adherence and analysis of an *rfb* locus. Infect. Immun. 64: 4795-4801.

Black, F.T., Andersen, P.L., Orskov, J., Orskov, F., Gaarslev, K. and Laulund, S. 1989. Prophylactic efficacy of lactobacilli on traveller's diarrhea. In Travel Medicine ed. Steffen, R. Conference on International Travel Medicine 1. Zurich, Switzerland, Berlin. 333-335.

Boerlin, P. 1999. Evolution of virulence factors in Shiga toxin-producing *Escherichia coli*. Cell. Mol. Biol. Life Sci. 56: 735-741.

Boerlin, P., McEwen, S.A., Boerlin-Petzold, F., Wilson, .B., Jonson, R.P. and Gyles, C.L. 1999. Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. J. Clin. Microbiol. 37: 497-503.

Boylston, T.D., Vinderola, C.G. Ghoddusi, H.B. and Reinheimer, J.A. 2004. Incorporation of bifidobacteria into cheese: challenges and rewards. Int. Dairy J. 14: 375-387.

Breidt, F., JR., Hayes, J.S. and McFeeters R.F. 2004. Independent effects of acetic acid and pH on survival of *Escherichia coli* O157:H7 in simulated acidified pickle products. J. Food Prot. 67(1): 12-18.

Brunder, W., Schmidt, H. and Karch, H. 1997. EspP, a novel extracellular serine protease of entero-hemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. Mol. Microbiol. 24: 767-778.

Buchanan R.L. and Doyle M.P. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli*. Food Tech. 51(10): 69-76.

Buchanan, R.L. and Bagi, L.K. 1994. Expansion of response surface models for the growth of *Escherichia coli* O157:H7 to include sodium nitrite as a variable. Intl. J. Food Microbiol. 23: 317–322.

Buchanan, R.L. and Klawitter, L.A. 1992. The effect of incubation temperature, initial pH, and sodium chloride on the growth kinetics of *Escherichia coli* O157:H7. Food Microbiol. 9: 185–196.

Calderwood, S.B., Acheson, D.W.K., Keusch, G. T., Barrett, T.J., Griffin, P.M., Strockbine, N.A., Swaminathan, B., Kaper, J.B., Levine, M.M., Kaplan, B.S., Karch, H., O'Brien, A.D., Obrig, T. G., Takeda, Y., Tarr, P.I. and Wachsmuth, I.K. 1996. Proposed new nomenclature of SLT (VT) family. ASM News. 62: 118-119.

Centers for Disease Control and Prevention (CDC). 2006. *Escherichia coli* O157:H7. Retrieved January 15, 2006, from http://www.cdc.gov/ncidod/dbmd/diseaseinfo/escherichiacoli_g.htm

Centers for Disease Control and Prevention (CDC). 2005. Outbreaks of *Escherichia coli* O157:H7 associated with petting zoos--North Carolina, Florida, and Arizona, 2004 and 2005. MMWR Morb. Mortal. Wkly Rep. 54(50): 1277-1280

Chart, H. 2000. VTEC enteropathogenicity. J. Appl. Microbiol. Sym. Suppl. 88:12S-23S.

Cheng, H.Y., Chou, C.C. 2001. Acid adaptation and temperature effect on the survival of *Escherichia coli* O157:H7 in acidic fruit juice and lactic fermented milk product. Int. J. Food Micorbiol. 70: 189-195.

Cheng, H.Y., Yang, H. and Chou, C.C. 2002. Influence of acid adaptation on the tolerance of *Escherichia coli* O157:H7 to some subsequent stresses. J. Food. Prot. 65(2): 260-265.

Chiang, B.L., Sheih, Y.H., Wang, L.H., Liao, C.K. and Gill, H.S. 2000. Enchancing immunity by dietary consumption of a probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune response. Eur. J. Clin. Nutr. 54: 849-855.

Crittenden R. 2004. An update on probiotic bifidobacteria. In: Salminen, S., Wright, A. and Ouwehand, A. eds. Lactic acid bacteria: microbiology and functional aspects, 3rd ed. Marcel Dekker Inc. New York. 125-172.

Dave, R.I. and Shan, N. P. 1996. Evaluation of media for selective enumeration of *Streptococcus thermophhilus*, *Lactobacillus delbrueckii* spp. *Bulgaricus*, *Lactobacillus acidophilus*, and bifidobacteria. J. Dariy Sci. 79: 1529-1536

De Boer, E. and Heuvelink, A.E. 2000. Methods for the detection and isolation of Shiga toxin-producing *Escherichia coli*. Symp. Ser. Soc. Appl. Microbiol. 29: 133S-143S.

De Man, J.C. Rogosa, M., Sharp, M.E. 1960. A medium for the cultivation of Lactobacilli. J. Appl. Bacteriol. 23: 130-135.

Deguchi, Y., Morishita, T. and Mutai, M. 1985. Comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria. Agric. Biol. Chem. 49(1): 13-19.

del Rosario, B. and Beuchat, L. 1995. Survival and growth of enterohemorrhagic *Escherichia coli* O157:H7 in cantaloupe and watermelon. J. Food Prot. 58: 105-107.

Dineen, S.S., Takeuchi, K., Soudah, J.E. and Boor, K.J. 1998. Persistence of *Escherichia coli* O157:H7 in dairy fermentation systems. J. Food Prot. 61(12): 1602-1608.

Ding, W., Wang, H. and Griffiths, M.W. 2005. Probiotics down-regulate *flaA* σ^{28} promoter in *Campylobacter jejuni*. J. Food Prot. 68(11): 2295-2300.

Douglas, A.S. and Kurien, A. 1997. Seasonality and other epidemiological features of haemolytic uraemic syndrome and *Escherichia coli* O157 isolates in Scotland. Scott. Med. J. 42: 166-171.

Doyle, M.P. 1991. *Escherichia coli* O157:H7 and its significance in foods. Int. J. Food Microbiol. 12: 289-302.

Doyle, M.P. and Schoeni, J.L. 1984. Survival and growth characteristics of *Escherichia coli* associated with hemorrhagic colitis. Appl. Environ. Microbiol. 48: 855– 856.

Embley, T.M. and Stackebrandt, E. 1994. The molecular phylogeny and systematics of the actinomycetes. Annu. Rev. Microbiol. 48: 257-289.

Fooks, L.J. and Gibson, G.R. 2002. Probiotics as modulators of the gut flora. Br. J. Nutr. 88(S1), S39-S49.

Frankel, G., Lider, O., Hershkoviz, R., Mould, A.P., Kachalsky, S.G., Candy, D.C.A., Cahalon, L., Humphries, M.J. and Dougan, G. 1996. The cell-binding domain of intimins from enteropathogenic *Escherichia coli* binds to β_1 integrins. J. Biol. Chem. 271: 20359-20364.

Fratamico, P.M. and Cooke, P.H. 1996. Isolation of Bdellovibrios that prey on *Escherichia coli* O157:H7 and *Salmonella* species and application for removal of prey from stainless steel surfaces. J. Food Safety. 16: 161-173.

Gagnon M., Kheadr E.E., blay, G.L., Fliss I. 2004. In vitro inhibition of *Escherichia coli* O157:H7 by bifidobacterial strains of human origin. Int. J. Food Microbiol. 92: 69-78.

Gavini, F., Cayuela, Ch., Antoine, J.M., Lecoq, C., Lefebvre, B., Membré, J.M., Neut, C.H. 2001. Differences in the distribution of bifidobacterial and enterobacterial species in human faecal microflora of three different (children, adults, elderly) age groups. Micro. Ecol. Health. Dis. 13: 40-45

Gavini, F., Esbroeck, M.V., Touzel, J.P., Fourment, A. and Goossens, H. 1996. Detection of fructose-6-phosphate phosphoketolase (F6PPK), a key enzyme of the bifid-shunt, in *Gardnerella vaginalis*. Anaerobe. 2: 191-193.

Gibson, G.R. and Wang, X. 1994. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J. Appl. Bacteriol. 77: 412-420.

Glass, K.A., Loeffelholz, J.M., Ford, J.P. and Doyle M.P. 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride in fermented, dry sausage. Appl. Environ. Microbiol. 58: 2513-2516.

Goodson, M., Rowbury, R.J., 1989. Resistance of acid-habituated *Escherichia coli* to organic acids and its medical and significance. Letters in Applied Microbiology. 8: 211–214.

Gorden, J. and Small, P.L. 1993. Acid resistance in enteric bacteria. Infect. Immun. 61: 364-367.

Gagnon, M., Kheadr E., Gwenaëlle, L.B. and Fliss, I. 2004. In vitro inhibition of *Escherichia coli* 0157:H7 by bifidobacterial strains of human origin. Int J Food Microbiol. 92: 69–78.

Guessous, F., Marcinkiewicz, M., Polanowska-Grabowska, R., Kongkhum, S., Heatherly, D., Obrig, T. and Gear, A.R. 2005. Shiga toxin 2 and lipopolysaccharide induce human microvascular endothelial cells to release chemokines and factors that stimulate platelet function. Infect. Immun. 73(12): 8306-8316.

Harmsen, H. J.M., Wildehoer, A.C.M., Raangs, G.C., Wagendorp, A.A., Klijn, N., Bindels, J.G. and Welling, G.W. 2000. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J. Pediatr. Gastroenterol. Nutr. 30: 61-67

Harrison, J. and Harrison M. 1996. Fate of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella typhimurium* druing preparation and storage of beef jerky. J. Food Prot. 59: 1336-1338.

Hengge-Aronis, R., Lange, R., Henneberg, N. and Fischer, D. 1993. Osmotic regulation of *rpoS*-dependent genes in *Escherichia coli*. J. Bacteriol. 175: 259–265.

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U. and Huis in't Veld JHJ. 1998. Overview of gut flora and probiotics. Int. J. Food Microbiol. 41: 85-101.

Ishibashi, N. and Shimamura S. 1993. Bifidobacteria: Research and development in Japan. Food Tech. June, 126-135.

Jiang, T., Mustapha, A. and Savaiano, D.A. 1996. Improvement of lactose digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*. J. Dairy Sci. 79: 750-757.

Kandel, G., Donohue-Rolfe, A., Donowitz, M. and Keusch, G.T. 1989. Pathogenesis of *Shigella* diarrhea. XVI. Selective targeting of shiga toxins on the intestinal electrolyte transport. J. Clin. Invest. 84: 1509-1517.

Karmali, M.A., Petric, M., Lim, C., Fleming P.C., Arbu, G.S. and Lior, H. 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J. Infect. Dis. 151: 775-782.

Keene, W.E., Sazie, E., Kok, J., Rice, D.H., Hancock, D.D., Balan, V.K., Zhao T. and Doyle, M.P. 1997. An outbreak of *Escherichia coli* O157:H7 infections traced to jerky made from deer meat. JAMA 277: 1229-1231.

Kim, S.H., Yang, S.J., Koo, H.C., Bae, W.K., Kim, J.Y., Park, J.H., Baek, Y.J., Park, Y.H. 2001. Inhibitory activity of *Bifidobacterium longum* HY8001 against vero cytotoxin of *Escherichia coli* O157:H7. J. Food Prot. 64(11): 1667-1673.

Konowalchuk, S., Speir, J.I. and Stavric, S. 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. 18: 775-779.

Leahy, S. C., Higgins, D. G., Fitzgerald, G. F. and Van Sinderen, D. 2005. Getting better with bifidobacteria. J. Appl. Microbiol. 98(6):2303-1315.

LeBlanc J.J. 2003. Implication of virulence factors in *Escherichia coli* O157:H7 pathogenesis. Crit. Rev. Micorbiol. 29(4): 277-296.

Lee, S.M. and Chen, J. 2004. Survival of *Escherichia coli* O157:H7 in set yogurt as influenced by the production of an exopolysaccharide, colanic acid. J. Food Prot. 67(2): 252-255.

Leenanon, B. and Drake, M. A. 2001. Acid stress, starvation and cold stress affect poststress behavior of *Escherichia coli* O157:H7 and nonpathogenic *Escherichia coli*. J. Food Prot. 64: 970-974.

Levine, M.M. 1987. *Escherichia coli* that cause diarrhea: Enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. 155, 377-389.

Leyer, G.J., Wang, L.L. and Johnson, E.A. 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl. Environ. Microbiol. 51: 3752-3755.

Li, Y., Yang, H., Kang, L., Xu, Y., Bai, B. and Song, W. 2005. Bifidobacteria DNA induces murine macrophages activation in vitro. Cell Mol. Immunol. 2(6): 473-478.

Masaki, H., Tokumaru, Y., Itaya, T., Aoki, A., Saiitoh, A., Andoh, K., Nose, N. and Katabami, J. 1992. Investigation of pathogenic *Escherichia coli*. Pollution of well water and its survival in various kinds of water. Japan. J. Food Microbiol. 9: 51-57.

Matsumoto, M., Ohishi, H. and Benno, Y. 2004. H+-ATPase activity in *Bifidobacterium* with special reference to acid tolerance. Int. J. Food Microbiol. 93:109-113

Mayer, J.B.; Moser, L. 1950. die Typendifferenzierung des thermobakterium Bifidum. Z. Kinderheilk. 455-468.

Moro, E. 1900. Über die nach Gram-färbbaren Bacillen des Säuglingsstuhles. Wien. Lin. Wschr. 13: 114.

Mutai, M., and Tanaka, R. 1987. Ecology of *Bifidobacterum* in the human intestinal flora. Bthd. Micro. 6: 33-41.

Nataro, J.P. and Kaper, J.B. 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11(1): 142-201.

O'Brien, A.D. and Holmes, R.K. 1987. Shiga and Shiga-like toxins. Microbiol. Rev. 51: 206-220.

Ogwaro, B. A., Gibson, H., Whitehead, M. and Hill, D.J. 2002. Survival of *Escherichia coli* O157:H7 in traditional African yoghurt fermentation. Int. J. Food Microbiol. 15, 79(1-2): 105-112.

Orban, J.I., Patterson, J.A. 2000. Modification of the phosphoketolase assay for rapid identification of bifidobacteria. J. Microbiol. Meth., 40: 221-224

Ouwehand A.C. and Vesterlund S. 2004. Antimicrobial components from lactic acid bacteria. In: S. Salminen, A. von Wright and A. Ouwehand eds. Lactic acid bacteria: microbiological and functional aspects. 3rd ed. Marcel Dekker, Inc., New York. 375-389.

Padhye, N.V. and Doyle, M.P. 1992. *Escherichia coli* O157:H7: Epidemiology, pathogenesis, and methods for detection in food. J. Food Prot. 55: 555-565.

Palumbo, S.A., Call, J.E., Schultz, F.J., and Williams, A.C. 1995. Minimum and maximum temperatures for growth and verotoxin production by hemorrhagic strains of *Escherichia coli*. J. Food Protect. 58: 352–356.

Park, S., Worobo, R.W. and Durst, R.A. 2001. *Escherichia coli* O157:H7 as an emerging foodborne pathogen: a literature review. Crit. Rev. Biotech. 21(1): 27-48.

Phillip, I., Tarr, M.D. Marguerite, A. and Neill, M.D. 2001. *Escherichia coli* O157:H7. Infectious Diarrhea. 30(3): 735-751.

Plummer, S., Weaver, M.A., Haris, J.C., Dee, P. and Hunter, J. 2004. *Clostridium difficile* pilot study: effect of probiotic supplementation on the incidence of *C. difficile* diarrhea. Int. Microbiol. 7: 59-62.

Proesmans, W. 1996. Typical and atypical hemolytic uremic syndrome. Kidney Blood Press. Res. 19: 205-208.

Raghubeer, E.V. and Matches, J.R. 1990. Temperature range for growth of *Escherichia coli* serotype O157:H7 and selected coliforms in *E. coli* medium. J. Clin. Microbiol. 28: 803-805.

Rajkowski, K.T. and Marmer, B.S. 1995. Growth of *Escherichia coli* O157:H7 at fluctuating incubation temperatures. J. Food Prot. 58:1307–1313.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzanch, F., Edberg, S., Medzhitov, R. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 118: 229-241.

Riley, L. W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Herbert, R.J., Olcott, E.S., Hohnson, L.M., Hargrett, N.T., Blake, P.A. and Cohen, M.L. 1983. Hemorrhagic colitis associatd with a rare *Escherichia coli* serotype. N. Engl. J. Med. 30: 681-685.

Rosberg-Cody, E., Ross, R.P., Hussey, S., Ryan, C.A., Murphy, B.P., Fitzgerald, G.F., Devery, R. and Stanton, C. 2004. Mining the microbiota of the neonatal gastrointestinal tract for conjugated linoleic acid-producing bifidobacteria. Appl. Environ. Microbiol. 70: 4635-4641.

Rogosa M. 1974. Genus III, *Bifidobacterium* Orla-Jensen. In: Buchanan R.E., Gibbons N.E., eds, Bergeys's Manual of Determinative Bacteriology, 8th edn., Williams & Wilkins, Baltimore, 669-676.

Rowbury, R.J. 1995. An assessment of environmental factors influencing acid tolerance and sensitivity in *Escherichia coli*, *Salmonella* spp. and other enterobacteria. Lett. Appl. Microbiol. 20: 333–337.

Salminen S., Deighton, M.A., Benno, Y. And Gorbach S.L. 1998. Lactic acid bacteria in health and disease. In: Salminen S., Von Wright A, eds. Lactic acid bacteria: microbiology and functional aspects, 2nd ed. Marcel Dekker Inc. New York. 211-53.

Sandvig, K., Garred, O., Prydz, K., Kozlov, J.V., Hansen, S.H. and van Deurs, B. 1992. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature. 358: 510-512.

Sandvig, K. and van Deurs, B. 1996. Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. Physiol. Rev. 76: 949-966.

SAS. 2004. Statistical analysis software. Version 9.0. SAS Institute Inc., Cary, NC, USA.

Servin, A. L. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbioal pathogens. FEMS Microbiol. Rev. 28: 405-440.

Scardovi, V. 1986. Genus *Bifidobacterium*. In: Bergey's Manual of Systematic Bacteriology, Vol 2. Baltimore, Williams and Wilkins, 1418-1434.

Scardivu, V. and Trovatelli, L.D. 1965. The fructose-6-phosphate shunt as peculiar pattern of hexose degradation in the genus *Bifidobacterium*. Ann. Microbiol. 15: 19-29.

Scardovi, V., Sgorbati, B. and Zani, G. 1971. Starch gel eletrophoresis of fructose-6-phosphoketolase in the genus *Bifidobacterium*. J. Bacteriol. 106: 1036-1039.

Schets, F.M., During, M., Italiaander, R., Heijnen, L., Rutjes, S.A., van der Zwaluw, W.K. and de Roda Husman A.M. 2005. *Escherichia coli* O157:H7 in drinking water from private water supplies in the Netherlands. Water Res. 39(18): 4485-4493.

Schmidt, H., Maier, E., Karch, H. and Benz, R. 1996. Pore-forming properties of the plasmid-encoded hemolysin of enterohemorrhagic *Escherichia coli* O157:H7. Eur. J. Biochem. 241: 594-601.

Scotland, S.M., Willshaw, G. A., Smith, H.R. and Rowe, B. 1987. Properties of strains of *Escherichia coli* belonging to serogroup O157 with special reference to production of Vero cytotoxin VT1 and VT2. Epidemiol. Infect. 99: 613-624.

Sebaold, M., Gasser, F. and Wermer, H. 1965. DNA base composition and classification. Application to group of bifidobacteria and to related genera, Ann. Inst. Pasteur. 109: 251-269.

Sghir, A., Gramet, G., Suau, A. Rochet, V., Pochart, P. Dore, J. 2000. Quantification of bacterial groups within the human fecal flora by oligonucleotide probe hybridization. Appl. Environ. Microbiol. 66: 2263-2266.

Shah, N.P. 2000. Probiotic bacteria: Selective enumeration and survival in dairy foods. J. Dairy Sci. 83: 894-907.

Shelef, L.A. 1994. Antimicrobial effects of lactates: a review. J. Food Prot. 19: 87-108.

Shimamura, S., Abe, F., Ishibashi, N., Yaeshima, T. A. and Tomita, M. 1992. Relationship between oxygen sensitivity and oxygen metabolism of *Bifidobacterium* species. J. Dairy Sci. 75: 3296-3306

Silk, T.D. and Donnelly C.W. 1997. Increased detection of acid-injured *Escherichia coli* O157:H7 in autoclaved apple cider by using nonselective repair or trypticase soy agar. J. Food Prot. 60: 1483-1486.

Simpson, P.J., Ross, R.P., Fitzgerald, G.F. and Stanton, C. 2004. *Bifidobacterium psychraerophilum* sp. nov and *Aeriscardovia aeriphila* gen. nov., sp. nov., isolated from a procine caecum. Int. J. Syst. Evol. Microbiol. 54: 401-406.

Sinclair, J.F., Dean-Nystrom E.A. and O'Brien A.D. 2006. The established intimin receptor Tir and the putative eucaryotic intimin receptors nucleolin and beta1 integrin localize at or near the site of enterohemorrhagic *Escherichia coli* O157:H7 adherence to enterocytes in vivo. Infect. Immun. 74(2): 1255-1265.

Stanley, K.D., Golden, D.A. Williams, R.C. and Weiss, J. 2004. Inactivation of *Escherichia coli* O157:H7 by high-intensity ultrasonication in the presence of salts. Foodborne Pathog. Dis. 1(4): 267-280.

Tamin, A.Y., Marshall, V.M.E. and Robinson, R.K. 1995. Microbiological and technological aspects of milks fermented by bifidobacteria. J. Dariy Res. 62: 151.

Tarr, P.I., Besser, T.E., Hancock, D.D., Keene, W.E. and Goldoft, M. 1997. Verotoxigenic *Escherichia coli* infection: U.S. overview. J. Food Prot. 60: 1466-1471.

Tavan E., Cayuela, C., Antonie, J.M. and Cassand, P. 2002. Antimutagenic activities of various lactic acid bacteria against food mutagens: heterocyclic amines. J. Dairy Res. 69: 335-341.

Tissier M.H. 1900. Recheriches sur la flore intestinale normale et pathologique du nourisson. Thesis, University of Paris.

Tsai, Y.W., Ingham, S.C. 1997. Survival of *Escherichia coli* O157:H7 and *Salmonella* spp. in acidic condiments. J. Food Prot. 60: 751–755.

Ventura, M., van Sinderen, D., Fitzgerald, G., and Zink, R. 2004. Insights into the taxonomy, genetics and physiology of bifidobacteria. Anton. Leeuw. Int. J. G. 86(3):205-23.

Vlková, E., Medková, J., Rada, V. 2002. Comparison of four methods for identification of bifidobacteria to the genus level. Czech J. Food Sci., 20: 171-174

Werner, H., Gasser, F. and Sebaold, M. 1966. DNA-baser bestimmunigen an 28 Bifidus Stämmen und Anstämmen morphologishähnlicher Gattungen. Zbl. Bak. I. Brig. 198S: 504-516.

Yamamoto, Y., Togawa, Y., Shimosaka, M. and Okazaki, M. 2003. Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11. Appl Environ Microbiol., 69(10): 5746-5753.

Zhao, T. and Doyle, M.P. 1994. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in commercial mayonnaise. J. Food Protect. 57: 780–783.

Zhao, T., Doyle, M.P., and Besser, R.E. 1993. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in apple cider without preservatives. Appl. Environ. Microbiol. 59: 2526–2530.

Zhao, T., Doyle, M.P., Shere, J., and Garber, L. 1995. Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. Appl. Environ. Microbiol. 61: 1290-1293.

APPENDIX

ADDITIONAL TABLES

Table 1 Habitats of the species of the genus *Bifidobacterium*

Species	Habitat
B. adolescentis	Feces of human adult; bovine rumen; wewage; human vagina
B. angulatum	Sewage; feces of human adult
B. animalis	Feces of rat, chicken, rabbit calf, and guinea pig; sewage
B. asteroides	Intestine of *Apis melligera*
B. bifidum	Feces of human adult, infant and suckling calf; human vagina
B. boum	Bovine ruman; feces of piglet
B. breve	Feces of infant and suckling calf, human vagina; sewage
B. catenulatum	Feces of infant and human adult; sewage
B. choerinum	Feces of piglet; sewage
B. coryneforme	Intestine of *Apis melligera*
B. cuniculi	Feces of rabbit
B. denticolens	Human dental caries
B. dentium	Human dental caries and oral cavity; feces of human adult; abscess and appendix
B. gallicum	Human feces
B. gallinarum	Chicken cecum
B. indicum	Intestine of *Apis cerana* and *A. dorsata*
B. infantis	Feces of infant and suckling calf;
B. inopinatum	Human dental caries
B. lactis	Fermented milk
B. longum	Feces of human adult, infant, and suckling calf; human vagina; sewage
B. magnum	Feces of rabbit
B. merycicum	Bovine rumen
B. minimum	Sewage
B. pseudocatenulatum	Feces of infant and suckling calf; sewage
B. pseudolongum	
ssp. *pseudolongum*	Feces of pig, chicken, bull, calf, rat, and guinea pig
ssp. *globosum*	Feces of piglet, suckling calf, rat, rabbit, and lamb;
B.psychraerophilum	Pig caecum
B. pullorum	Feces of chicken

Table 1 Habitats of the species of the genus *Bifidobacterium* (continued)

Species	Habitat
B. ruminantium	Bovine rumen
B. saeculare	Feces of rabbit
B. scardovii	Adult feces
B. subtile	Sewage
B. suis	Feces of piglet
B. thermacidophilum	Anaerobic digester
B. thermophilum	Feces of pig, chicken, and suckling calf; bovine rumen; sewage
B. thermacidophilum spp. *porcinum*	Piglet faeces

Adapted from Biavati *et al.* (2000) and Leahy *et al.* (2005)

Table 2 Absorbance (OD_{435nm}) value for the detection of enzyme F6PPK to identify bifidobacteria strains (n=3)

Bifidobacteria strain	OD_{435nm} value (± SD)
B. longum VMKB S2B	0.384±0.024
B.bifidum 15696	0.253±0.024
B.bifidum VMKB 221	0.529±0.024
B.longum VMKB 211	0.335±0.024
B.bifidum VMKB 213	0.378±0.012
B. longum 15708	0.391±0.026
Bifidobacterium spp. VSB1	0.405±0.034
B.bifidum 791	0.328±0.032
Bifidobacterium lactis/animalis Bb12	0.447±0.016

Table 3 Growth curve (absorbance, OD_{600} value) of bifidobacteria in MRS broth for 16 h of incubation at 37°C under anaerobic conditions (n=3)

Bifidobacteria strain	Incubation Time (h) (± SD)					
	0	3	6	9	12	15
B. longum VMKB S2B	0.006±0.002	0.017±0.007	0.093±0.024	0.195±0.038	0.839±0.021	0.892±0.079
B.bifidum 15696	0.008±0.004	0.017±0.011	0.034±0.004	0.085±0.033	0.276±0.198	0.713±0.196
B.bifidum VMKB 221	0.008±0.003	0.045±0.002	0.458±0.136	1.016±0.011	1.358±0.136	1.591±0.062
B.longum VMKB 211	0.008±0.002	0.022±0.010	0.089±0.026	0.169±0.014	0.615±0.152	0.829±0.126
B.bifidum VMKB 213	0.011±0.009	0.019±0.012	0.101±0.014	0.209±0.034	0.797±0.014	1.060±0.156
B. longum 15708	0.008±0.002	0.023±0.011	0.066±0.024	0.132±0.059	0.507±0.126	0.588±0.049
Bifidobacterium spp. VSB1	0.009±0.005	0.021±0.007	0.210±0.088	0.259±0.016	0.759±0.144	0.979±0.151
B.bifidum 791	0.007±0.002	0.021±0.008	0.146±0.040	0.264±0.019	0.926±0.103	1.074±0.093
Bifidobacterium lactis/animalis Bb12	0.005±0.002	0.018±0.011	0.075±0.032	0.110±0.031	0.477±0.125	0.703±0.158

Table 4 pH value of bifidobacteria in MRS broth for 16 h of incubation at 37°C under anaerobic conditions (n=3)

Bifidobacteria strain	Incubation Time (h) (± SD)					
	0	3	6	9	12	15
B. longum VMKB S2B	6.46±0.070	6.44±0.064	6.32±0.089	6.11±0.038	6.06±0.157	5.54±0.148
B.bifidum 15696	6.45±0.069	6.43±0.084	6.37±0.052	6.23±0.033	6.00±0.150	5.66±0.025
B.bifidum VMKB 221	6.45±0.072	6.42±0.087	5.96±0.201	5.56±0.011	5.36±0.110	5.04±0.176
B.longum VMKB 211	6.46±0.067	6.44±0.076	6.35±0.095	6.16±0.014	5.93±0.118	5.45±0.100
B.bifidum VMKB 213	6.42±0.079	6.40±0.078	6.19±0.181	6.00±0.034	5.78±0.110	5.42±0.150
B. longum 15708	6.42±0.061	6.40±0.070	6.27±0.150	6.15±0.059	5.83±0.111	5.71±0.030
Bifidobacterium spp. VSB1	6.42±0.059	6.41±0.067	6.22±0.127	5.99±0.016	5.76±0.076	5.24±0.120
B.bifidum 791	6.44±0.064	6.41±0.067	6.24±0.104	5.96±0.019	5.85±0.159	5.28±0.090
Bifidobacterium lactis/animalis Bb12	6.43±0.067	6.42±0.074	6.30±0.123	6.20±0.031	5.95±0.146	5.79±0.015

Table 5 Growth curve of *Escherichia coli* O157:H7 (ATCC 43890) and media pH in TSB for 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation Time (h)	pH value (± SD)	OD_{600} value (± SD)	Plate count (log CFU/ml) (± SD)
0	7.30±0.238	0.002±0.002	6.079±0.040
1		0.008±0.002	6.676±0.333
2		0.051±0.017	7.766±0.599
3	6.44±0.044	0.258±0.023	8.173±0.278
4		0.710±0.043	8.990±0.363
5		0.779±0.043	9.050±0.416
6		0.850±0.040	10.270±1.062
7	5.86±0.091	0.770±0.016	9.204±0.372
8		0.845±0.014	9.055±0.190
9		0.821±0.025	9.052±0.105
10		0.851±0.011	9.166±0.251
11		0.776±0.072	8.903±0.037
12	6.06±0.057	0.783±0.054	9.079±0.389
24	5.98±0.295	0.800±0.056	8.930±0.303

Table 6 Growth curve of acid-adapted *E. coli* O157:H7 (ATCC 43890) and media pH in TSB supplemented with 10g/l glucose (final pH following incubation: 4.8±0.007) and incubated at 37°C for 24 hours with aerobic shaking at 100 rpm (n=3)

Incubation Time (h)	pH value (± SD)	OD_{600} value (± SD)	Plate count (log CFU/ml) (± SD)
0	4.80±0.010	0.007±0.001	5.95±0.118
1		0.013±0.001	6.11±0.147
2		0.015±0.001	6.60±0.072
3		0.038±0.007	7.05±0.205
4	5.04±0.053	0.148±0.033	7.87±0.121
5		0.333±0.001	8.37±0.126
6		0.485±0.007	8.54±0.085
7		0.563±0.018	8.56±0.015
8	4.57±0.036	0.568±0.015	8.66±0.085
9		0.563±0.011	8.58±0.130
10		0.554±0.038	8.62±0.093
11	4.47±0.006	0.548±0.035	8.75±0.208
18	4.16±0.025	0.535±0.034	8.40±0.068
24	4.12±0.020	0.529±0.031	8.06±0.038

Table 7 Survival of *E. coli* O157:H7 (ATCC 43890) in acid-adjusted or unadjusted media (11% NFDM and MRS broth) during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	Unadjusted pH		Adjusted pH	
	11% NFDM (pH 6.39)[a]	MRS broth (pH 6.46)[a]	11% NFDM (pH 3.81)[ab]	MRS broth (pH 3.94)[b]
	(log CFU/ml) (± SD)			
0	5.97±0.07[D, a]	5.96±0.08[C, a]	6.06±0.13[A, a]	6.03±0.05[A, a]
4	8.03±0.05[C, a]	7.57±0.04[B, a]	5.94±0.11[A, b]	5.99±0.10[A,b]
8	8.52±0.17[B, a]	8.75±0.08[A, a]	5.72±0.09[A, b]	5.06±0.16[B, c]
24	8.87±0.03[A, a]	8.81±0.05[A, a]	1.20±1.05[B, b]	0[C, c]
Error Mean Square (EMS) [1]	0.0094	0.0041	0.28	0.0091
$LSD_{0.05}$ [1]	0.18	0.12	1.00	0.18
Coeff Var (C.V.) [1]	1.23	0.82	11	2.2
EMS	0.077 [2]		12.99 [3]	
$LSD_{0.05}$	0.46 [2]		3.21 [3]	
C.V.	4.50 [2]		4.50 [3]	

[A B C D] — Means (± standard deviations) with the same letter within each column are not different (P>0.05)

[a b c] — Means (± standard deviations) with the same letter within each row are not different (P>0.05)

[1] — Judge the difference among four incubation time in each treatment

[2] — Judge the difference among treatments in each incubation time

[3] — Judge the difference among treatments as a whole

Table 8 Survival of *E. coli* O157:H7 (ATCC 43890) in 11% NFDM combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	Bifidobacteria mix +10 µl *E.coli* O157:H7	10 µl *E.coli* O157:H7
	(log CFU/ml) (± SD)	
0	5.97 ± 0.05^{A}	6.06 ± 0.24^{C}
4	5.77 ± 0.15^{A}	7.63 ± 0.14^{B}
8	0.55 ± 0.95^{B}	8.32 ± 0.09^{A}
24	0^{B}	8.72 ± 0.26^{A}
EMS	0.14	
$LSD_{0.05}$	0.64	
C.V.	6.84	

$^{A\,B\,C}$ — Means and standard deviations with the same letter within each column are not different (P>0.05)

Table 9 Survival of *E. coli* O157:H7 (ATCC 43890) and media pH in MRS broth combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	Colony number (log CFU/ml) (± SD)		pH value (± SD)	
	Bifidobacteria mix + 10µl *E.coli* O157:H7	10µl *E.coli* O157:H7	Bifidobacteria mix + 10µl *E.coli* O157:H7	10µl *E.coli* O157:H7
0	6.07±0.13^{A}	5.99±0.10^{C}	4.94±0.12	6.55±0.050
1			4.41±0.096	6.54±0.061
2			4.22±0.042	6.53±0.061
3			4.11±0.050	6.52±0.055
4	4.9±0.31^{B}	7.58±0.17^{B}	4.05±0.046	6.51±0.070
5			4.02±0.038	6.43±0.069
6			3.99±0.026	6.28±0.091
7			3.95±0.044	6.06±0.12
8	**0**^{C}	8.80±0.032^{A}	**3.95±0.031**	5.90±0.12
24	0^{C}	8.85±0.16^{A}	4.04±0.14	5.02±0.45
EMS		0.027		
$LSD_{0.05}$		0.28		
C.V.		3.12		

$^{A\,B\,C}$ — Means and standard deviations with the same letter within each column are not different (P>0.05)

Table 10 Survival of *E. coli* O157:H7 (ATCC 43890) in 11% NFDM or MRS broth combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	Bifidobacteria mix + 10µl *E.coli* O157:H7 in 11% NFDM[b]	Bifidobacteria mix + 10µl *E.coli* O157:H7 in MRS broth[b]	10µl *E.coli* O157:H7 in 11% NFDM[a]	10µl *E.coli* O157:H7 in MRS broth[a]
	(log CFU/ml) (± SD)			
0	5.97±0.05[a]	6.07±0.13[a]	6.06±0.24[a]	5.99±0.095[a]
4	5.77±0.15[b]	4.89±0.31[c]	7.63±0.14[a]	7.58±0.16[a]
8	0.55±0.95[b]	0[c]	8.32±0.09[a]	8.80±0.032[a]
24	0[b]	0[b]	8.72±0.26[a]	8.85±0.16[a]
EMS[1]		0.081		
$LSD_{0.05}$ [1]		0.47		
C.V. [1]		5.37		
EMS [2]		17.89		
$LSD_{0.05}$ [2]		3.76		
C.V. [2]		5.37		

[a b c] — Means and standard deviations with the same letter within each row are not different (P>0.05)

[1] — Judge the difference among treatments in each incubation time

[2] — Judge the difference among treatments as a whole

Table 11 Survival of *E. coli* O157:H7 (ATCC 43890) when combined with bifidobacteria mix in the media (11% NFDM and MRS broth) and *E. coli* O157:H7 in acid-adjusted media during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	Bifidobacteria mix + 10µl *E.coli* O157:H7		10µl *E.coli* O157:H7 in acid-adjusted media	
	11% NFDM	MRS broth	Acid-adjusted 11% NFDM	Acid-adjusted MRS broth
	(log CFU/ml) (± SD)			
0	5.97±0.05[a]	6.07±0.13[a]	6.06±0.13[a]	6.03±0.05[a]
4	5.77±0.15[a]	4.89±0.31[b]	5.94±0.11[a]	5.99±0.10[a]
8	0.55±0.95[d]	0[d]	5.72±0.09[a]	5.06±0.16[b]
24	0[b]	0[b]	1.20±1.05[a]	0[b]
EMS	0.14			
$LSD_{0.05}$	0.62			
C.V.	10.06			

[a b c d] — Means and standard deviations with the same letter within each row are not different (P>0.05)

Table 12 Survival of acid-adapted *E. coli* O157:H7 (ATCC 43890) in 11% NFDM combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	Bifidobacteria mix +10 µl acid-adapted *E.coli* O157:H7	10 µl acid-adapted *E.coli* O157:H7
	(log CFU/ml) (± SD)	
0	5.53±0.05^{A}	5.52±0.18^{D}
4	5.48±0.02^{A}	7.44±0.07^{C}
8	0^{B}	8.33±0.09^{B}
24	0^{B}	8.62±0.15^{A}
EMS	0.0089	
$LSD_{0.05}$	0.16	
C.V.	1.84	

$^{A\,B\,C\,D}$ — Means and standard deviations with the same letter within each column are not different (P>0.05)

Table 13 Survival of acid-adapted and unadapted *E. coli* O157:H7 (ATCC 43890) in 11% NFDM combined with bifidobacteria mix during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	Bifidobacteria mix + 10µl acid-adapted *E.coli* O157:H7[b]	Bifidobacteria mix + 10µl unadapted *E.coli* O157:H7[b]	10µl acid-adapted *E.coli* O157:H7[a]	10µl unadapted *E.coli* O157:H7[a]
	(log CFU/ml) (± SD)			
0	5.53±0.05[b]	5.97±0.05[a]	5.52±0.18[b]	6.06±0.24[a]
4	5.48±0.02[b]	5.77±0.15[b]	7.44±0.07[a]	7.63±0.14[a]
8	0[c]	0.55±0.95[b]	8.33±0.09[a]	8.32±0.09[a]
24	0[b]	0[b]	8.62±0.15[a]	8.72±0.26[a]
EMS[1]		0.072		
$LSD_{0.05}$ [1]		0.45		
C.V. [1]		5.11		
EMS [2]		17.96		
$LSD_{0.05}$ [2]		3.77		
C.V. [2]		5.11		

[a b c] — Means and standard deviations with the same letter within each row are not different (P>0.05)

[1] — Judge the difference among treatments in each incubation time

[2] — Judge the difference among treatments as a whole

Table 14 Survival of *E. coli* O157:H7 (ATCC 43890) and media pH in the supernatant of bifidobacteria during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	Colony number (log CFU/ml) (± SD)		pH value (± SD)	
	MRS supernatant (adjusted pH 6.50)	MRS supernatant (unadjusted pH 4.38)	MRS supernatant (adjusted pH 6.50)	MRS supernatant (unadjusted pH 4.38)
0	6.01 ± 0.12^{D}	6.06 ± 0.075^{A}	6.59±0.06	4.20±0.09
2			6.61±0.11	4.19±0.07
4	7.14 ± 0.10^{C}	0^{B}	6.68±0.09	4.22±0.08
6			6.61±0.06	4.20±0.08
8	8.41 ± 0.13^{B}	0^{B}	6.34±0.08	4.20±0.07
24	8.60 ± 0.01^{A}	0^{B}	5.08±0.34	4.10±0.08
EMS		0.0058		
$LSD_{0.05}$		0.13		
C.V.		1.678		

$^{A\,B\,C}$ — Means and standard deviations with the same letter within each column are not different (P>0.05)

Table 15 Survival of *E. coli* O157:H7 (ATCC 43890) in pH adjusted MRS supernatant or fresh MRS broth during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	MRS supernatant (adjusted pH 6.50)[a]	Fresh MRS broth[a]
	(log CFU/ml) (± SD)	
0	6.01±0.12[a]	5.993±0.095[a]
4	7.14±0.10[b]	7.580±0.165[a]
8	8.41±0.13[b]	8.803±0.032[a]
24	8.60±0.01[b]	8.847±0.162[a]
EMS[1]	0.018	
$LSD_{0.05}$ [1]	0.23	
C.V. [1]	1.75	
EMS [2]	4.77	
$LSD_{0.05}$ [2]	2.18	
C.V. [2]	1.75	

[a b] — Means and standard deviations with the same letter within each row are not different (P>0.05)

[1] — Judge the difference among treatments in each incubation time

[2] — Judge the difference among treatments as a whole

Table 16 Survival of *E. coli* O157:H7 (ATCC 43890) in MRS supernatant or acid-adjusted (fresh) MRS broth during 24 h of incubation at 37°C with aerobic shaking at 100 rpm (n=3)

Incubation time (h)	pH value of MRS supernatant (± SD)	MRS supernatant (log CFU/ml) (± SD)	Acid-adjusted (fresh) MRS broth (log CFU/ml) (± SD)
0	4.20±0.09	6.06±0.075[a]	6.03±0.05[a]
2	4.19±0.07		
4	4.22±0.08	0[b]	5.99±0.10[a]
6	4.20±0.08		
8	4.20±0.07	0[b]	5.06±0.16[a]
24	4.10±0.08	0[a]	0[a]
EMS		0.0051	
$LSD_{0.05}$		0.12	
C.V.		2.47	

[a b c] — Means and standard deviations with the same letter within each row are not different (P>0.05)