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BOLU ABANT İZZET BAYSAL UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
Department of Biology

MOLECULAR CLONING AND CHARACTERIZATION OF BILE SALT HYDROLASE FROM *LACTOBACILLUS FERMENTUM*

MASTER OF SCIENCE

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ABSTRACT

MOLECULAR CLONING AND CHARACTERIZATION OF BILE SALT HYDROLASE FROM *LACTOBACILLUS FERMENTUM*
MSC THESIS
Halkawt ALI OTHMAN OTHMAN

BOLU ABANT İZZET BAYSAL UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
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BOLU, AUGUST 2022
xviii + 79

The gastrointestinal normal flora plays a vital role in human physiology, as it controls the host's nutritious, physiological, and immunological activities. In the human intestine, bile salt hydrolase (BSH), produced mainly by intestinal bacteria, catalyzes the hydrolysis of taurine or bile acid bound to glycine binding to amino acid and deconjugated bile acid. Deconjugated bile acids play an important role in the developing certain gastrointestinal diseases, such as gallstone formation, cholestasis, and colon cancer as well as blood cholesterol levels. However, the LAB are a group of microorganisms that mainly essensial for fermentative of dietary food in the market today. Because of the correlation with both BSH and human health, the structural and biochemical properties of BSHs require considerable research in order to understand their role in the regulation bacterial and host metabolism. The catalytic activities and substrate specificities of BSHs vary more. Some amino acids reported to be responsible for catalytic activity were also entirely kept intact across all BSH members of the same family. As an outcome, knowing and ability to understand the mechanism of action of the BSH enzymes of the probiotic microorganisms used in those treatments and fermentation is very vital. Despite the fact that *Lactobacillus fermentum* is likely one of the most beneficial probiotic bacteria strain on the market in the world today, little information is known about the mechanism of action and substrate preferences of this microorganism's BSH enzyme. In this study, the *bsh* gene encoding 326-amino acids from *L. fermentum* strain was cloned, expressed and characterized in *Escherichia coli* BLR(DE3) strain. An *E. coli* BLR(DE3)-pET22b expression system was used to produce rcombinant BSH. The

recombinant BSH activity of *L. fermentum* was not determined qualitatively using the Direct Plate Assay with two different bile salts, glydeoxucocholic acid and taurodeoxycholic acid. In addition to this, 37 kDa BSH protein was not visible on SDS-PAGE analysis. Multiple sequence alignment revealed that BSH enzyme of *L. fermentum* had a one mutation (one codon deletion) on *bsh* gene. This mutation can affect the stability of BSH enzyme of *L. fermentum*. In this respect, *L. fermentum* cannot be a potential probiotic candidate since the capability of BSH activity is one of the criteria for the selection of the probiotics.

KEYWORDS: *Lactobacillus fermentum*, Probiotics, Purification, Bile Salt Hydrolase (BSH), Lactic Acid Bacteria, Polymerase Chain Reaction (PCR), Cloning Vector, SDS-PAGE.

ÖZET

LACTOBACILLUS FERMENTUM'DAN SAFRA TUZU HİDROLAZININ MOLEKÜLER KLONLANMASI VE KARAKTERİZASYONU
YÜKSEK LİSANS TEZİ
HALKAWT ALİ OTHMAN OTHMAN

BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ
BİYOLOJİ ANABİLİM DALI

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Gastrointestinal mikrobiyota insan fizyolojisinde önemlidir ve konağın beslenme, fizyolojik ve immünolojik işlevlerinden sorumludur. İnsan bağırsağında, çoğunlukla bağırsak bakterileri tarafından üretilen safra tuzu hidrolazı (BSH), taurin veya glisine bağlı safra asidinin aminoasit ve dekonjuge safra asidine hidrolizini katalize eder. Dekonjuge safra asitleri, kan kolesterol seviyelerini sınırlamada ve safra taşı oluşumu, kolestaz ve kolon kanseri gibi bazı gastrointestinal hastalıkların gelişiminde önemli bir rol oynayabilir. Bununla birlikte, LAB, bugün piyasada diyet gıdaların fermentatifi için gerekli olan bir mikroorganizma grubudur. Hem BSH hem de insan sağlığı ile korelasyonu nedeniyle, BSH'lerin yapısal ve biyokimyasal özellikleri, bakteri ve konak metabolizmasının düzenlenmesindeki rollerini anlamak için önemli araştırmalar gerektirir. BSH'lerin katalitik aktiviteleri ve substrat spesifiklikleri daha fazla değişiklik gösterir. katalitik aktiviteden sorumlu olduğu düşünülen bazı aminoasitler, BSH ailesinin tüm üyelerinde tamamen korunmuştur. Sonuç olarak, bu tedavilerde ve fermantasyonda kullanılan probiyotiklerin BSH enzimlerinin etki mekanizmasını bilmek ve anlamak hayati önem taşımaktadır. *Lactobacillus fermentum* muhtemelen bugün piyasadaki en faydalı probiyotik türlerden biri olmasına rağmen, bu mikroorganizmanın BSH enziminin etki mekanizması ve substrat tercihleri hakkında çok az şey bilinmektedir. Bu çalışmada, *L. fermentum*'den 326 aminoasidi kodlayan *bsh* geni *Escherichia coli* BLR(DE3) suşuna klonlanmış eksprese edilmiş ve karakterize edilmiştir. *E. coli* BLR(DE3)-pET22b ekspresyon sistemi, rekombinant BSH üretmek için kullanılmıştır. *L.*

fermentum'un rekombinant STH aktivitesi, iki farklı safra tuzu, glideoksukokolik asit ve taurodeoksikolik asit ile Doğrudan Plaka Testi kullanılarak kalitatif olarak belirlenememiştir. Buna ek olarak 37 kDa BSH proteini SDS-PAGE analizinde de görülmemiştir. Çoklu dizi hizalaması, *L fermentum*'un BSH enziminin *bsh* geninde bir mutasyona (bir kodon silinmesi) sahip olduğunu ortaya çıkardı. Bu mutasyon, *L. fermentum*'un BSH enziminin stabilitesini etkileyebilir. Bu bağlamda, Bu mutasyon, *L. fermentum* BSH enziminin stabilitesini etkileyebilir. Bu bağlamda, *L. fermentumu* potansiyel bir probiyotik adayı olamaz çünkü probiyotik adaylarının BSH aktivitesine sahip olmaları probiyotiklerin seçimindeki kriterlerden biridir.

ANAHTAR KELİMELER: Safra tuzu hidrolazı (BSH), *Lactobacillus fermentum*, Probiyotikler, saflaştırma, laktik asit bakterileri, Klonlama vektörü, SDS-PAGE, Polimeraz zincir reaksiyonu (PCR).

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LIST OF ABBREVIATIONS AND SYMBOLS

Amp^R : Ampicilline Resistance

AGA : American Gastroenterological Association

BLAST : Basic Local Aalignment Search Tool

bp : Base Pair

BSA : Bovin Serum Albumin

BSH : Bilesalt Hydrolase

CVDs : Cardiovascular Diseases

CHD : Coronary Heart Disease

DTT : Dithiothreitol

FAO : Food/agriculture Organization

FDA : Food/drug Administration

EDTA : Ethylenediamine Tetraacetic Acid

G+ : Gram Positive

GCA : Glyocolic-Acid

GCDCA : Glycochenodeoxycholic Acid

GDCA : Glycodeoxycholic Acid

GIT : Gastro Intestinal Tract

GRAS : Generally Recognized/As Safe

HDL : High-Density Lipoprotein

IgA : Immuno globulin A

IBD : Inflamatory Bowel Disease

IBS : Irritable Bowel Syndrome

IPTG : Isopropyl β-D-1-thiogalactopyranoside

Kbp : Killo Basepair

Kda : Killo Dalton

L : Litter

LAB : Lactic Acid Bacteria

LB : Luria Bertani-Broth

LDL : Low density Lipoproteins

ml : Mili-Litter

MRS : de MAN, ROGOSA and SHARP

µl : Mikro-Litter

µM : Micro-Molar

Ni+2 : Nickel (II)

ng : Nano-Gram

Ntn : N-terminal Nucleophilic

OD : Optic Density

PEG : Poly-Ethylene Glycol

PCR : Polymerase Chain Reaction

rpm : Rotation Per Minute

SCFA : Short Chain/Fatty Acids

SDS : Sodium Dodecyl Sulfate

PAGE : Polyacrylamide Gel Electrophoresis

SIB : Swiss Institute of Bioinformatics

SCORAD : Ability to Score Atopic Dermatitis

TCA : Taurocholic Acid

TCA : Trichloro AceticAcid

TCDCA : Tauro-Chenodeoxycholic Acid

TDCA : Tauro-deoxycholic Acid

Tween20 : Polyethylene-Glycol Sorbitan-Monolaurate

U : Enzyme Unit

UTI : Urinary-Tract Infection

UV : Ultra Violet

WHO **:** World Health Organization

Xgal **:** 5-Bromo-4-Chloro 3-Indolyl-β-D-Galacto Pyranoside

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1. INTRODUCTION

1.1 History and Definition of Probiotics

The term of “normal microflora” first appeared at the end of last century ever since microbiologists and physiologists both learned such microorganisms and their role in our life, the meaning of the concept “probiotic” is a combination of the Latin pro and the Greek bios, which means “for life” which was first used in 1954 and The Greek meaning of the term (‘for life’) exactly 1ractice1e with the period ‘antibiotics’ meaning “against life”.

A decade later, Lilly and Stillwells defined the term probiotic as “substances yield by microorganisms which stimulate the growth of all others” (1). Also, Parker used the word probiotics in a different way, referring to animal feed supplements that were specifically designed to improve health. He added a new term: “organisms and substances that influence to the GIT of microbial balance” (2). Hence, one would say that the origins of probiotics started and, either paralleled with the use of fermented foods by mankind (3). However, in the early twentieth century, Metchmikoff found the therapeutic benefits of probiotics. He suspected that the longer and/or healthier life span of Bulgarian peoples seems to be owing to their usage of fermented milk products (1).

In this regard, numerous definitions have been proposed for the word probiotics over the time. Probiotics were defined by Marteau and his colleagues as “microbial preparations or elements of microbial cells which also improve a beneficial effect on health and human well-being (4).” This was also coincided which multiple discoveries throughout the century. Hull discovered the first probiotic species, *Lactobacillus acidophilus*, in 1984, however, Holcombh later identified *Bifidobacterium bifidum* in 1991(5). Furthermore, A probiotic is a live microbial addition which enhance the host’s respond diseases by modifying its host’s microbial diversity. (6). Since then (i.e., 1984), the most successful and perfect description of probiotics it has been started by Roy fuller’s description it says “a live microbial feed supplement that benefits the host animal by improving its intestinal microbial balance”. The principle can also be subjected to human nutrition and medicine (2).

Even now the United Nations' Food and Agriculture Organization (FAO) and the World Health Organization (WHO) in the year (2002), discussed the new field of probiotics are defined as "live micro-organisms that, once prescribed in sufficient amounts, provide such a health benefit on the host ". All probiotics must be "healthy for their intended usage" (7). Ever since, the above definition has become the most widely accepted and used version throughout the world (7–10). These efforts resulted in gradual enhancement in our understanding of the microbiological and physiological aspects of this group of microorganisms as whole and impacted our approaches in finding more about them.

A vast number of bacteria can be clearly found in the different parts of the human body, including both of the surface and deep layers, skin, mouth, lungs, intestine, and all surfaces exposed to the outside world. Over the years, a number of microbes have been utilized as probiotics for humans and these are presented in Figure 1. While bacteria, yeasts, and fungi can all be used as probiotics, still only bacterial strains, exclusivily Lactic acid bacteria (LAB), are widely used. Therefore, the strain of LAB was selected in this study, LAB genera, such as lactobacilli, streptococcus, and bifidobacterial, are the most widely accepted micro-organisms that have a long story of safe use in the human gastro intestinal tract (GIT). Additionally, to LAB, the GIT contains some yeast species such as *Saccharomyces boulardii* as well as non- LAB such as Bacillus in Table 1. The almost all of such micro-organisms enjoy living in the colon. However; they can be present in the GIT. The microbiota performs a key role in the healthy as well as immune system within human body. Probiotics generate a variety of metabolites such a result of their metabolic activity.

The majority of these bacteria are considered beneficial bacteria because they antagonist's pathogenic bacteria, modification of the gut microbiota can help patients benefiting from liver disease and increase the immune response.
In quite some time, antibiotics and chemotherapies have proven to be effective treatment options, but in the last few decades, pro-, pre-, as well as symbiotic, are widely applyied as complementary methods against additional health effects such as cancer prevention, IBD, immune modulation, and so on are also discussed

infectious for human disease. Prebiotics are a type of non-digestible special plant of soluble fibres by our gut. They serve as food for some genera of probiotics which are tiny living microorganisms, that mainly lactobacilli and bifidobacterial and therefore, provide us as a source of nutrition for such colon's own cells. The word prebiotic is less well known by the general publics.

The term symbiotic corresponds to" a mixture comprising live micro-organisms and/or Substrates differentially beng used by host micro-organisms that confer a benefit to the host". A symbiotic product benefits the host by trying to enhance the preservation but rather integration of live microbial supplements there in GIT, particularly by accelerating the production but instead metabolic activity rather than one or a small number including well bacteria. *Lactobacillus*, *Bifidobacterium* spp., *S. boulardii*, and *Bifidobacterium coagulants* are some of the probiotic strains used in symbiotic formulations. In organisms' probiotics, prebiotic, and symbiotic improve both specific and nonspecific defence system. The microbiota's defences system is activated by direct interaction with harmful bacteria and by controlling the immune system. Immune response has been successfully increased by probiotics, prebiotics, and also symbiotic, which have well defence and minimize the risks related with either stress factors or human disease. These substances have several advantages, including promoting host growth and immune responses to harmful bacteria, as well as to protect environmental consistency and health, and their uses are well known (6,7,11–13).

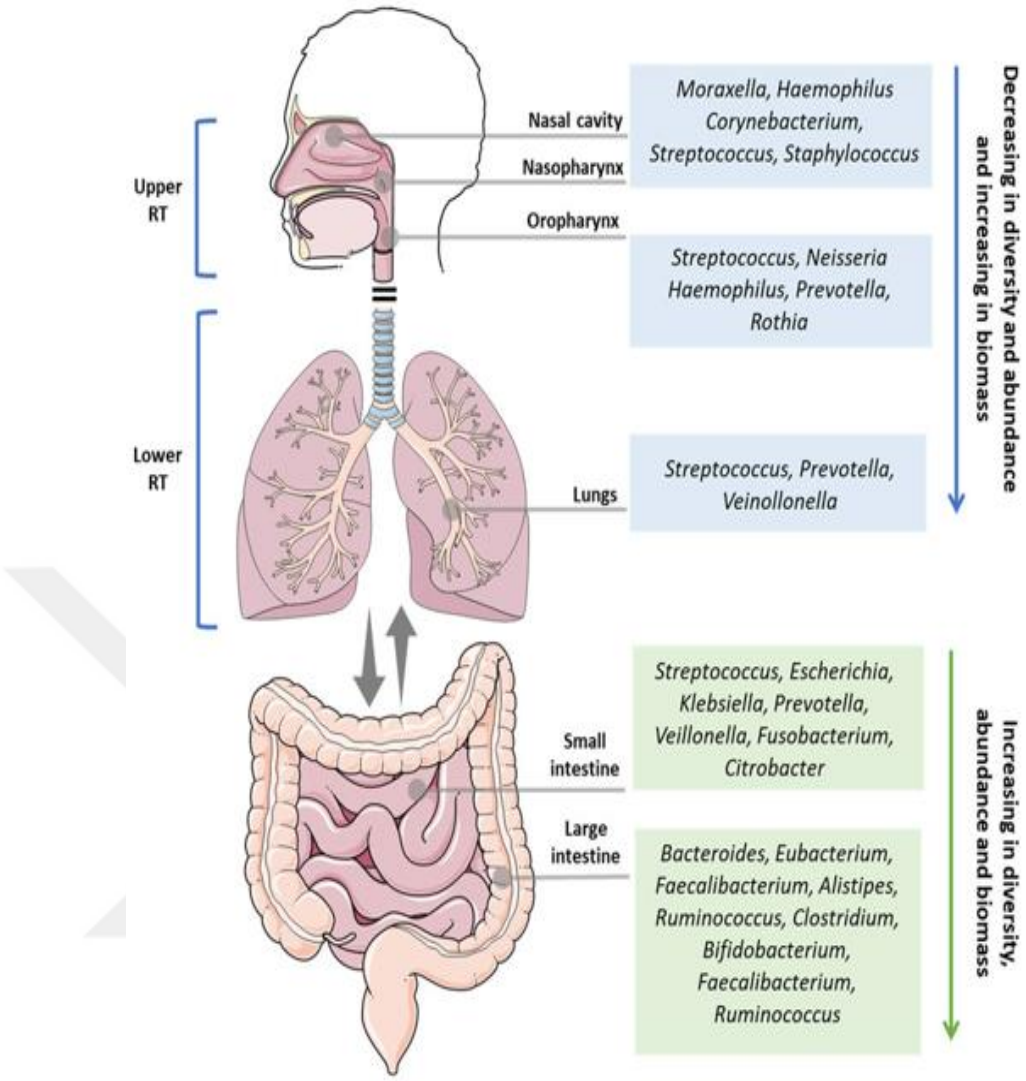

Figure 1.1 Diversity of various microbiome in human body (14).

Table 1.1. Common microorganisms used as probiotics for humans.

Lactobacilli	**Bifidobacterium**	**Other lactic acid bacteria**	**Non lactic acid bacteria**
Lactobacillus fermentum	*Bifidobacterium bifidum*	*Streptococcus thermophilus*	*Bacillus cereus var. toyoi*
Lactobacillus reuteri	*Bifidobacterium Infants*	*Lactococcuslactics ssp lactis*	*Propionibacterium*
Lactobacillus acidophilus	*Bifidobacterium adolescentis*	*Enterococcus faecalis*	*Saccharomyces boulardii*
Lactobacillus plantarum	*Bifidobacterium longum*	*Enterococcus faecium*	*Escherichia colistrain nissle*
Lactobacillus casei	*Bifidobacterium breve*	*Sporolactobacillus inulinus*	*Saccharomyces cerevisiae*
Lactobacillus rhamnosus	*Bifidobacterium antimalis*	*Pediococcus acidilactici*	*Escherichia colistrain nissle*
Lactobacillus delbrueckii	*Bifidobacterium lactis*	*Leuconstoc mesenteroides*	
Lactobacillus johnsonii			

1.1.1. The Selection Criteria of Probiotics

Probiotics have made a great impression on researchers in the past few years due to their positive effects by both the newly manufactured product found in the market either the host benefit health. The bright future probiotic strain is required to have a variety of favorable properties and the ability to achieve its beneficial effects on the host cell. Knowing the taxonomic classification of the microorganism is the initial step in selecting a probiotic strain since identifying the environment, source and physiochemical characteristic of the strain will affect our definition of the probiotics usability (13). To be considered as a potential probiotic, for every bacterial strain should really possess some unique characteristics. Per the to the FAO/WHO ®, this product is generally recognized

as safe (GRAS) (15). The mainly critical selection factor used for choosing probiotic strains includes that the bacteria or other organism must be:

5.2.1.6 **Safety of Probiotics**

The determination of safety is the first step in the selection and evaluation of probiotic strain they must not be harmful (friendly to the host cell and deadly to the cancer and pathogenic bacteria) which means should be meet the US Food and Drug Administration (FDA) Isolated from the same species as the intended host cell, selection requirement for individual use or be regarded as generally recognized as safe (GRAS) status the gastro intestinal tract and also breast milk are both good sources for identifying potential probiotic strains for humans (16).

1.1.1.2 Acid and Bile Salt Tolerance:

When choosing probiotic strains, one of the most critical properties that are looked for is acid resistance. The ability to tolerate acid and bile is requirement for a probiotic strain (17). Tolerance is needed not just for survival, but also for easy colonization of the GIT (16). It has been shown that bile salt hydrolase activity gives tow great benefits to the bacteria which are increasing their resistance to Conjugated bilesalt and enhancing their survival in the GIT to colonization (18,19).

1.1.1.3 Adherence to the Human Intestinal Mucosal Cell Wall

Attachment of bacteria to epithelial cells is indeed a complex interplay one which links two cell membranes, microbial or rather human, and therefore is predicated mostly on chemical but also physiochemical structure of said probiotic strain's cell surface (13).

1.1.1.4 Antioxidant Activity

A successful probiotic allows be able to show viable antioxidant ability, and probiotics may show antioxidant activity in a variety of ways. Probiotics may

have a potential therapeutic effect in lowering reactive oxygen species and treating GI disorders, according to various studies (16).

1.1.1.5 Cell Viability

The viability of probiotics is always important for reaching the action parts of the GIT during preparation and passaging. However, it has been shown that Probiotics lose their positive effects on human health after entering the action phase during some change in (biological, chemical and physical) condition (13, 21).

1.1.1.6 Immune Stimulating Activity Against Pathogenic Bacteria

This is another critical factor to remember for candidate Probiotics strains in the GI Tract. Probiotics must have secreted substance such as (organic acid, bacteriocins and hydrogen peroxide and others), either Antibiotic tolerance in microorganisms these has increased due to the uncontrolled use of antibiotics in human and veterinary medicine against pathogen because these elements are able to limiting the harmful bacteria ability. These beneficial effects are usually due to probiotics' ability to control intestinal permeability and normalize host intestinal microbiome. Furthermore, a candidate Probiotics cytotoxicity is important to strain selecting as a probiotic because toxic product and free radicals may cause free radical chain reaction through destroying biological elements (14, 17).

1.1.2 The Impact of Probiotics on Human Health

The positive impacts of probiotics on human health and nutrition have become increasingly evident, probiotics may also be used as a dietary source to 7ractice the gut health and essencial for human self. Human health is dependent on the gut microbiota strains. Probiotics, antibiotics, prebiotics, and the immune status of the host all are these factors can make a difference in the composition of the gut microbiome. An increasing number of studies have been performed to investigate the impact of the gut microbiota on immune system, and also suggest a possible relationship between the gut microbiota also Multiple sclerosis, diabetes, IBD Inflammatory Bowel Disease, but instead arthritis are all instances of autoimmune disorders.

Probiotics have been widely used as an immune-modulator, and some probiotics, for instance both *L. acidophilus* and NCFM *L. rhamnoses* GG, can improve the disease-related indicators of diabetes. For example, *L. acidophilus* NCFM may modulate the levels of interleukin-1 (IL-1), IL-6, IL-12, and IL-18, which are related to diabetes. *L. plantarum* may modulate the cytokine level of the colonic mucosa of streptozotocin-induced diabetic rats. *L. rhamnoses* GG has been reported to inhibit the production of TNF-α, IL-1, and interferon (IFN)-γ, and to enhance IL-10 production in intestinal epithelial cells, thus protecting against DSS-induced colitis. Beneficial outcome about probiotics is increasing day by day. Some specific probiotic bacteria have been related to beneficial effect on the human host cell, such as the promotion of gastro intestinal maturation and honesty (21). Probiotics have been shown to increase lactose absorption, firstly by slowing gastric digestion and intestinal movement, thus resulting in delaying lactose transferring toards the intestine, improving the action of residual β-galactosidase in the small intestine but instead lowering lactose massive 8racti. The activity of β-galactosidase of probiotics is 8ractic being the 2nd vital factor that responsible for improvement of lactose digestion.

American Gastroenterological Association (AGA) tested that using the combination of tow or mor different strains of probiotic can treat of many GI diseases such as: in infants less than 37 weeks GI age and premature (low birth-weight) infants, Crohn's disease, IBD, and colitis (23, 24). It has been shown that human have a healthy gut fully of normal flora, this is related low systemic inflammation, hence, this may case COVID-19 frequency should be reduced. The immune system is important and may provide the secret to defeating corona virus. Acknowledging the target cytokine pathways and gut flora associations to cytokine responses throughout (SARS-CoV-2) disorder is crucial for developing novel therapeutic strategies. Moreover, these viruses can affect many human system or organs for instant: respiratory system, GIT, CNC, and also cardiovascular system (CVS) (23). There have been over 20 million confirmed cases globally, with over 740,000 deaths as a result (23).

Prevention and management of antibiotic associated diarrhea, along with verification of several strains' affect the quality for both adult and childhood

(24,25). More research shows that probiotics may help people with type 2 diabetes, auto-immune diseases. Improvement of rheumatoid arthritis is a type of chronic inflammatory diseases. Treatment of inflammatory bowel disease IBD (21). As per the recent research, the microbiota has an effect on gestational health and performance (22). Increased immunoglobulin IgG and IgA serum levels also boost flu immunization. There has since been a positive effect in the management of atopic disorders, with a decrease in the SCORAD (Ability to score Atopic Dermatitis) caused by atopic dermatitis or atopic allergies, asthma eczema, and food intolerances (glutin and/or fructose intolerance) (24). Probiotics were shown in prior reports to have a potential beneficial impact on the respiratory system, notably in the prevention and management of the duration and frequency of acute respiratory infections, and even an increase in IgA-secreting cells within the 9racti mucosa (24).

1.2 Lactic Acid Bacteria (LAB)

Lactic acid bacteria are regarded as the main group of probiotic bacteria (26). Historically they consist of numerous of bacterial species of the genus with in Lactobacillales order, which is the most well-known contains, *Lactococcus*, *lactobacillus*, *Streptococcus*, *Carnobacterium*, *Enterococcus* and also, *Leuconostoc*, *Weisella*, *Tetragenococcus*, *Vagococcus*, *Oenococcus*, and *Pediococcus* (27, 28). These genera differ in terms of morphology, optimal temperature, pH and salt tolerance, pathogenic potential and habitats (27–29). They are typically a non-respiratory and therefore don't yield catalase,They mainly ferment glucose and other sugars to lactic acid and/or to (lactic acid, CO_2, and ethanol) (30, 31). There are two basic metabolic groups of LABS based on sugar fermentation patterns: homofermentative and heterofermentative.

1. Numerouse bacterial species, each with *L. acidophilus*, *L. casei,* and *L. plantarum*, have homofermentative glucose metabolism because lactic acid is the major product, accounting for at least 85 % of end metabolic products, Embden Meyerhof (E-M) pathway.
2. Across many bacterial species, like as *L. fermentum* either *L. brevis*, glucose metabolism seems to be heterofermentative, of lactic acid financial

reporting for 10racti half of metabolic by-products and ethanol, acetic acid, and carbon dioxide accounting for the other half.

The presence of the key enzymes of something like the E-M pathway (fructose 1,6-diphosphate) and 10ract the (PK) pathway is the vital difference between these two above categories at the enzyme level (phosphor ketolase) (32–36). At a pH of less than (5.0) in a meat product, the conditions seem to be more suitable for heterofermentative *Lactobacillus* spp. growth than homofermentative *Lactobacillus* spp. growth. To ferment compounds like sugars, several *Lactobacillus* spp. need an Aw higher than 0.95 (37)**.** Aw values less than 0.95 severely limit growth, while values less than 0.92 completely halt growth. Growth necessitates a pH of more than 3.2 (37)**.**

LAB all grow anaerobically, But unlike other anaerobes, they grow as "aerotolerant anaerobes" in the presence of air or oxygen (O_2) (38). However, if they have little or no catalase, they still have superoxide dismutase and just a diversity from other detoxification processes, a greater part among which require peroxidase enzymes (39). LAB members are non-pathogenic organisms apart from a few species which are assumed Generally Recognized as Safe Status (GRAS) and are generally recognized as safe food-grade microorganisms (26,32).

Even though many bacteria genera are yield lactic acid (LA) as a primary or secondary final product of fermentation; they are restricted to sugar-containing environments because sugar metabolism provides all of their energy (40). One of the most popular kinds of microorganisms found in food fermentation is lactic acid bacteria (27,37,41). Those who make a significant contribution to the taste and texture of fermented foods by generating growth-suppressing chemical compounds and significant amounts of lactic acid, and further inhibiting harmful bacteria germs (27).

As fermentation agent, LAB is widely used in the production of yogurt, cheese, cultured butter, sour cream, sausage, cucumber pickles, olives, and sauerkraut, despite the fact that a few varaiety of species can degrade beer, wine, and processed meats (42). LAB was among the first living species in the world, appearing around three billion years ago, during the transition from anaerobiosis

to aerobiosis (40). The *Lactobacillus* or otherwise *Bifidobacterium* genera have received the most attention and/or documented lactic acid bacteria strains in addition to significant their potential probiotic properties (40, 43).

Yogurt produced in Europe and other parts of the world over the last few decades is the direct consequence fermentation of cow milk by something like a gut flora one which incorporates *Delbrueckii bulgaricus* as well as *Streptococcus thermophilus*. Fermentation requires three hour shifts at 42 °C, and in store packaging either in bulk, generating set or stirred yoghurt. Lactic acid makes up 1.2–1.4% of the final product. Yogurt can also be made with goat's milk. In Greece, Historically, sheep's milk would be used to make yoghurt, with something like a tiny portion of a particular batch allowed to serve as a booster (44).

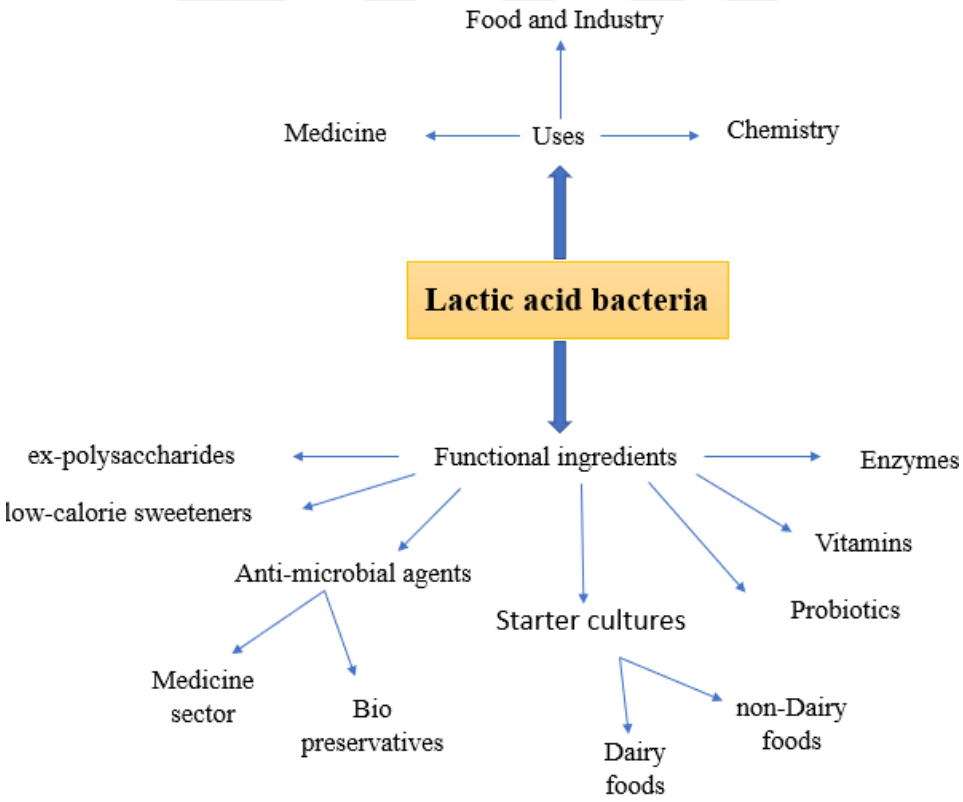

Figure 1.2. Functional ingredients and uses of lactic acid bacteria (LAB)with some mofications (45).

1.2.1 The Genus of *Lactobacillus*

Beijerinck proposed the genus *Lactobacillus* in 1901. It is a very diverse genus with species exhibiting a wide range of morphological, biochemical, and physiological characteristics (46, 47). *Lactobacillus* spp. do seem to be participants upon Lactobacillaceae family and even the lactobacillus genus is just a group of Gram+, anti-spore formation (cocci, coccobacilli), and/or rod-shaped either less than (53Mol % - G+C) contents base pair in their DNA compositions (25, 26). *Lactobacillus* spp. genome sizes vary greatly, ranging from around 1 to more than 4 M band they are lack the enzyme catalase (48). The Lactobacillus genera generally found in a variety of animal feeds, water, soil, silage, dung, milk, and dairy products (34, 38). Lactobacillus are commensal bacteria that live in the gastrointestinal systems of animals and humans, as well as genital tract and the human mouth (46, 47).

Various species of Lactobacillus are used commercially in the production of sour milks, yogurt, and cheeses, as well as they have a vital role during the production of fermented vegetables (sauerkraut and pickles), beverages (juices and wine), sourdough breads, and some sausages (49, 50). The genus has been classified into three main sub-groups, with approximately 261 species recognized (51). Lactobacilli of Category I are obligately homofermentative, producing lactic acid as a significant end product (>85%) from glucose. *L. delbrueckii* and *L. acidophilus* are the species that 12racticent them. They can grow at 45 degrees 12ractic but not at 15 degrees 12ractic. Class II, is likewise homofermentative, grows at 15ºC and exhibits fluctuating growth at 45ºC. *L. casei* and *L. plantarum* can generate more oxidized fermentations If oxygen (O_2) is present, (e.g., acetate). Group III Lactobacilli are heterofermentative. They yield lactic acid from glucose, and also carbon dioxide (CO_2) as well as ethanol. Aldolase is still not present, although phosphoketolase is. *Lactobacillus fermentum*, *Lactobacillus brevis*, and *Lactobacillus keferi* are examples of representative species (40, 46–48, 52). The majority of lactobacilli species are homofermentative, although some are heterofermentative (46, 53).

Lactobacillus, is characterized from other species of the genus in the family, by its capacity to make lactic acid as a by product of glucose metabolism

and they are generating appropriate amounts of lactic acid LA (40, 47, 54). *Lactobacillus* have always been non-motile bacterial strains which may persist during both aerobic and/or anaerobic conditions, instantces are including *L. delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus casei*, and *L. sanfranciscensis* (47, 49, 53).

1.2.2 *Lactobacillus fermentum*

L. fermentum is a facultatively heterofermentative species, so called since it causes fermentation (55). It can be utilized as a probiotic and is present in many types of cheese for instant (Comté, Ragusano) like a part of the Non-starter lactic acid bacteria (NSLAB) community (50,55). *L. fermentum* belongs to the *L. reuteri* group (37). These bacteria can be found in milk, milk processing plants, living or decaying plants, and the intestines of mammals, mainly humans (55, 56). The length of single or pairs of *L. fermentum* rods varies in length greatly (55, 57).

L. fermentum utilizes a variety of carbohydrates (trehalose cellobiose, galactose, maltose, mannose, arabinose, raffinose, xylose, sucrose, ribose, and melibiose), but the proceses of fermentation is related specific strain (55, 57). *L. fermentum* has a (G+C) concentration range of (52–54 mol %) and a peptidoglycan of the d-aspartyl-l-ornithine type. Actual catalase activity for hydrogen peroxide removal is shown (50, 57, 58).

One of the most common species found in natural whey cultures is the *Lactobacillus fermentum*, for a sort of stretched-curd cheese prepared from sheep's or cow's milk, cheeses undergo a unique stretching process, and hard, granular cheese that is cooked but not pressed (50, 57, 59). Like other food processes, Fermentation process, as lead to and/or creation of metabolites (60).

L. fermentum is widely found in fermented foods and is classified a "generally regarded as safe" (GRAS) organism by the US Food and Drug Administration FDA (60–62). All were noticed that it was fully secure for infants and kids. Even so, in immunodeficient individuals, it can sometimes give way to bacteremia. Probiotics must be avoided in patients who've had organ failure, an immunocompromised state, or a faulty gut mucosal system (61, 62).

The Whole-genome sequencing, and also standard microbiological, biochemical, and/or phenotypic techniques, was used to identify the *L. fermentum* strain LfQi6 at the species and strain levels. This identification meets the EFSA QPS status for the *L. fermentum* species (61). *L. fermentum* LFQi6 has the ability to produce ethanol in cheese. The rate-limiting step in the formation of ethyl esters of the short-chain fatty acids that provide pleasant fruity flavors in certain hard Italian-style cheeses is probably ethanol generation (55, 61). *L. fermentum* LFQi6 has greater estero-lytic activity than other dairy lactobacilli, which may add to the fruity flavors found in cheese (55, 61)**.** *L. fermentum* is a virtually unknown probiotic strain that already illustrated guarantee in diagnostic clinical studies on cholesterol but rather immunity (55). The following purposes advantages are supported by clinical studies. There's too much data to justify use of certain L. fermentum for all of the other following applications.

In a clinical investigation, *L. fermentum* improved cholesterol slightly (63). *L. fermentum* decreased total (blood cholesterol, either triglyceride levels) and low-density lipoprotein (LDL) cholesterol (63). It also reduced body weight and the ratio of liver weight to body weight in mice (64). In highly trained distance runners, *L. fermentum* reduced the duration and severity of respiratory disease. In male , *L. fermentum* reduced the intensity of gastrointestinal and respiratory disease symptoms, but Less in female (65). In infants, *L. fermentum* reduced gastrointestinal and upper respiratory tract infections (66). *L. fermentum* also lowers the load of *Staphylococcus* in the breast feeding-milk of women who have difficult nursing (67). *L. fermentum* was shown to continue increasing its bio-activity of calcium, phosphorus, but rather zinc throughout fermented goat milk (68).

In laboratory trials, live either dead *L. fermentum* is also used to shown reduce inflammation but instead inflammatory mediators (69). *L. fermentum* destroys S1-casein and reduces its identification and binding to IgE in the blood of people with cow's milk allergy (68, 69). *L. fermentum* adjusts gut microbial community and lesenes ampicillin-induced colon inflammation (70). *L. fermentum* alleviates ampicillin-induced memory loss and lowers nervousness behavior (71).

In alcoholic liver disease, *L. fermentum* greatly reduces liver damage. Green tea extract and *L. fermentum* protect liver cells against ethanol. *L. fermentum* boosts the liver-protective effects of Ssanghwa-tang (SHT), (SHT) is a traditional herbal medicine formula used for fatigue reduction, pain relief and physical strength development (63). By stimulating the Th1 response and increasing IgA synthesis, *L. fermentum* enhances resistance to fatal influenza infection (72). Staphylococci, enterotoxigenic enterobacteria, and *Candida albicans* are all inhibited by *L. fermentum.* It was shown to be effective against *S. aureus* and *P. aeruginosa*, which are prevalent bacteria in hospital-acquired infections (73).

Table 1.2. Scientific classification of *Lactobacillus fermentum.*

LACTOBACILLI : SCIETIFIC CLASSIFICATION	
DOMAIN	Prokaryota
KINGDOM	Bacteria
DIVISION	Firmicutes
CLASS	Bacill
ORDER	Lactobacillales
FAMILY	Lactobacillaceae
GENUS	Lactobacillus
SPECIES	Fermentum
SUB SPECIES	LFQi6

1.3 Cholesterol

Cholesterol is an essential macronutrient for public health. Lengthy elevated blood cholesterol levels can cause to atherosclerosis, which tends to increase the risk of cardiovascular disease (CVDs). According the World Health Organization (WHO), cardiovascular diseases (CVDs) contribute for 30% points

over all deaths in the world and will be estimated to remain this same main cause of death over the next twenty years. By 2030, CVDs will affect approximately 23.3 million people around the world. And per the WHO, a 10%points lowering in blood serum-cholesterol through men over 40 could really start reducing its incidence of heart disease besides 50% points within only five years. To limit blood cholesterol levels, neither drug therapy nor nonpharmacologic approaches like dietary intervention, behaviour modification, or regular exercise were also most often was using (74).

Cholesterol is a sterol that is biosynthesized in all animal cells because it is a structural component of all animal cell membranes that was shown in Figure 1.3 (75). In mammalian cells, Cholesterol can be one of the main crucial elements for regulating its features of cellular membrane (76). Cholesterol is an essential to enter the bio-synthesis of bile acid, as well as in several biochemical pathways, vitamin D, and all steroid hormones such as aldosterone, progesterone, testosterone, cortisol, and others (75).

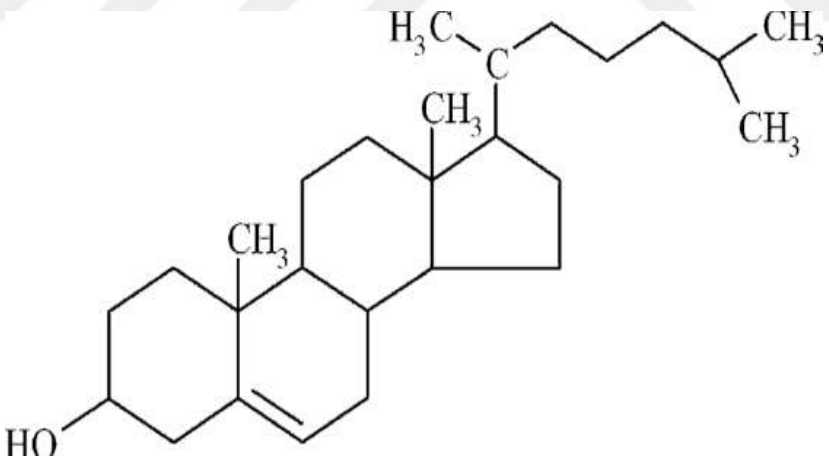

Figure 1.3. Chemical structure of cholesterol (77)

1.3.1 Cholesterol Lowering Mechanism

Lactobacilli affects the mechanism of cholesterol lowering by many mechanisms. Firstly, elevating the number of bile salt-hydrolyzing

microorganisms has the ability of increasing the production of bile acid. In addition, the level of cholesterol in the intestine is declined because of the rise in bile acid-binding capacity of the bile salt, the decrease in the absorption of cholesterol and bile acid into the intestine, and the decrease within the absorption of cholesterol from the intestine to the liver. Additionally, cholesterol-lowering effect of probiotic Lactobacilli has been reported as it improves blood lipid profiles and increase fecal lipid-degradation.

1.3.2 Cholesterol Lowering Effect of Bile Salt Hydrolase Inhibitors

Cholesterol lowering effect of bile salt hydrolase inhibitors (BSHIs) and a new BSHI. The production and hydrolytic and biological properties of a new bile salt hydrolase (BSH) inhibitor, tauroursodeoxycholate bile salt hydrolase (TDCA-BSH) is reported. TDCA-BSH (5-8 mg/kg, i.p.) significantly declined the cholesterol levels of rats which are fed with cholesterol-rich chow, in parallel with a decrease in fecal cholesterol excretion. The bile acid deconjugating activity of TDCA-BSH was almost twice as high as the activity of the reference BSHI, taurine-conjugated urso deoxycholate (TUDCA-BSH).

1.4 Bile

Bile is a greenish-yellow fluid that is made in the liver then either concentrated in the gallbladder or delivered straightly to the intestinal lumen (Figure 1.4). Bile is composed mainly of (95% points H_2O) where a wide range of intrinsic composition such like bile salts, cholesterol, amino acids, bilirubin phospholipid, steroids, enzymes, vitamins, porphyrins, and heavy metals, along with exogenous drugs, xenobiotics, ” or “ toxins, were being solubilized (78).

Bile performs a variety of critical functions. (i) Cholesterol is mainly eliminated through the bile. (ii) Bile is the primary secretion path for possibly harmful extracellular lipophilic substances as well as other entracellular substrates, including bilirubin and bile salts with molecular weights significantly larger than (300 – 500) Dalton’s which are hard to sort and/ or excreted by the kidney. (iii) Bile includes a variety of hormones and pheromones that aid in the development and growth of the intestine in some species, while also trying to

serve as attractants for non – human vertebrate weaning. (iv) Bile salts have either hydrophilic or hydrophobic properties. They are the most abundant organic solutes in bile and are responsible for emulsifying dietary fats and facilitating their intestinal absorption. (v) Bile is a necessary component of both the chole hepatic and enterohepatic circulations (78).

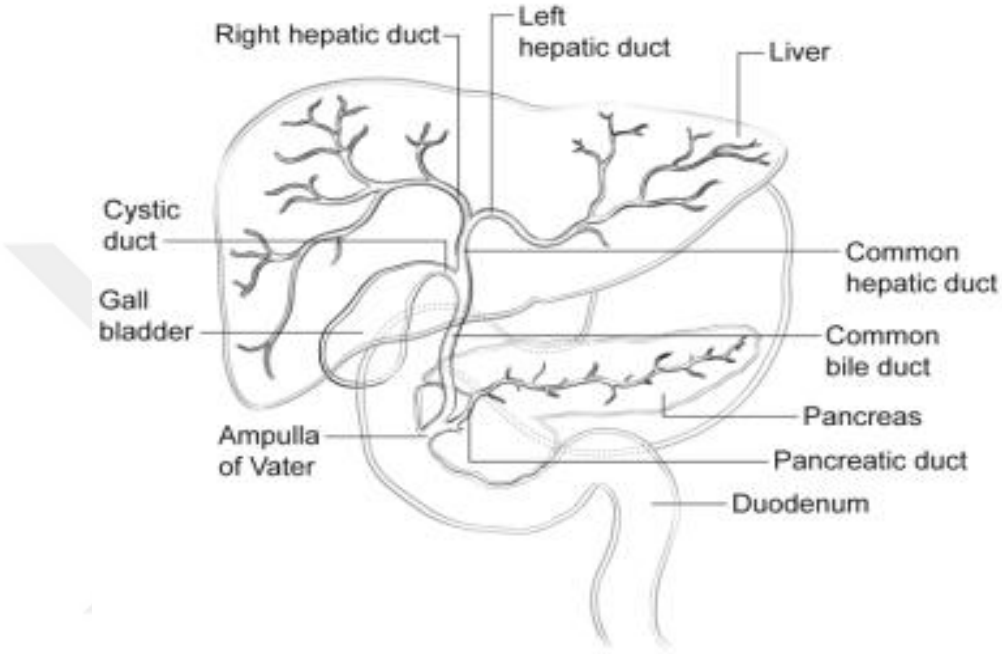

Figure 1.4. Anatomy of Biliary Track (79).

1.4.1 Bile Acid

Bile acids are indeed the two interacting elements: a rigid steroid nucleus and a short aliphatic side chain (80–83). Bile acids, which are reproduced from cholesterol, account for nearly half of the 100% organic component of bile (81,83). A significant portion of cholesterol in humans is converted to bile acids on a daily basis. Bile flow and biliary secretion of bile acids (BAs), phospholipids, drugs, cholesterol, and toxic metabolites are produced as a result of bile acid synthesis (78,80,81). Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the primary bile acids produced in humans and rodent (Figure 1.5). In rodents, a substantial portion of CDCA is transformed to muricholic acids, that are not present in humans, via 6-hydroxylation. Once primary bile acids access the GI

tract, they are decided to convert into further over 50 diverse chemical secondary bile acids (81). These primary bile acids are further metabolized to glycine or taurine molecule, producing conjugated bile acids such as taurocholic acid via N-acyl amidation TCA (81). Conjugation is important for bile acid solubility; unconjugated bile acids are only sparingly soluble at physiological pH. The glycine to taurine conjugated bile acid ratio varies between mammals (81).

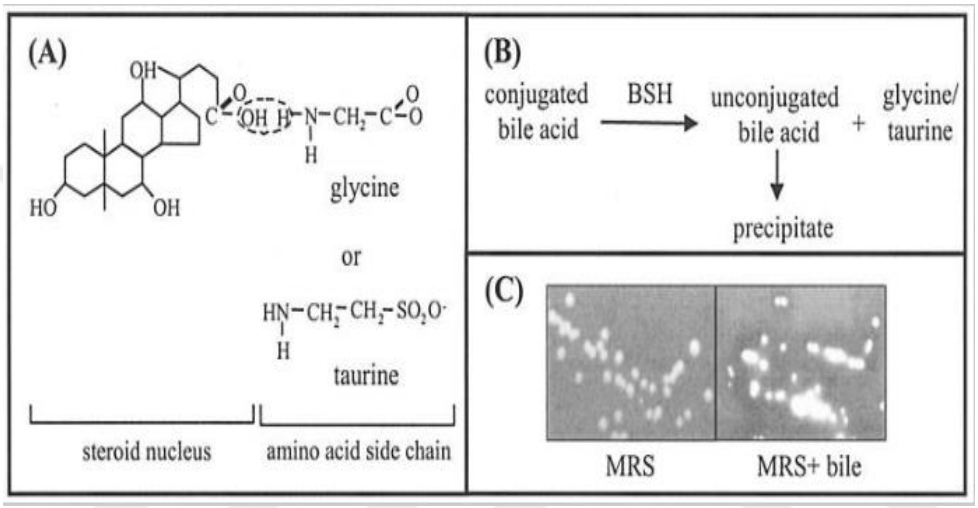

Figure 1.5. Bile acid chemical structure. Primary bile acids are 19ractice19ed in the liver from cholesterol and conjugated with either glycine or taurine without first being secreted. An amide bond attaches a bile acid carboxyl group or an amino acid amino group. The process is catalyzed by BSH enzymes. Once BSHs cleave its peptide connection of bile acids, an amino acid group is eliminated out from steroid core. The resultant unconjugated bile acids precipitate at low pH. ® BSH activity selection. *L. plantarum* was streaked on the MRS agar (A) or MRS agar supplemented with 0.2 % (w/v) GDCA (B) and incubated along 48 hours anaerobically (43,84)**.**

1.4.2 Bile Salt

Bile salts were also categorized into four: primary, secondary, conjugated, and unconjugated. They each start serving a different function throughout the human body. Bile acids are formed by hepatocytes from

cholesterol thru a sequence of seventeen various enzymes. Primary bile salts were being produced either by commenced operations of bile acids into bile salts that is satisfaction thru the complexation of potassium or sodium ions. Cholate and chenodeoxycholate are the most abundant primary bile salts in humans. Primary bile salts aid in the regulation of bile flow into the gallbladder. Another function is to keep cholesterol soluble and thus prevent the formation of gall stones (80).

Secondary bile salts are produced by bacteria in the intestines that convert primary bile salts to secondary bile salts by removing a hydroxyl group. Cholate becoming deoxycholate (DOC) and chenodeoxycholate becoming lithocholate are two examples of this conversion. Ursodeoxycholate is another common secondary bile salt in humans (80).

Conjugated bile salts are established because once primary bile salts combine to taurine and otherwise glycine amino acids well within hepatocyte, of cholate being more and more increasingly taurocholic acid rather than glycocholic acid. Conjugation tends to increase bile salts' water solubility, that either improves their skill to emulsify lipids; conjugated bile salts exceed the number unconjugated bile salts there in human body. The basic task of such bile salts mostly in intestine is to aid as in digestion of dietary fats via emulsifying, which occurs once fats initiate to split into micelles until being digested further (shown image below). Passive reabsorption through the ileum wall is also avoided by conjugated bile salts (85). Secondary bile salts are established when bacteria start removing taurine or glycine from conjugated bile salts. Bile salts that seem to be unconjugated are primary or secondary salts found in the biliary system. Associated with microbial activity or an absence of these two major amino acids, unconjugated bile salts are not bonded to taurine or glycine. Even before to bile excretion in the faeces, bacteria will remove taurine and glycine, preserving these important amino acid sources. As a result, taurine supplements are commonly advertised as a treatment for liver disease and high cholesterol (85, 86).

Bile salts are formed while lipid-soluble bile acids are conjugated either to glycine and taurine molecules to create water-soluble primary conjugated bile acids. Bile salts are hydrophobic only on one hand and/or hydrophilic on the

other, as well as they tendency to cluster micelles across small drops of lipids, with their hydrophobic sides tackling the fat molecule as well as their hydrophilic sides facing outwards (Figure 1.6) (87, 88) and they are soluble in water. Bile salts form mixed micelles with phospholipids and cholesterol and are stored in the gallbladder and secreted into the intestinal tract to aid digestion and nutrient absorption. The negative charges on the hydrophilic hand prevent the fat droplets from re-aggregating (86, 89).

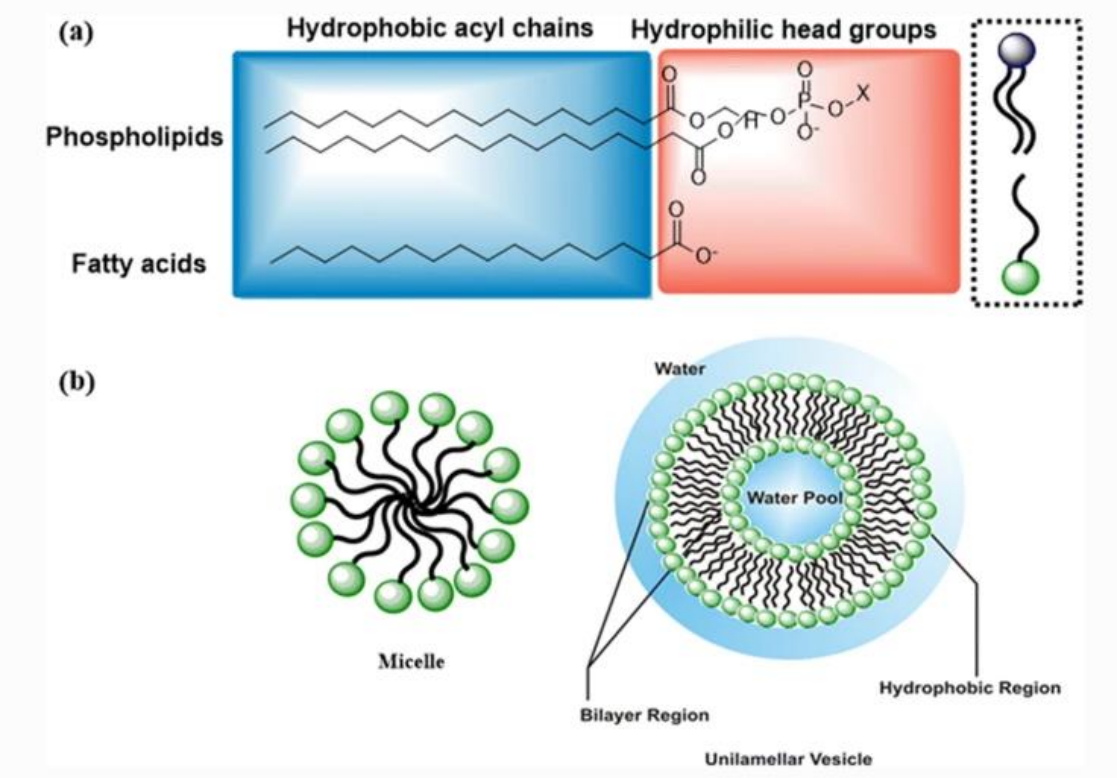

Figure 1.6. Chemical Structure of lipid and Bile acid. The role of fatty acids as a model protocell membrane is revealed by studying the dynamics of vesicles made up of fatty acids or other amphiphile mixtures (90).

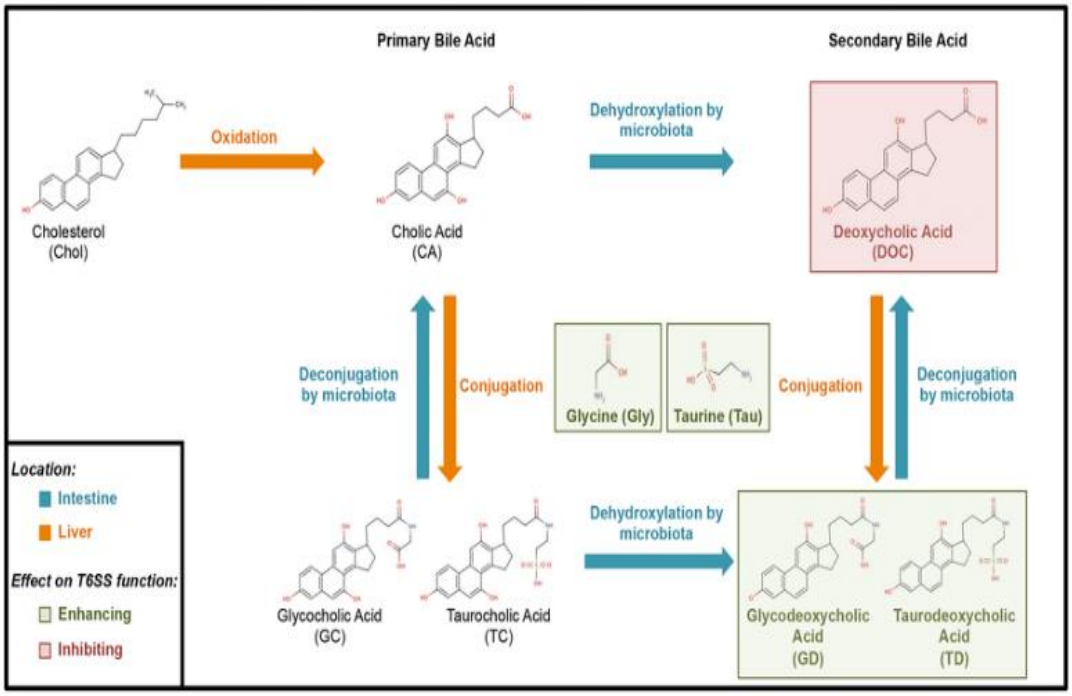

Figure 1.7. Bile salts influence the pandemic vibrio cholerae mucin-activated type vi secretion system (91).

1.5 Bile Salt Hydrolase Enzyme (BSH)

The gut microbiome produces bile salt hydrolase (BSH; EC 3.5.1.24), also known cholylglycine hydrolase an enzyme that can catalyzes the hydrolysis of amide bonds in conjugated bile acids (BAs) there by inhibiting the balance of (BAs) metabolism, releasing free amino acids (92). In the metabolism of bile acids, it plays a key role. A bacterium's probiotic potential has long been determined by the presence of an active BSH enzyme, BSHs have been successfully isolated and characterized from a wide range of microorganisms and have been noticed to be intracellular, oxygen (O_2) unresponsive, even with a slightly acidic pH optimum (normally between pH 5.0 and 6.0) (43). A diverse range of BSH enzyme variants have been isolated from gastrointestinal and environmental microorganisms. Lactobacilli were found to be responsible for the majority of bile salt hydrolase activity in germ-free mice microorganism studies. BSH has been classified into five groups based on evolutionary analysis of BSH gene sequences: BSH-A, BSH-B, BSH-C, BSH-D, and also E, with BSH-A and BSH-B subtypes having high hydrolysis activity than others groups of BAHs (93).

Active Sitte: BSHs are members of the choloylglycine hydrolase family, which also includes penicillin amidases (EC 3.5.1.11), which hydrolyze penicillin to produce 6-aminopenicillinic acid (6-APA) (94). Both BSHs and penicillin amidases are N-terminal nucleophilic (Ntn) hydrolases with an N-terminal cysteine residue, which would be frequently used during industrial manufacture of semi – synthetic derivative antibiotics. Since an autoproteolytic method to avoid the initiation formyl methionine, that is a feature held in common by the Ntn hydrolase superfamily, this Cys-1 becomes a catalytic centre point (95).

Cys-1's thiol (SH) group has been shown to be required for BSH catalysis. Bifidobacterium bifidum BSH Cys-1 replacement with the nucleophilic amino acids serine or threonine with a hydroxyl group instead of a thiol group abolishes BSH activity, and *Bifidobacterium longum* BSH Cys-1 replacement with alanine results in an inactive protein. Agencies which oxidize thiol groups for instant (p-mercuribenzoate, iodoacetamide, $Hg2^{+}$, $Cu2^{+}$, and $Cd2^{+}$) were shown to closely inhibit BSH activity in (*Lactobacillus reuteri*, *B. longum* and *Clostridium perfringens).* Several amino acids reported to be involved in BSH catalysis involve Asp-20, Tyr-82, Asn-175, and Arg-228. Sequence alignments reveal that, aside from Cys-1, these amino acids are strictly conserved in all BSHs (43).

1.5.1 Taxonomy of BSH

In gut bacteria, bile salt hydrolase (BSH) may hydrolyze conjugated bile salts to unconjugated bile acids and amino acids. As signaling molecules, they play a vital role in host health by lowering blood cholesterol levels, maintaining bile acid balance, and regulating different metabolic processes (96). It's also believed that (99%) of functional genes throughout the human body are derived from microbes (81). The taxonomic identification of (BSHs) in gut microbiome is seen here, as well as the abundance and activity differences of various bacterial (BSHs) across 11 diverse populations (77, 93). Bile salt hydrolase gene (*bsh*) taxonomically belongs to the *Lactobacillus* genera and to the *Enterococcus* or *Streptococcus* genera. It is used in food and feed processing and can produce an antimicrobial compound. It is a Gram-positive bacterium with high cell wall content, and its cell wall structure is unique compared to other Gram-positive

organisms. Researchers utilized mathematical and biological approaches to analyze the taxonomic, populational, and operational characteristics of BSHs in human gut microbiota, also with following aims.

1) to determine which bacteria may actually responsible for BSHs in microbiome.

2) to research how the structural, sequential, and biological activity variations of BSHs from different bacteria vary.

3) to identify what factors can affect BSH trends within human gut microflora (92,97).

The taxonomic identification of BSH in the human microbiota is introduced, including the availability and activity differences of various bacterial BSH between 11 different populations from six continents. Firstly, it was discovered that bile salt hydrolase protein sequences BSHs are found in 591 gut bacterial strains from 117 genera in the human microbiota, with 27.52 percent of these bacterial strains containing BSH paralogs. Huge changes in BSH distribution patterns were evidenced among various populations. Therefore, based on phylogenetic tree, researchers reclassified these BSHs in to the eight phylotypes and investigated the abundance trends of such phylotypes between many different populations. The results revealed 7 main phylotypes (BSH-T1, BSH-T2, BSH-T3, BSH-T4, BSH-T5, BSH-T6, BSH-T7 and finally, minor type BSH-T0). However, from the foundation of 24 strains BSH-T1 (including 27 BSHs), 17 strains BSH-T2 (including 19 BSHs), five strains BSH-T3 (including six BSHs), 14 strains BSH-T4 (including 14 BSHs), 23 strains BSH-T5 (including 23 BSHs), 39 strains BSH-T6 (including 46 BSHs), and 14 strains BSH-T7 (including 14 BSHs). Furthermore, BSH-T0 (including seven BSH from one to seven strains) (92,95). Moreover, based on enzyme activity comparisons recent studies showed that BSH-T3 showed higher activity in both biological and virtual binding enzyme activity assays, and all BSHs were confirmed in *Lactobacillus*. Several studies have been conducted on BSH because of its important role in human metabolism. Most earlier experiments, however, focused on cloning BSH

from specific bacteria strains, such as *Lactobacillus*, *Listeria*, *Bacteroidetes* strains, *Bifidobacterium*, *Clostridium* and *Enterococcus* (89, 92, 95, 97).

1.6 Inflammatory Bowel Diseases (IBD)

Inflammatory bowel disease (IBD) is a complex, multi factorial disease mainly used to describe two main (Crohn's and ulcerative colitis) diseases that are characterized by chronic inflammation of the gastrointestinal tract (GIT). Prolonged inflammation causes gastrointestinal tract (GIT) destruction (98). Both Ulcerative Colitis and CD are widespread public health hazards, long term situation, with incidence rates of 12.7 and 24.3 per 100,000 person-years in Europe and prevalence rates of 0.5 and 1.0 %, respectively (99). Recent studies shown that there are some differences between both Crohn's disease and ulcerative colitis were shown in Table 1.3. Some of the differences between Crohn's disease and ulcerative colitis (100).

Table 1.3. Some of the differences between Crohn's disease and ulcerative**.**

Crohn's Disease (CD)	**Ulcerative Colitis (UC)**
Crohn's Disease can affect any part of the digestive system, from the mouth to the anus). It usually affects the small intestine just before the colon/ large intestine.	Ulcerative Colitis only affects the colon (large intestine).
Damaged areas occur in clusters next to healthy tissue sections.	Damaged areas are continuous (rather than patchy), typically begin in the rectum and expanding further into the colon.
Inflammation can spread across the various layers of the GI tract's walls.	Inflammation can spread across the various layers of the GI tract's walls.

1.7 Colitis

Inflammatory bowel disease (IBD) can affect people of any age, however it is most commonly can diagnosed between the ages of 15 and 40 (100). Once, we know some previous studies showed The Use of Fecal Calprotectin (calcium- and zinc-binding protein) in Inflammatory Bowel Disease, that the level of calprotectin shows the number of neutrophils involved in IBD inflammation. This was adequately revealed in inflammatory diseases of the intestine by the strong relation among fecal (calcium- and zinc-binding protein) levels and other measures of acute inflammation, like indium-labeled white cell excretion or quantitative histopathologic assessment of inflammation in colonic biopsies of controls and ulcerative colitis patients. The quantity of calprotectin shows the number of neutrophils present in this inflammation. Calprotectin is then measured using an enzyme-linked immunosorbent assay (ELISA) in a stool extract with less than 1 g. Most studies suggest a normal range of (10 to 50 or 60 g/mg) if quantitative assays are utilized (101). Although the actual cause is unknown, current research has shown that human genetic component, environment, microbiomes, and immune system all play a role in the pathogenesis of IBDs.Changes in the composition of the gut microbiota result in metabolite changes that are believed to play a critical role in the pathogenesis of IBD (99,100). The microbial generation of metabolites may have various effects on the host that are relevant to IBD pathogenesis.

Based on some researches, Bile acid signaling via the nuclear farnesoid-activated X receptor (FXR, also referred as the bile acid receptor) has been found to be protective in DSS and 2,4,6-trinitrobenzenesulfonic acid colitis models via suppressing NF-B signaling (98,102). Dysbiosis may have a direct effect on FXR signaling since BSH in gut bacteria play a vital role in bile acid modification.

The relative abundance of BSH in the gut flora is much lower in IBD patients versus healthy persons, with the greatest decline seen in Firmicutes from Crohn’s disease patients. Secondary bile acid levels are reduced in IBD patients, particularly in flares (103). Both of IBD and IBS problems are persistent and produce abdominal pain, cramps, and frequent bowel movements. Despite the fact

that they have similar acronyms and symptoms, these two illnesses are fundamentally distinct (87). Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal illness characterized by chronic stomach pain and irregular bowel movements. IBS causes physical symptoms (104). Pain involved with IBS contains both visceral pain, that originates in bodily organs, and functional pain, which has no known bodily source (105). Inflammatory bowel disease is diagnosed using a marker called fecal calprotectin, which is released during inflammation. This test is also used to differentiate between inflammatory and irritable bowel diseases (100).

1.8 Colon Cancer

Colorectal cancer (CRC) was the second most common cancer death worldwide in 2018, and/or is the third most common type of cancer diagnosed in men, and/or the second most common in women (87,106). There are an estimated 135,430 new cases of colorectal cancer in the United States (U.S.) per year, with an approximate 50,260 total deaths to the disease. Morbidity is already trying to improve as in U.S. by about 2.5% to 3 % per year since 1990, but while survival stay poor in advanced disease, early-stage colon cancer can possibly be treated, with an estimated 74 % five-year survival rate for patients with stage I disease (87, 106). A variety number of recent studies show that the microbiota can have a massive effect on colon cancer (CC) prevention and ability to heal during intestinal inflammation. Molecular events by which the gut bacterial community influences intestinal diseases may serve as a catalyst for novel preventive and therapeutic approaches (107).

Manipulation of the microflora with probiotic strains has been researched to improve the safety and Gastro intestinal adverse reactions profile of cancer treatment, with some research evaluating the possible clinical benefit of probiotic strains with surgical procedure, radiation therapy, and chemotherapy. Probiotics are enticing as a potential treatment supplement because they are cheap and have few, if there is any, significant side effects (108).

Numerous frameworks were found in past few years, its most obvious being its role of specific reactive oxygen species (ROS) production and activation

of specific peptide receptors in regulating intestinal homeostasis. Recent evidence showed that particular gut microbes, such like Lactobacillus spp., can help to positively regulate such processes (109). Evidently, it was scientifically proven that epigenetic changes and gene regulation can take place during the duration of colon cancer (CC) development. Along with genetics, diet, lifestyle, , and oncogenic infection, specific microorganisms or microbiome variability have recently been linked to this tumor (109).

Colon tumorigenesis is definitely associated to the role of some microbial metabolites as an initiator or inhibitor of pro-carcinogenic or antioncogenic activities (109). These days, trying to identify a specific bacterial population or a change in their vast quantities or majority of single strains responsible for increasing cancer cell sensitivity and development is tricky. A few bacteria, with not just *Fusobaterium nucleatum* and *P. gingivalis*, but also *Escherichia coli*, *Enterococcus faecalis* and *Bacteroides fragilis*, were found in colon cancer (CC) patients, whereas the Lactobacillus genus, Bifidobacterium, Blautia, Faecalibacterium and Clostridial were missing (104,105).

Recent studies showed that dysbiosis is an imbalance or distress of the microbiomes specific pathways that, in concept, can result to tumor development. Dysbiosis in the Intestinal tract can inhibit the immune systems as well as mucosal wall's homeostasis, due to increased mucosal wall's permeability and a continuous state of inflammation. The above, throughout turn, can stimulate growth factors and cytokines such as tumor growth factor beta (TNF-), tumor necrosis factor (TNF), interleukin-6 (IL-6), but rather vascular endothelial growth factor (VEGF), that can lead to the growth and survival of dysplastic cells. Dysbiosis caused by bacterial biofilm production can often result in increased bile acid metabolism, cell proliferation via Toll-like receptor sites, or other processes, all of which can contribute to malignant transformation, however, free iron and heme were connected to the development of colitis-associated colorectal cancer (CRC) even though they enable the Fenton reaction, which produces hydroxyl radical ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$), that also causes oxidative damage (94–96, 99).

1.9 Giardiasis Treatment with BSH

Giardia duodenales (also known as *Giardia lamblia* or *Giardia intestinalis*) is a flagellate replicative form protozoan parasite responsible for giardiasis, a disease that causes intestinal malabsorption, abdominal pain, acute or chronic diarrhea, and weight loss that infects humans, mammals, reptile, and a variety of animals via Oro-fecal transmission and/or from contaminated water and soil (89,110). A disease is one of the most common worlds wide spread towards the environment in the form of a resistant cystic form; when ingested, these cysts make a distinction into trophozoite by excystation in the proper Gastro Intestinal tract conditions (92). It mainly affects children, even those people are malnourished or immunocompromised (111). In recent study that's well documented that some probiotic bacteria can provide both in vitro and in vivo protection against this parasite (111).

Recent studies have found a direct correlation between *G. duodenales* and the gut microbiota. Through turn, *G. duodenalis* colonization changes the composition of the gut microbiota. Giardia coexists with the host's microbiota in the gut, and lots of studies have shown the value of this gut environment and/or certain probiotic bacteria in giving protection against *G. duodenalis* infection (112). To treat giardiasis, several anti-infectious drugs have been developed mainly metronidazole, a 5-nitroimidazole (113). Earlier research focused on the use of probiotic bacteria (*L. johnsonii* La1, *L. gasseri* CNCM I-4884) with in treatment of giardiasis. Using murine models, neither strain increase immunological response, protect against Giardia-mediated tissue damage, and inhibit trophozoite growth (113). In the current study, a varied collection of lactobacilli strains from various ecological sources were tested for anti-giardial activity. The anti-parasitic actions of several of the strains studied were shown to be highly linked with the expression of BSH-like activities. The *L. gasseri* CNCM I-4884 strain were able to greatly inhibit parasite development by reducing the trophozoite burden in the small intestine. Furthermore, after 5 days of treatment, this strain reduced dramatically fecal discharge of Giardia cysts, which might contribute to the parasite's transmission (108,110).

In vitro, *Lactobacillus johnsonii* La1 inhibits Giardia growth. Morever, Researchers showed that the supernatant of this strain (*Lactobacillus johnsonii* La1) includes high active principles that may not directly toxic to Giardia but can transform non-toxic bile components into highly toxic Giardia components. The parasite growth suppression by *L. johnsonii* La1 culture wasted supernatant is performed by released bacterium bile-salt hydrolase-like activities that generate deconjugated bile salts from bile in the medium. Deconjugated bile salts, as opposed to conjugated bile salts, the primary components of bile, were shown to be toxic to *G. duodenalis* (107,110).

1.10 Importance of Bile Salt Hydrolase Gene Cloning and Analysis of it

Recent works showed that BSH has a role in regulating host metabolisms. However, the specific in vivo effects of bile acid metabolites on host metabolism have yet to be uncovered. Moreover, there are a lot of studies reported conflicting results regarding BSH activity and their amount to achieve metabolic benefits for the host (114). BSHs are widely distributed in members of the gut microbiota, though most recent studies have focused on BSH activity characterization from *Lactobacillus* species. Finding out the exact BSH enzymes that work in relation to bile acids (BAs) efficiently has proven to be challenging since many strains carry multiple gene copies, so many studies are needed to identify the relationship between both BA toxicity and BSH activity (81,115). To identify the activity mechanism of BSH in probiotic strains, functional studies in vitro, correlative relationships between BSH activities, and investigation of metabolic effects have been the focal point in researches recently (111,112). Given the important role of BSH genes, it is essential to systematically identify the BSH coding genes, find the evolutionary relationship, the correlation between the gene characteristics and BA hydrolyzation, however, study the variation in sequence and activity. With the increase in availability of genomic data of Lactobacillaceae, it is possible to further explore the gene biodiversity, various works aim to use biological and computational approaches to explore the patterns of BSH and the effect of various factors on it, as well as the association between BSH abundance and diseases (81,111).

2 AIM AND SCOPE OF THE STUDY

Cardiovascular disease (CVD) is among the top causes of mortality risk today. In advanced nations, high blood lipid levels have been identified as the leading cause of cardiovascular disease and perhaps other disabilities. An association respectively cholesterol levels and the risk of coronary heart disease have been noticed throughout many animal and clinical research findings. Low-fat/low-saturated-fat diets have been generally approved for cardiovascular disease (cvd) prevention. Though there is no doubt that low-fat diets are effective in lowering blood cholesterol levels in laboratory settings, they appear to be less effective in 31ractice due to poor compliance, that could be attributed to the diets' low palatability and suitability among consumers. Due to low consumer compliance, efforts have been made to identify additional dietary components that can lower blood cholesterol levels. Dietary supplementation with fermented dairy products and/or lactic acid bacteria containing dairy products has been shown to lower serum cholesterol levels. Various approaches, including the use of probiotics, particularly Bifidobacterium spp. and Lactobacillus spp., have been used to address this issue.

There have been numerous studies on cloning genes, characterization, and functional enzyme activity. In general, the bile salt hydrolase enzyme exhibits genetic variation between several species and strains of the same species. According to the publications, amino acid differences between species may cause BSH activity. These findings demonstrate that there is a link between protein structure, amino acid sequence, enzyme activity and substrate specificity.

BSHs are enzymes produced by liver hepatocytes that aid in the absorption of dietary lipids in the small intestine. It has been reported to play an important role in the enterohepatic circulation of bile salts and secondary metabolites derived from BSH deconjugation. BSH activity is an important factor in selecting probiotic bacteria that reduce cholesterol in the blood.

Because of the consequences on human health, BSH is a potential candidate for the advancement of probiotics. Because of changes in hepatobiliary functions and fat metabolism, unconjugated BAs have a significant impact on host

physiology. As a result, BSH has a critical impact on the host's lipid metabolism, cholesterol level, and body weight. On the other hand, many recent researches indicate that there is strong link between BSH activity and certain human diseases such as bowl diseases, gallstone formation, colon cancer, giardiasis, diabetis and colitis.

BSH activity, like other probiotic properties, is strain specific. Even within the same species, the substrate specificities of BSH enzymes vary. According to some studies, there is a link between substrate specificity and the amino acid sequence of BSHs. There are many types of BSHs and we do not know much about these BSHs. This is a problem and cloning and analysis of BSHs from different lactobacillus species are required.

This study aims to obtain nucleotide and amino acid residues of BSH of *L. fermentum* and to detect the hydrolysis activity of this enzyme at optimum conditions (37°C and pH: 6.5) in the presence of two different bile acids (GDCA and TDCA) by direct plate assay. This study also aims to analyze the stability of the BSH enzyme of the *L. fermentum* by SDS-PAGE. To achieve these aims the *bsh* gene was amplified and cloned into *Escherichia coli* XL1-Blues strain, expressed and characterized in *E. coli* BLR(DE3) by using medos which were defined in Mataerial and Medhods section.

3 MATERIALS AND METHODS

3.1. Bacterial Growth and Genomic DNA Isolation

Lactobacillus fermentum strain used in this research is the commonly accessible *L. fermentum* (LFQi6) strain. The streak plate technique was used to isolate the *L. fermentum* on a fresh MRS medium (de MAN ROGOSA and SHARP) plate (116).

The streaked plate with *L. fermentum* was incubated in an anaerobic jar at 37ºC. Eventhough the bacterial growth slow, we made sure to exceed the 18 hours growth. After 18 hours of incubation, we started the first liquid culture with 4ml of MRS broth culture and a colony from the previously streaked plate and incubate it alongside a MRS broth culture in an anaerobic jar at 37ºC. The following day, the overnight culture was retrieved to later on start a second liquid culture with 2% of the previous overnight culture. The pellet of this second culture was then collected by first centrifuging at (4000rpm) at 25ºC along 4 minutes and then (6000rpm) along 3 minutes after re-suspending the pellet in 1ml of MRS broth. Using the obtained *L. fermentum* pellet, the genomic DNA isolation protocol on the (fermentas genomic DNA isolation kit/ EU) was used.

3.2. DNA Amplification by PCR

Bsh DNA was amplified by PCR (Thechne TC-3000) using species specific primers which were shown in Table 2.1. The reaction was performed in a 50 µl solution containing 25 mM of the forward and revers primers of *L. fermentum*, 5 mM dNTP mix, the genomic DNA as a template and the Pfu DNA polymerase enzyme (Table 2.2). The PCR conditions are shown in Table 2.3. The Pfu DNA polymerase was preferred because of it is proofreading activity there by reducing the chance of removing contaminating adversaries and/or unwanted sequences (111,113).

Table 3.1. Primers used for amplification of the desired BSH gene.

Primers	Sequences (5' to 3')	Primer length (bp)	Tm
LBFER(F)	5' -ATGTGCACGAGCATCAA -3'	17 bp	50°C
LBFER®	5' -TTAGTTCAATTTCACCGGTG -3'	20 bp	

Table 3.2. PCR reaction ingradients53°C

Reactants	Quantities
Up (ultra-pure) H_2O	33µl
LB. FER F (25mM)	1µl
LB. FER R (25mM)	1µl
dNTP mix (2.5 mM/each)	4µl
Template DNA (2.5 u/ µl)	5µl
Pfu DNA polymerase buffer (10X PCR) w/15mM $MgCl_2$)	5µl
Pfu DNA polymerase Enzyme (2.5 U/ mL)	1µl
Total volume	**50µl**

Table 3. 3. PCR Reaction Condition.

Cycles	Temperatures	Time
Initiation Denaturation	94°C	2 min
Denaturation	94°C	20 sec
Annealing	50°C	10 sec
Extension	65 – 72°C	1min
Final Extension	72°C	5 min
Number of Cycles	**30**	

3.3. Purification of the PCR Products

For extraction and purification, The PCR products became loaded and kept from separated from definitive products in (1% w/v) agarose gel (Sigma, Europian Economic Community). After being separated by electrophoresis at 75 volts for along 1 hour by using the TAE buffer (1X) and approximately 1 kb PCR products, the PCR products were purified using the protocol mentioned in the Thermo Scientific Gene Jet PCR Purification Kit (EU).

3.4.Preparation of pHAL1 Construct

3.4.1. DNA Isolation of pBluescript Vector

From a previously streaked plate of pBluescript/XL-Blue, single colony was inoculated into liquid LB medium and incubated overnight for nearly 14 to 16 hours at 37^{o}C and 175 rpm shaker. The pellet of this liquid culture was then collected by first centrifuging at 4000rpm at 25^{o}C for 4 minutes and then at 6000rpm during 3 minutes after re-suspending the pellet within 1 ml of LB (Luria Broth) medium. Using the obtained cell pellet the plasmid DNA isolation were proceeded using the protocol mentioneded on the Amplicon plasmid DNA isolation Kit (EU).

3.4.2. Digestion of pBluescript with *Sma*I Fast Digest Restriction Enzyme

Since our PCR products are blunt ended, the circular pBluescript vector was digested by *Sma*I fast digest restriction enzyme (Fermentas, thermoscienntific EU) in reaction conditions that was shown in Table 2.4 for future ligation reactions condition as followed:

Table 3.4. Reaction Condition for pBluescript digestion

Reactants	Quantities
Up (ultra-pure) H_2O	21µl
pBluescript DNA isolation	20µl
Tango Buffer (10X)	6µl
sma i enzyme (10 u/ µl)	3µl
Total volume	**50µl**

3.4.3 Insertion of *L. fermentum* BSH Gene into pBluescript Vector

The *L. fermentum* BSH DNA and pBluescript vector DNA were ligated at 22ºC for two hours using the T4 DNA ligase fast digest enzyme (Fermentas) with appropriate buffer in the reaction conditions which were shown in Figure 2.1 and Table 3.5. The new construct was designated as pHAL1. Polyethylene glycol (PEG) (Fermentas, thermoscientific EU) was also added to the reaction to improve the ligation efficiency. T4 DNA Ligase was inactivated after ligation process by incubating at high temperature (65°C for 10 minutes). The exact amount of vector and insert DNA to be used in this ligation reaction was determined by the following formula:

Ng of Vector X kb size of insert / kb size of vector X molar ratio of insert/vector = ng of insert

Table 3.5. Ligation reaction of bsh DNA to plasmid pBluescript Vector.

Reactants	Quantities
Up H_2O	9 µl
PEG (Poly-ethylineglysin)	2 µl
pBluescript Vector/SmaI	1 µl
L. fermentum bsh DNA	5 µl
T4 Ligase Buffer (10X)	2 µl
T4 DNA ligase enzyme (5 u/ µl)	1 µl
Total volume	**20 µl**

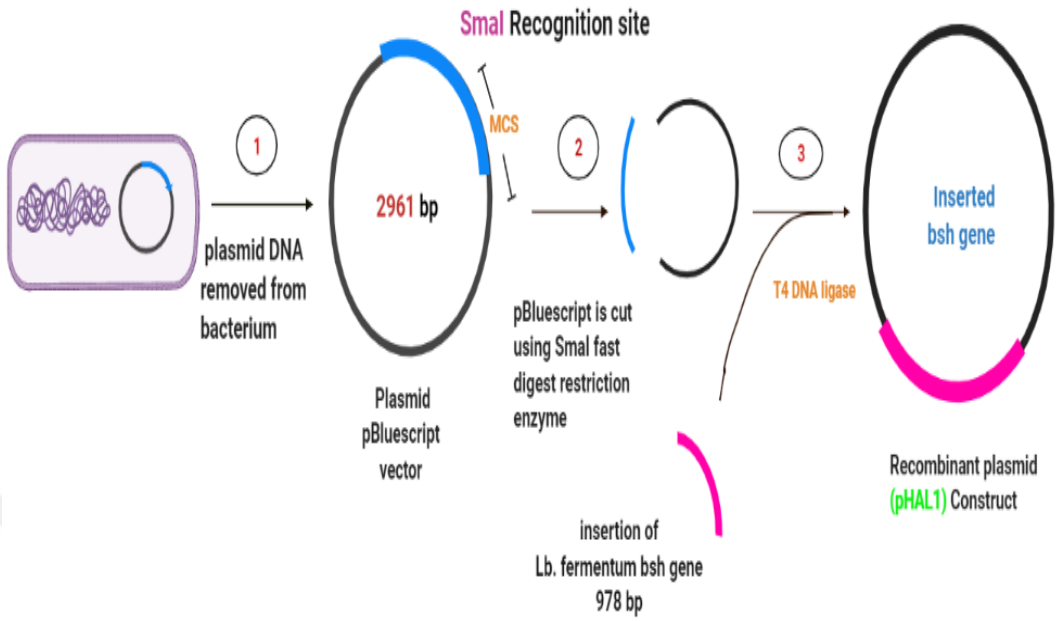

Figure 3.8. Plasmid pBluescript vector and recognition site of SmaI fast digest restriction enzyme.

3.5.Preparation of the XL-1Blue and BLR(DE3) (Competent Cells)

Transformation reactions were obtained thought a three days process. The XL1-Blue (Strata gene, USA) and BLR(DE3) (Nova gene, USA) were streaked on solid LB medium and incubated at 37 ºC overnight. Single colony of each XL1-Blue and BLR(DE3) was inoculated in 3 ml LB medium and incubated overnight at 37ºC and 175rpm shaking for around 14 - 16 hrs. Then 1% of this culture was transferred into a 50 ml LB and re-incubated at 37ºC at 125rpm shaking for 2-2.5 hours until the optic density (OD) of the culture at 600 nm wavelength reached 0.04-0.06. Once the desired OD, 40 ml of the culture was separated into 2 sterile falcon tubes of 20 ml each. They were centrifuged at 4ºC and 4000 rpm for 5 minutes. The pellets were re-suspended in 10 ml of $CaCl_2$ (100 mM, pH: 7.0) and centrifuged at 4ºC and 4000 rpm for along 10 minutes. The newly obtained pellets were re-suspended in 1 ml of calcium chloride ($CaCl_2$) containing 10% glycerol and allocated into 100 ml culture in a different Eppendorf tube and stored at -80º C cold ice freezer.

3.6. Transformation of pHAL1 into *Escherichia coli* XL1-Blue Strains

Ten µl of the ligation reaction product (purified gel) was transferred into 100 µl of competent cells XL1-Blue *E. coli* cells. The sample has been subjected a heat shock strategic of 45 minutes of incubation on ice and then five minutes incubation at 37ºC and finally five minutes incubation on cold ice. After heat shock processes, 1 ml of LB Broth was added to the sample in an Eppendorf tube and incubated at 37ºC and 175rpm shaking along 1 hr. After that, the sample was again centrifuged at 6000rpm for around three minutes and the pellet was re-suspended in a 100 ml LB broth.

The spread plate technique was used to inoculate a LB agar plate containing IPTG (Isopropyl β-D-1 thiogalactopyranoside), X-gal (5 bromo 4 Chloro 3 indolyl β–D galactopyranoside) and also ampicillin (25 mg/ml) concentration. The plate was incubated overnight at 37ºC and 175 rpm shaking for around 14-16 hrs (117). After that, we selected 4 random/white colonies from transformation products of LB plates and grown in an overnight culture of 5 ml LB with ampicillin. Then, once the pellet of the liquid culture was collected, we mentioned to the DNA isolation using the protocol examined on the fermentas plasmid DNA isolation Kit (EU).

3.7. Stock Preparation

Once we know that our insert is in the right orientation, we proceed to start four liquid cultures from the cultured plates of the sample that were sent for sequencing. After the cultures were incubated overnight at 37ºC and 175rpm shaking during 14-16 hours, the LB liquid cultures were retrieved and the once for stock preparation were transferred into glass tube to be centrifuged at 25ºC at 5000rpm around 4 minutes. Then, the obtained pellets were resuspended in 4 ml of LB containing 30% frozen glycerol and devoted within 2 different cryogenic tubes and kept at -80ºC frozen.

3.8. DNA Sequencing of *Lactobacillus fermentum bsh* Gene

Approximately 1 kb DNA from XL1-Blue/pHAL1 transformant was sequenced by Macro Gene (Seoul, Korea) using the universal primers that are

M13/pUC forward (5' -GTAAAACGACGGCCAGT-3') and M13/pUC reverse (5'- CAGGAAACAGCTATGAC - 3').

3.9. Preparation of pHAL2 Construct

3.9.1. pET22b^{+} Plasmid DNA Isolation

Since pET2b is a low copy plasmid, it was inoculated into a 10 ml of LB medium containing ampicillin (25 mg/ml) and incubated overnight at 37^{o}C and 175rpm for around 14-16 hours. The pellet of this liquid culture with then collected by first centrifuging at 4000rpm and 25^{o}C for 4 minutes and then at 6000rpm for three minutes after re-suspending the pellet in 1 ml of LB medium. Using the obtained pellet, the plasmid DNA isolation described by (fermentas Gene Jet plasmid Miniprep Kit EU).

3.9.2 Insertion of *L. fermentum bsh* gene into pET22b Vector

Insertion of *L. fermentum bsh* gene into pET22b Vector the *L. fermentum bsh* DNA was inserted into pET22b expression vector DNA at 22°C for 2 hours using the T4 DNA ligase enzyme (Fermentas EU) and its appropriate buffer (Figure 3.2 and Table 3.6.). The new construct was designed as pHAL2.

Table. 3.6. Ligation reaction of *L. fermentum bsh* gene and pET22b vector DNA.

Reactants	Quantities
Up H_2O	10.5µl
pET22b vector	5 µl
L. Fermentum bsh DNA	1.5 µl
T4 Buffer	2 µl
T4 DNA ligase fast digest enzyme	1 µl
Total volume	**20 µl**

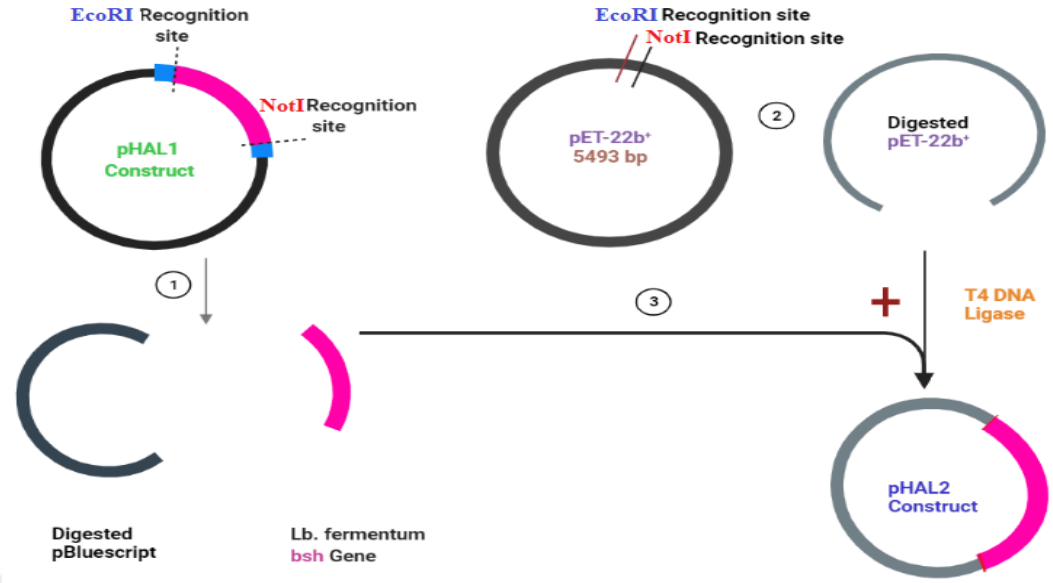

Figure 3.9. Preparation of pHAL2 Construct.

3.10.1 Transformation of pHAL2 into *E. coli* BLR(DE3) Strains

10 µl of the ligation reaction product (pET22b + insert *L. fermentum bsh* gene) was transferred into 100 µl of *E. coli* BLR(DE3) competent cells. The sample has undergone a heat shock stratergies for 45 minutes of incubation on cold/ice then 5 minutes incubation at room temperature (37°C) and finally 5 minutes incubation on cold/ice. After that, 1 ml of LB was added to the sample and incubated at 37°C and 175 rpm during 1 hour. Later, the sample was centrifuged at 6000 rpm, 3 minutes and the pellet were re-suspended in 100 µl LB. The spread plate technique was used to inoculate a LB Agar plate containing Ampicillin (25mg/ml). The plate was incubated (16-18 hours) over night at 37°C and 175 rpm shaker. Plate, 5 random colonies were detected and grown in an overnight culture of 10 ml LB and also on an Ampicillin containing LB Agar plate. Then once the pellet of that liquid culture was then collected. We mentioned to the DNA isolation using the protocol previousely explained on the fermentas plasmid DNA Isolation Kit (EU).

3.10.2. Detection of The Transformants that had Desired Insert BSH Gene

Even before we got to know which of the five cultures had carried an insert bsh gene, we digested the DNAs isolated from the five transformants with EcorI/NotI fast digest restriction enzymes (Fermentas EU) in the reaction conditions shown in (Table 3.7).

Table 3.7. Digestion Conditions of pHAL2 DNA with EcoRI/NotI Fast Digest Restriction Enzymes**.**

Reactants	Quantities
Up H_2O	5µl
pHAL2 plasmid DNA	3 µl
Fast digest green buffer(10x)	1 µl
Enzyme EcoRI fast digest	0.5 µl
Enzyme NotI fast digest	0.5 µl
Total volume	**10 µl**

Once we know that our pHAL2 transformant has only taken only one insert *bsh* gene in the correct orientation, we proceed to start two liquid cultures from the sample's culture plates that were used for stock storage. For overnights, nearly 16-18 hours, the cultures were incubated at 37°C and 175 rpm shaking. The liquid cultures were then extracted and transferred to a glass tube, where they were centrifuged for 4 minutes at 25°C and 5000 rpm. The pellets were then re-suspended in 4 ml of LB containing 30% glycerol, separated into two cryogenic tubes, and stored at -80°C cold /ice refregrator.

3.11 Direct Plate Assay

To determine the activity of recombinant BSH enzymes qualitatevily, LB agar plates with ampicillin ((25 mg/ml), GDCA (2 mM) or TDCA (5 g/lt), IPTG (0.1 M), CaCl2 (0.35 g/L), The plates was streaked with a positive control

(BLR(DE3), a negative control (pET22b$^+$/BLR(DE3), and (pHAL/BLR(DE3) and incubated at 37°C near 72 hours before the samples were compared for their opaque appearance, The formation of granular, opaque, and white colonies was caused by the accumulation of free salts as halos around colonies.

3.12 Overexpression and Determination of BSH Activity

3.12.1 Preparation of Cell Extract

After gained the successful transformation of (pHAL) construct into *E. coli* BLR(DE3) transformants was incubated individually in a 10 ml LB containing Ampicillin and incubated overnight at 37°C and 175 rpm shaking for approximately 16-18 hours. Five ml of such an overnight culture was transferred into a 250 ml LB supplemented with Ampicillin. This freshly prepared culture was then incubated at 37°C and 175rpm until the culture's OD_{600} values reached 0.45 - 0.6. When the culture's OD_{600} values reached 0.5, 0.3mM of Isopropyl -D-1-thiogalactopyranoside (IPTG) was added, and the culture was re-incubated at 37°C and 175rpm until the OD_{600} values again reached at 1.5 - 3.0. Once those values were determined, 100 ml of culture was transferred into 250 ml falcon tubes and centrifuged for 15 mins at 4°C, 8000 rpm. The pellet was re - suspended in 2 ml of binding buffer at pH 7.8 before being transferred to a single tube. The mixture also was incubated along 10 mins on something like a rocking platform at 4°C with lysozymes at quite a desired concentration of 1mg/ml. Following the incubation, Tween 20 (Polyethylene glycol sorbitan monolaurate), DNase, and RNase were added to the tube at final concentrations of 1%, 5g/ml, and 5g/ml, respectively, and incubated the tube again for 10 minutes, at 4°C on the something like a shaking scaffold. The insoluble debris was then removed by centrifugation at 14000 rpm, 4°C while 30 mins. The supernatant was then collected in a new tube after passing it through a 0.45 μm filter. The protein concentration of the cell extract was detected using the Lowry method protocols with Bovine Serum Albumin (BSA) as a control protein.

3.12.2 Partially Purification of the BSH Enzyme and SDS PAGE Analysis

The samples were grown in the same way that they were grown to obtain a cell extract in order to purify the BSH protein. However, after transferring the supernatant through a 0.45 μm filter, their absorbance at 600nm and 280nm were measured. The gel is divided into two parts: separating gels and stacking gels. First, a separating (running gel) solution was prepared and poured into the gel apparatus. Staking gel was prepared and poured in after 45 minutes. The polymerization of the gel takes at least two hours, and samples were not loaded until the polymerization was complete. The purified sample was then incubated for 10 minutes at 37°C with a protein loading dye and β-Mmercaptoethanol. The mixture was then loaded onto a 12% polyacrylamide gel (PAGE) and electrophoresed at 120 volts for along 3 hours according to the Laemmli protocol. The gel was then immersed in a tank containing Coomasie Brilliant Blue dye and incubated for along 20-30 minutes at room temperature on a rocking platform. After the dye was removed and the gel was soaked in a de-staining solution overnight to effectively remove any remaining stain, the gel was visualized.

4. RESULTS

4.1. Amplification of *bsh* Gene by PCR

The genomic DNA of *L. fermentum* strain (LFQi6), isolated from infant faeces, was used for preparation of insert bile salt hydrolase gene, and then blunt ended PCR product was amplified using species specific primers. The PCR amplicons were loaded on % 8 agarose gel and electrophoresed at 75 V for along one hour and ten minutes. The DNA band of *bsh* gene was visualized approximately (1.0 kbp) under the UV transilluminator after staining of the agarose gel with Ethidium Bromide (EtBr) (Figure 3.1).

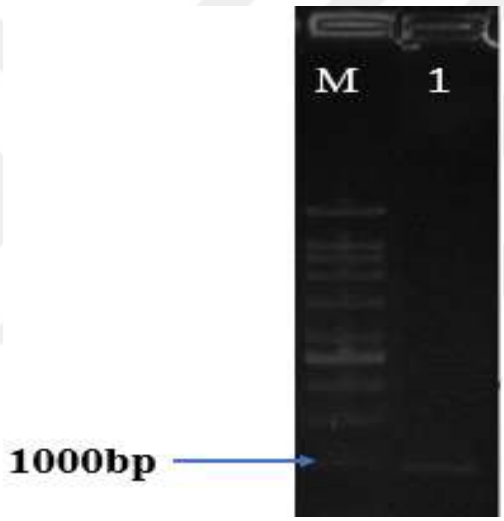

Figure 4.10. DNA amplification of the BSH gene of *L. fermentum* LFQi6 strain by PCR. M represents marker with 1 kb DNA vivantas ladder while line 1 is the PCR product.

4.2. Purification of the PCR Product

To remove unwanted, undigested methylated genomic DNA and non-specific PCR products of *bsh* gene was purified from %1 agarose gel after electrophoresis at 75Volts during 1 hour (Figure 4.2).

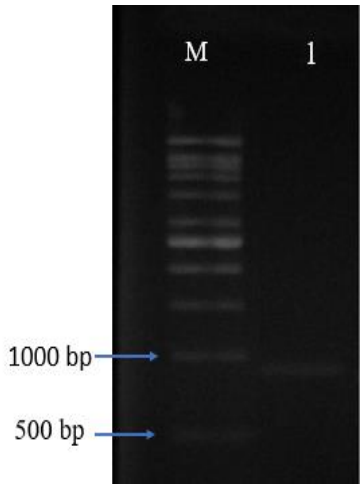

Figure 4.11. Purification of the PCR product. M represents the marker with vivantas 1kb ladder, while line 1 is the purified PCR product, *bsh* DNA.

4.3. Preparation of pHAL1 Construction

4.3.1. DNA Isolation of the pBluescript KS$^+$ Cloning Vector

To clone *bsh* gene into plasmid pBluescript KS$^+$ vector (2966 bp) pBluescript KS$^+$ vector DNA was isolated from *E. coli* XL1 strain by using fermentas plasmid DNA isolation kit according to its protocol. A DNA was loaded on a 0.8% agarose gel and separated by electrophoresis then gel was stained with Etbr to observe DNA on a UV-transilluminator (Figure 4.12).

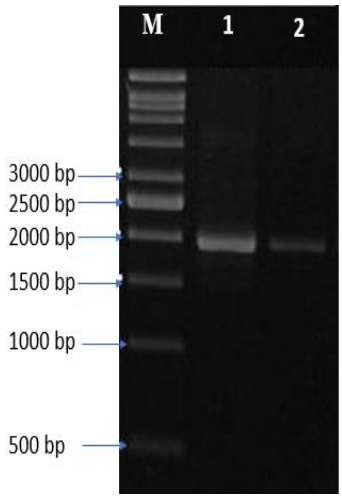

Figure 4.12. DNA isolation of the pBluescript Vector. M represents the marker with vivantas 1 kb ladder. line 1 is circular pBluescript vector DNA first elution, while line 2 is circular pBluescript vector DNA second elution.

4.3.2. Digestion of pBluescript DNA by *Sma*I Restriction Enzyme

Because the PCR products are blunt-ended, before ligation we must digest the pBluescript vector with the *Sma*I fast digest restriction enzyme. The digestion made pBluescript vector linear, resulting in a DNA band that was usually larger than 2.9 kilobases. The pBluescript plasmid DNA was once again purified using 1% gel electrophoresis (Figure 4.13).

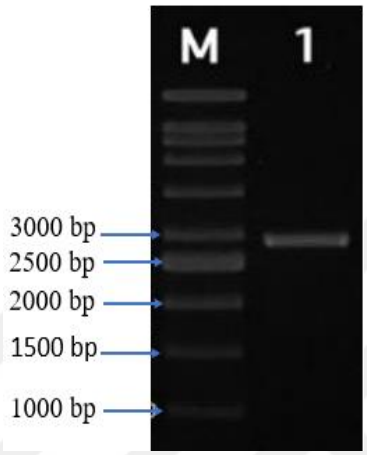

Figure 4.13. Digestion of pBluescript by SmaI fast digest restriction enzyme. M represents the marker with vivantas 1 kb ladder. While line 1 is the linear pBluescript DNA digested by SmaI fast digest restriction enzyme.

4.3.3. Transformation of pBluescript-*bsh* DNAs Constructs into *E. coli* XL1-Blue Strain

Ligation products were transformed into the XL1-Blue competent cell. Transformants were selected with using LB plates which contain ampicillin, IPTG and X – gal. Insert positive clones were selected via Blue and White Colony Assay having ampicillin (Figure 4.14.). To select the clones that have a bsh insert gene, a few white colonies from the plate in (figure 4.14) were selected and DNAs were isolated.

Immediately after the completion of the ligation process, 10µl of the ligation reaction sample was transferred into 100 µl of *E. coli* XL1-Blue cells, cured with calcium chloride ($CaCl_2$)$2H_2O$. A few random white colonies from the LB plate, supplemented with ampicillin, IPTG, and X-gal, has been selected and inoculated overnight in LB for plasmid DNA isolation. To find out which of

our selected randomly chosen colonies has a desigered insert *bsh* gene, the isolated DNA was loaded on a 0.8% agarose gel (Figure 4.15). The result of gel electrophoresis showed us that both construct DNAs isolated from different transformants had an insert *bsh* DNA.

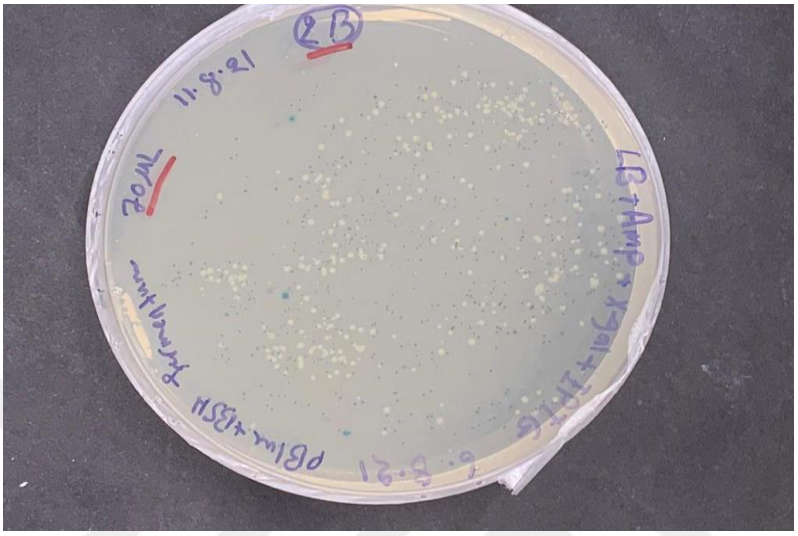

Figure 4.14. Blue colonies lack the *bsh* gene insert, whereas white colonies have insert *bsh* gene.

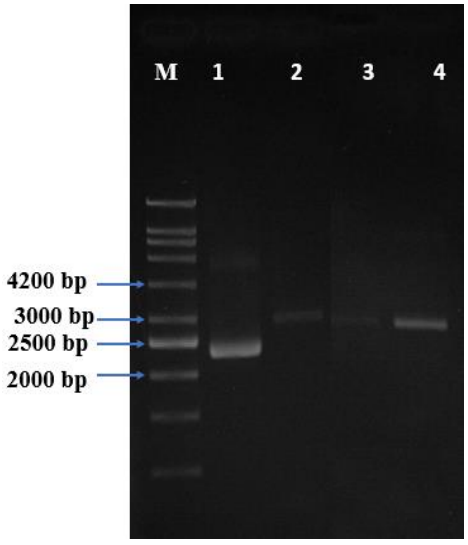

Figure 4.15. Transformation of pBluescript-bsh into *E. coli* XL1-Blue strains. M represents Marker with vivantas 1 kb ladder. line 1: circular pBluescript DNA, line 2: linear pBluescript DNA, line 3: pHAL1A DNA, line 4: pHAL1B DNA

4.4. DNA Sequence Analysis of *bsh* Gene

The *bsh* gene on pHAL1 construct was sequenced by Macrogen (Seoul, Korea) using the universal primers, M13/pUC Forward (5'-GTAAAACGACGGCCAGT-3') and M13/Puc Reverse (5'CAGGAAACAGCTATGAC-3'). The clone was analzied by the BioEdit Sequence Alignment Program and the SIB (Swiss Institute of Bioinformatics) ExPASy sequence translate tool (http://web.expasy.org/translate/). The acquired amino acid sequence was shown in Figure 4.16.

L. fermentum LFQi6 transitional termination codon (TAA) and encoded 326 amino acid protein with theoretical molecular mass of 36.5 KDa and theoretical pI of 4.85/ 36538.43 respectively, https: web.expasy.org/cgi-bin/copute_pi/pi_tool. John M. Walker (ed): The Proteomics Protocols Handbook, Humana Press (2005).

The amino acid characters of BSH enzymes from *Lactobacillus fermentum* LFQi6 strain are being aligned with its amino acid sequences of BSH enzymes from many other *Lactobacillus* species such as *Lactobacillus plantarum* (Gene bank >KU365160.1), *Lactobacillus rhamnosus* (Gene bank >KU365159.1), *Lactobacillus gasseri* (Gene bank >KY674913.1), *Lactobacillus acidophilus* (Gene bank >FJ439778.1), *Lactobacillus reuteri* (Gene bank >KT282080.1), *Lactobacillus johnsonii* (Gene bank >KT282081.1), *Clostridium perfringens* (Gene bank >U20191.1) *Lactobacillus salivarius* (Gene bank >FJ591070.1) obtained from NCBI GenBank using MEGA-X. program (Figure 4.16)(118) (Figure 3.7). The amino acid sequence of such *L. fermentum bsh* genotype is been acquired while was used the SIB ExPASy consequence translate tool. (https://web.expasy.org/translate/)

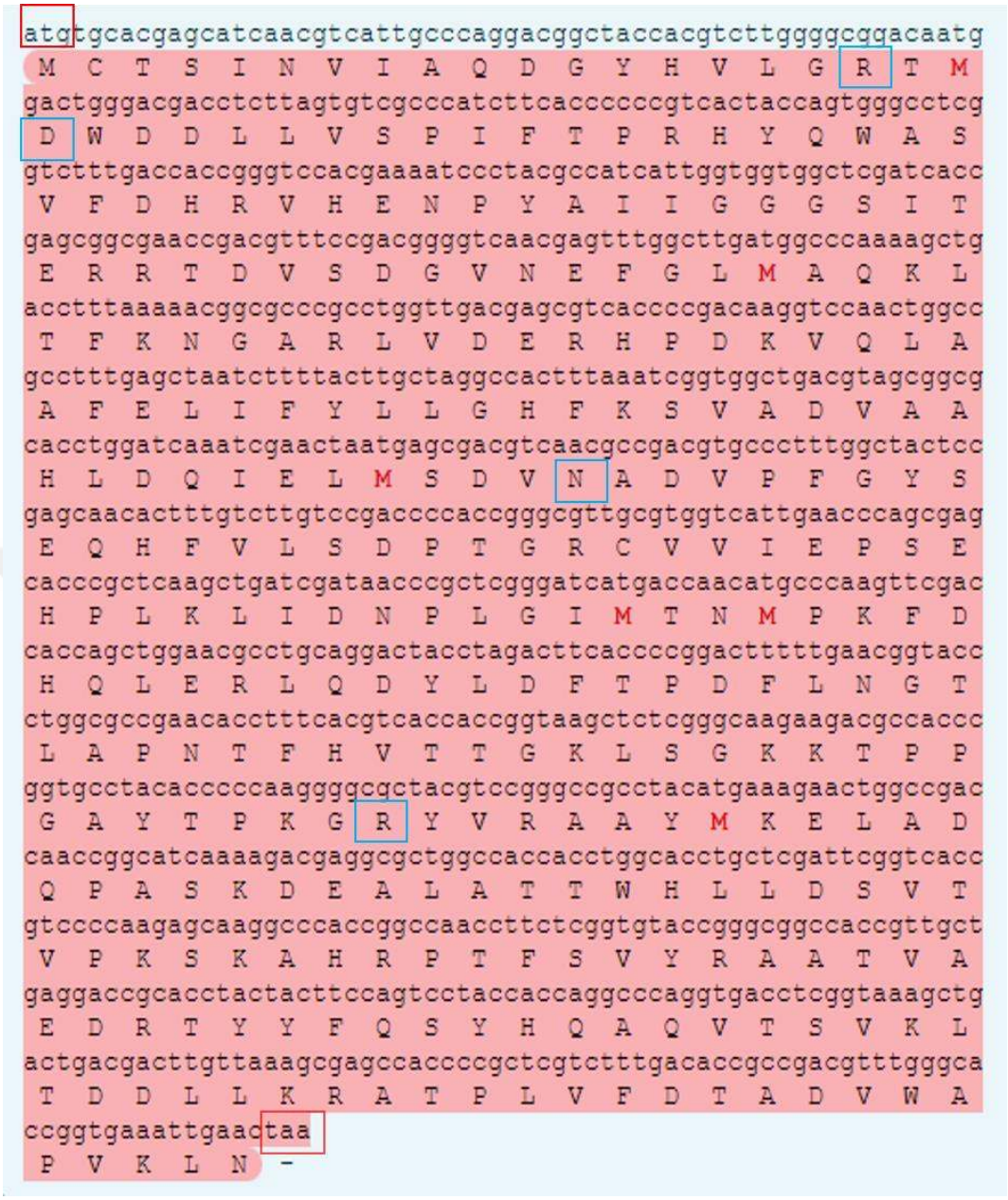

Figure 4.16. The amino acid sequence of the *L. fermentum bsh* gene was obtained using the SIB (Swiss Institute of Bioinformatics) ExPASy sequence translate tool. (http://web.expasy.org/translate/).

4.5. Phylogeny of BSH from *Lactobacillus fermentum* LFQi6

Analysis of the amino acid residues of BSH related enzymes (Figure 4.17.) and (Figure 4.18.) showed the Identity percentage analysis revealed the corelation between insert *bsh* gene of *L. fermentum* showed 99.9% similarity with BSH of *L. fermentum* LFQI6 using 2 types of phylogenetic tree, firstly the

similarity between of *L. fermentum* difeerent subspecies, finally the similarity between *L. fermentum* insert BSH gene and other strains of lactobacillus.

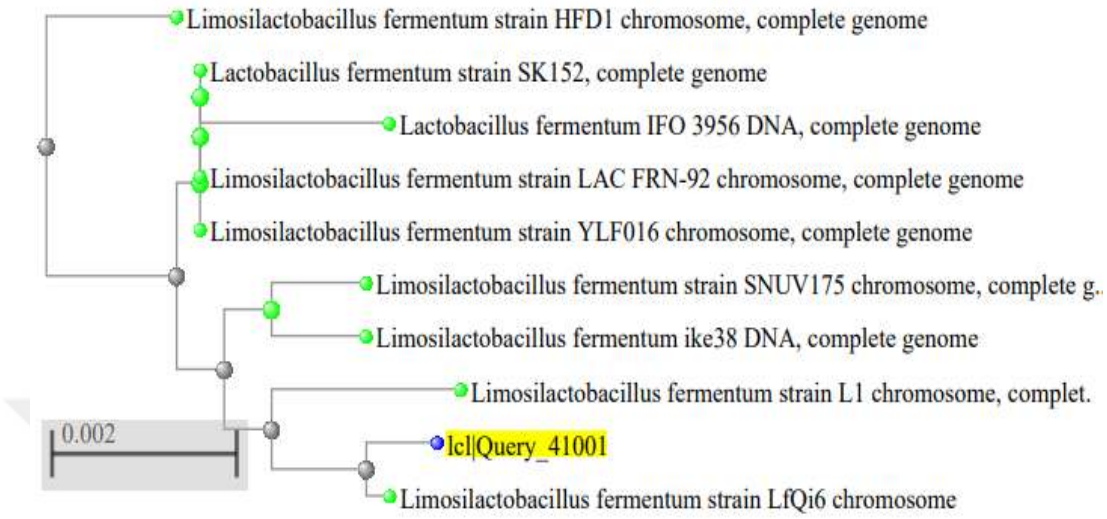

Figure 4.17. phylogenetic tree of different *L. fermentum* subspecies contains: Insert *bsh* gene of *L. fermentum* (unknown subspecies), *lactobacillus fermentum* HFD1 CP050919.1, *lactobacillus fermentum* SK152 CP016803.1, *lactobacillus fermentum* IFO3956 CP016803.1, *lactobacillus fermentum* LAC FRN-92 CP065522.1, *lactobacillus fermentum* YLF016 CP019030.1, *lactobacillus fermentum* SNUV175 CP076446.1, *lactobacillus fermentum* ike38DNA AP024320.1, *lactobacillus fermentum* L1 AP008937.1 , *lactobacillus fermentum* LFQi6 CP025592.1, the figure shown that the similarity between *lactobacillus fermentum* unknown strain and lactobacillus fermentum LFQi6 was 99%.

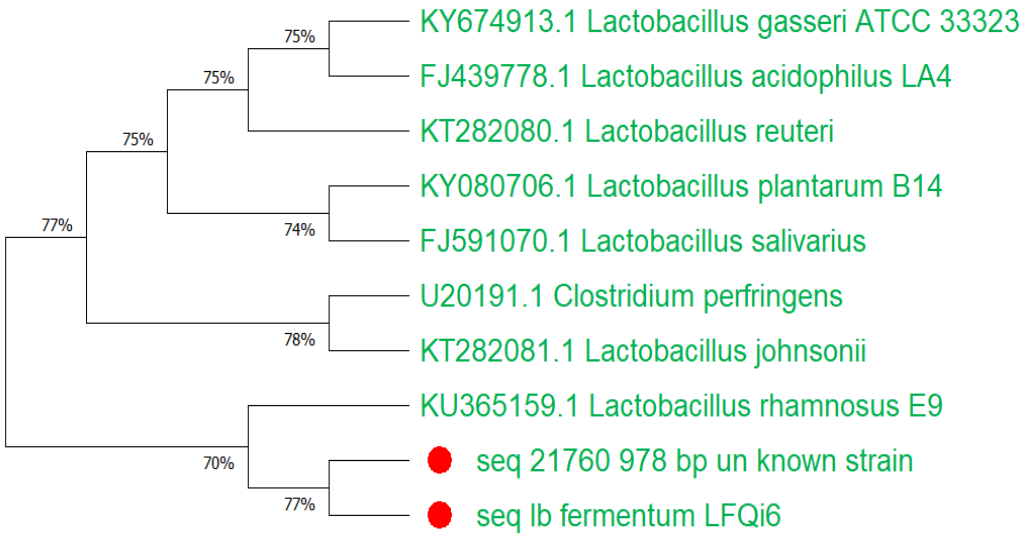

Figure 4.18. Phylogenetic tree of *L. fermentum* LFQi6 GeneBank: AUO26973.1 was represented in a tree structure with different strains of *L. fermentum* species, *Lactobacillus plantarum* (Gene bank >KU365160.1), *Lactobacillus rhamnosus* (Gene bank >KU365159.1), *Lactobacillus gasseri* (Gene bank >KY674913.1), *Lactobacillus acidophilus* (Gene bank >FJ439778.1), *Lactobacillus reuteri* (Gene bank >KT282080.1), *Lactobacillus johnsonii* (Gene bank >KT282081.1), *Clostridium perfringens* (Gene bank >U20191.1) *Lactobacillus salivarius* (Gene bank >FJ591070.1) The Phylogenetic tree indicated that the *L. fermentum* LFQi6 BSH enzyme related with BSH enzyme. https://www.ncbi.nlm.nih.gov/projects/treeview/tv.html.

4.6 Dotplot

Based on the BLAST results, this dot matrix view displays zones of similarity. The query sequence is shown on the X-axis, and also the numbers comprise the query's bases/residues. Just on Y-axis, the subject is represented, and the numbers represent the subject's bases/residues once again. The plot depicts alignments as lines. Plus, strand and protein match slant from the bottom left to the upper right corner, while minus strand matches slant from the upper left to the lower right. The plot's number of lines corresponds to the number of alignments found by BLAST.

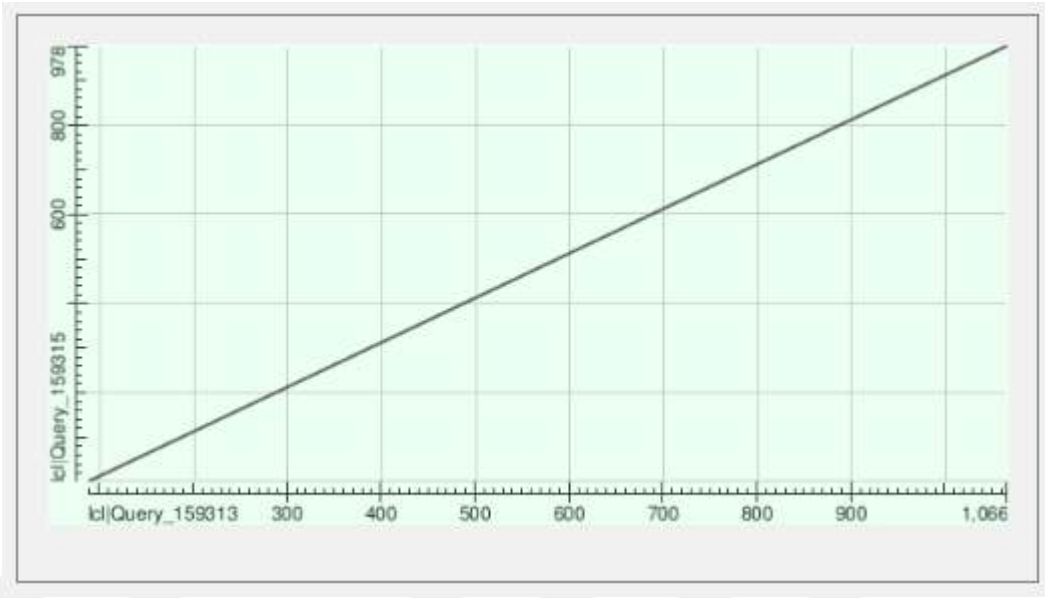

Figure 4.19. Dot matrix shows similarity between both strains of *L. fermentum* insert BSH gene (unknown) and *L. fermentum* LFQi6.

4.7 Amino Acid Sequence Alignment of BSH Enzymes

In this study, BLAST multiple sequence alignment revealed that BSH in *L. feremntum* belong to the Ntn hydrolase super family, members of which has strictly conserved catalytic sites, Cys-2, Arg-18, Asp-21, Asn-175 and Arg 228 and the amino acid motifs around these sites of BSH proteins are consistent in BSH of *L. fermentum* (Figure 4.20).

Figure 4.20. A. Alignment of amino acid residues of *L. fermentum* with BSH of mostly other Lactobacilus species (A-B) by T-COFFEE, (Version_11.00).

Figure 4.20. Continued B.

5. Preparation of pHAL2 Construct

5.1. pET22b DNA Isolation

Using the protocol mentionedon the Vivantis plasmid DNA Isolation Kit, we mentioned to the DNA isolation of the (5.5 kbp) low copy expression plasmid pET22b vector shown in (Figure 4.21).

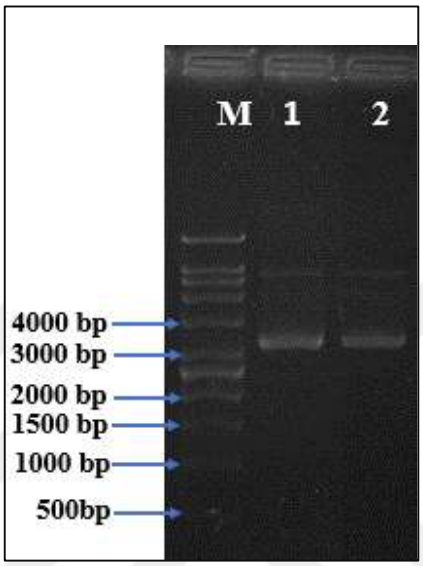

Figure 4.21. pET22b plasmid DNA Isolation. M represents the Marker with vivantas 1kb ladder. while line 1 represents the isolated pET22b plasmid DNA, line 2 represents the isolated pET22b plasmid DNA.

5.2. Digestion of pHAL1 and pET22b DNAs by EcoRI and NotI Fast Digest Restriction Enzymes

In order to retrieve our bsh gene stored in the pHAL1 transformant, we proceeded to the digestion of that transformant and plasmid DNAs with EcoRI and NotI fast digest restriction enzymes (Fermentas EU) at room temperature (37°C) for 30 minutes. The digestion products were then separated by agarose gel electrophoresis, even though the digestion of pHAL1 was not totally complete because of the low efficiency of oure restriction enzyme, it was visible that pHAL1 (Figure 4.22) and our desired *bsh* gene and the pET22b low copy expression vector were purified from the 1% agarose gel (Figure 4.22).

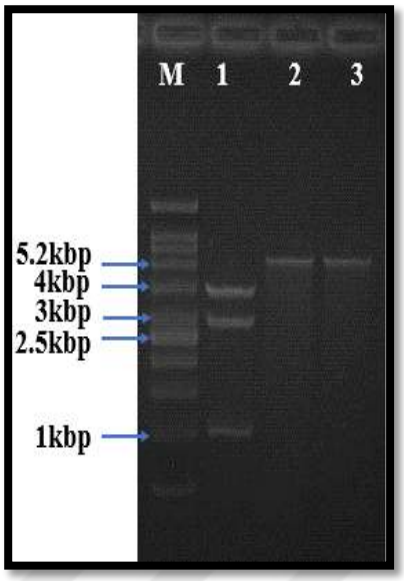

Figure 4.22. Digestion of pHAL1 construct and pET22b DNAs by EcoRI and NotI. M represents the marker with vivantas 1kb ladder. Line 1: Digestion of pHAL1 transformant by EcoRI /NotI before gel purification, Line 2: Digestion of pET22b by EcorI and NotI, Line 3: Digestion of pET22b by EcoRI and NotI.

5.2.1 Transformation of pHAL1 DNA into *E. coli* BLR(DE3) Strains

After the ligation reaction completed the insertion of bsh DNA into the pET22b expression vector, the ligation reaction product was transferred into Cells that are *E. coli* BLR(DE3) competent cells. Five random colonies were chosen and grown for overnight to isolate DNA. The isolated DNA was loaded onto a 0.8 percent agarose gel to detect which of the five randomly selected colonies had taken an insert (Figure 4.23). Lane 2 showed that this clone contained insert *bsh* DNA. Transformant may contain insert *bsh* with pET22b vector were shown in LB plates with ampiciline in Figure 4.24.

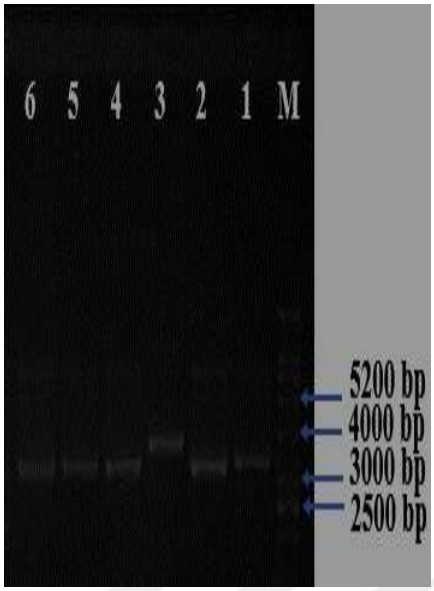

Figure 4.23. Transformation of pHAL2 into *E. coli* BLR(DE3) strains. M represents. The marker with vivantas 1kb ladder, Line 1: circular pET22b DNA, Line 2: pHAL2 A transformant DNA, Line 3: pHAL2 B transformant DNA, Line 4: pHAL2 C transformant DNA, Line 5: pHAL2 D transformant DNA, Line 6: pHAL2 E transformant DNA.

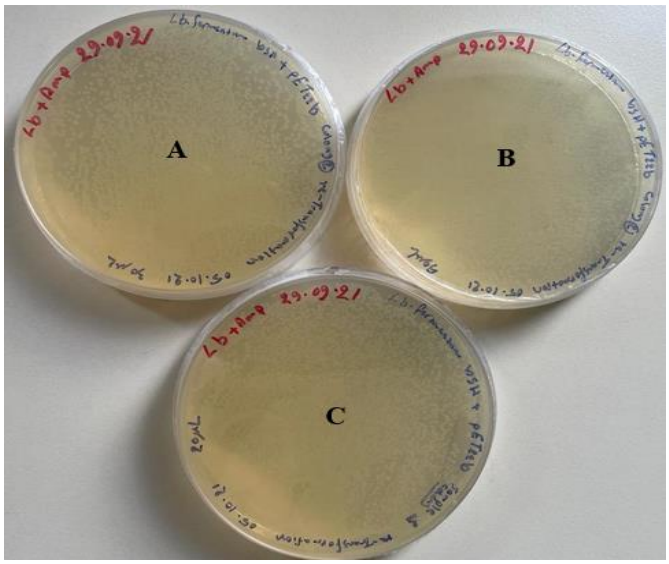

Figure 4.24. Insertion of pHAL2 construct into *E. coli* BLR(DE3). LB plates with Ampiciline. Regions labeled as letters (A) letter streaking on 30 microlitter, regions labeled as(B) letters streaking on 50 microlitter and region labeled as (C) letter streaking on 20 microlitter.

5.2.2 Detection of the Inserted bsh Gene from the Clones

The inserted bsh genes were selected by digesting the plasmid DNAs isolated from the transformants with EcorI and NotI fast digest restriction enzymes. The digested DNA products were loaded on an agarose gel 0.8% (Figure 5.24). Despit the fact that the digestion was not totally complete, it was visible that pHAL2/BLR(DE3) transformant DNA in lane 3 in Figure 4.25 had taken an insert BSH gene.

Figure 4.25 Detection of the bsh insert by EcorI/NotI fast digesting enzymes. M represents the marker with vivantas 1kb ladder and lane 1: represents the circular plasmid pET22b, lane 2: pHAL2 DNA (plasmid pET22b+ insert *bsh* gene), lane3: EcoRI /NotI digested DNA of pHAL1/BLR(DE3) transformants.

6 Analysis of BSH Enzyme Activity

6.1. Qualitative Analysis of rBSH Enzyme Activity (Direct Plate Assay)

To detecte the activity of r BSH enzymes qualitatevily, direct plate assay was used to compare the activity of the rBSH enzyme. In this assay, BSH activity was determined by white colonies with surrounding precipitation zones with halo/opaque growth, in the case of high activity, as shown in (Fig. 4.26). Direct plate assay results showed that the *E. coli* BLR(DE3) strains with rBSH from *L. fermentum* LFQi6 exhibited very low and/or not BSH activity against GDCA (Figure 4.26). On the other hand, the same strain failed to demonstrate convincing activity among TDCA in the same assay (Figure 4.26).

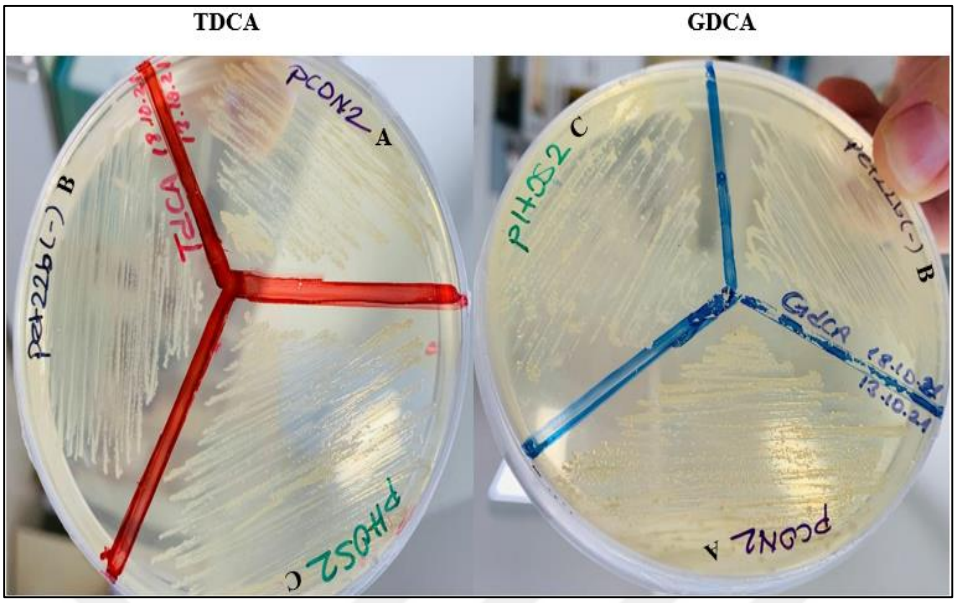

Figure 4.26. Determining of the rBSHs activities via direct plate assay proceses. The letters (a) in all plates represent phenotypes of positive controls (pCON2/BLR(DE3)), all regions labeled as (b) letters represent phenotypes of negative controls (pET22b /BLR(DE3), the (C) letter on the LB-plates containing GDCA represent phenotypes of pHAL2/BLR(DE3) respectively, the (C) in theLB plate containing TDCA represent phenotypes of pHAL2/BLR(DE3) respectively. BSH colonies have non halo/opaque of precipitated cholic acid phenotypes.

6.2 Partially Purified BSHs Enzymes SDS-PAGE Analysis

Partial purification of BSH protein of *L. fermentum* was visualized by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The nucleotide size of BSH gene is 978bp and the desired amino acid compositions and chain is 326 aminoacid. with theoretical molecular weight (5.94) of *bsh* gene protein is (36.5) KD The (36.5) kDa molecular mass of BSH proteins obtained by ExPASy program (http://web.expasy.org/protparam/) (Figure 4.27), This result suggests that there was not bsh protein band observed on SDS-PAGE, although there are some aminoacids essential for the catalytic activity and stability and/or folding of the BSH obtained by previous in silico analysis (Figure 4.28).

Number of amino acids:325

Molecular weight: 36538.43

theoretical pI of 4.85

Amino acid composition:

Ala (A) 27	8.3%	Phe (F) 16	4.9%
Arg (R) 15	4.6%	Pro (P) 20	6.2%
Asn (N) 10	3.1%	Ser (S) 17	5.2%
Asp (D) 27	8.3%	Thr (T) 25	7.7%
Cys (C) 2	0.6%	Trp (W) 4	1.2%
Gln (Q) 12	3.7%	Tyr (Y) 13	4.0%
Glu (E) 13	4.0%	Val (V) 27	8.3%
Gly (G) 17	5.2%	Pyl (O) 0	0.0%
His (H) 14	4.3%	Sec (U) 0	0.0%
Ile (I) 11	3.4%	Lys (K) 17	5.2%
Leu (L) 31	9.5%	Met (M) 7	2.2%

Total number of negatively charged residues **(Asp + Glu): 40**

Total number of positively charged residues **(Arg + Lys): 32**

Figure 4.27. Amino acid composition analysis of *L. fermentum* BSH protein via SIB (Swiss Institute of Bioinformatics) ExPASy Prot Param sequence analysis translate tool (http://web.expasy.org/protparam/).

Figure 4.28. Profile of partially purified SDS-PAGE result, M indicate the protein ladder. lane (1,3,5) indicates pHAL protein results, and Lane (2,4,6) indicates pCON2 as an (for positive control).

5. DISSCUSIONS

Bile salts hydrolyse ability of probiyotic strains is one of the specific requirements for probiotic strain selection. The biotransformation of bile salt by gut bacteria is a significant reaction in the gastrointestinal tract. BSH-producing probiotics have therapeutic properties, lowering serum cholesterol levels and lowering the risk of stroke, atherosclerosis, and coronary heart disease caused by hypercholesterolemia (119,120). Bacterial BSH activity, one on either hand, has been thought to be particularly toxic to host physiological changes due to the dehydroxylation and oxidation stages of primary bile salts in to the secondary bile salts by intestinal flora. Secondary bile salt manufacturing, in specific, has been related to cholesterol gallstone disease, colon cancer, and a variety of intestinal diseases (43). The Lactobacillus genera is a major component of the human GI microbiota, and it contains a diverse range of species that play important roles in gut health maintenance (121). Probiotic bacteria, bioprocessing (fermentation) has been used to produce a diverse range of foods and food ingredients in food preservation by humans. Bioprocessing technology has advanced this further, allowing for the specialized production of food and feed ingredients, as well as processing aids (63). BSH activity of enteric bacteria is important for persistent colonization in the GI tract and bile salt resistance is essential for probiotic species to survive and colonize in the GI tract and demonstrate their health-promoting effect on the host (122).

Probiotics are living organisms that improve the flora of the host's gastrointestinal system, regulates mucosal immunity, decrease inflammatory as well as allergic responses, relatively low blood cholesterol, and seem to have anti-colon cancer characteristics. Provided the above amazing list of possible medical benefits, it's really no great shock that using probiotics as biotherapeutic agencies keeps going to stimulate public's curiosity (69,109,123). Bacterial bile salt hydrolases (BSHs) are enzymes that cleave the conjugated glycine or taurine from bile acids, which is important step in the creation of deconjugated (BAs) and secondary (BAs). *Bsh* gene considered as desirable trait of probiotic bacteria due to its critical role in the BSH activity continues to bile resistance effects

(120,124). It is tempting to speculate that the increased BSH activity of LfQi6 biofilm cell mass may translate into clinically improved cholesterol lowering effect when using LfQi6 preformed biofilm cell mass rather than planktonic cells because the decomposition of bile salts by the BSH enzyme disrupts the formation of the cholesterol micelle, thereby preventing host cholesterol absorption (61). In this study, we cloned *bsh* gene and expressed the BSH enzyme of *L. fermentum in E. coli* BLR(DE3) strain to show the catalytic activity of this enzyme. Purified recombinant BSH of BLR(DE3)/pHAL from *L. fermentum* showed lack of bile salt hydrolase enzyme activity against both tauroconjugated bile salts (TDCA) and glycoconjugated bile salts (GDCA).

When the amino acid sequence of the recombinant BSH enzyme of *L. fermentum* was aligned with amino acid sequences of the other BSHs of Lactobacillus species obtained from National Center of Bioinformatics (NCBI), the alinment showed that one amino asid that is close conserved amino acids R18 and D20 was missing. Probaply due to one codon deletion from *bsh* gene of *L. ferementum,* BSH enzyme may lose its stability (folding) and activity. This mutation may disrupt either synthesis or formation of the BSH enzyme.

6. CONCLUSIONS AND RECOMMENDATIONS

In our study, we hoped to gain a better understanding of the structure and activity of the *bsh* gene in *L. fermentum* by cloning, expressing and characterizing it in *E. coli* BLR(DE3) strain. Our findings may support fallowing conclutions:

The BLAST analysis of the *bsh* gene sequences and multiple amino acid sequence alignment revealed that the BSH in *L. fermentum* belongs to the Ntn hydrolase super family.

Amino acid sequence alignment confirmed that five predicted catalytic sites, Cys2, Arg-18, Asp-21, Asn-175 and Arg-228, were strictly conserved in BSH enzyme of *L. fermentum*.

Even though *L. fermentum* has a *bsh* gene, our direct plate assay results obtained using partially purified rBSH enzyme demonstrated that the rBSH enzyme of *L. fermentum* was not able to deconjugate both glycine- and taurine-conjugated bile substrates.

Multiple sequence alignment revealed that BSH enzyme of *L. fermentum* had a one mutation (one codon deletion) on *bsh* gene. However, SDS-PAGE results of the partially purified BSH of *L. fermentum* confirmed the lack of BSH enzyme. This mutation may affect the stability or assamly of the BSH enzyme.

In this regard, this *L. fermentum* cannot be a potential probiotic candidate since the capability of BSH activity is one of the criteria for the selection of the probiotics. However, more research is needed, to completely understand the BSH enzyme's catalytic activity and substrate preferences.

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8. APPENDICES

APPENDIX 1

GROWTH MEDIA FOR BACTERIA

Liquid MRS (de MAN, ROGOSA and SHARPE) Medium

To make a liquid MRS medium (110), dissolve 52.2g of MRS in Ultra pure H_2O to a final volume of 1L. The pH of MRS broth does not need to be adjusted. The preparation is then sterilized for along 15-20 minutes at 121°C.

. MRS Liquid containing 30% Glycerol

To prepare a liquid MRS medium with 30% glycerol, 30 ml of glycerol is dissolved in 100 ml of MRS Broth. The preparation is then sterilized at 121^{O}C for along 15-20 minutes.

. MRS Plate

An MRS Plate is made by combining 15 g of agar-agar (Merck) with 1L of liquid MRS medium. The mixture is then sterilized for 15-20 minutes at 121°C.

. Liquid LB (Luria Bertani) Medium

Contents	for 1 litter
Tryptone (100 X 100)	10 g
Yeast Extract	5 g
NaCl	10 g

These contents are then dissolved in 900 ml Ultra pure H_2O, and the pH is set to 7.5 using NaOH. The final volume is then filled to 1L with Ultra pure H_2O and sterilized at 121 degrees Celsius for along 15 to 20 minutes.

. LB Plate

The amount of agar used to prepare an agar plate should be 1.5 percent of the amount of liquid LB medium. For example, in our work we added 15 grams of agar-agar (Merck) to 1L liquid LB medium and sterilized the preparation at 121°C along 15-20 minutes.

. Ampicillin

0.25 g ampicillin Sodium Salt

10 ml UpH_2O

The final solution is then sterilized by using a 0.22 micrometer (mm) filter and stored at -20^{O}C

. LB Plate with ampicillin

To make an LB Plate with ampicillin, 4 ml of ampicillin is added for 1L of sterilized liquid LB medium.

. LB Plate with IPTG, X-gal and Ampicillin

Following sterilization of the 1L LB plate medium, 1 mM IPTG (Isopropyl—D-1 thiogalacto pyranoside), 40 mg/ mL X-gal and 25 mg/ mL ampicillin (sodium salt) was added into the liquid medium.

Contents	**for 1 Litter**
IPTG	200mL
X-gal	400mL
Ampicillin	800mL

APPENDIX 2

Buffers and Solutions

For Genomic DNA Isolation

20 mM Tris HCl

In 10 mL of Ultra pure H_2O, 1.576 g of Trizma Hydrochloride (Sigma) was dissolved. The solution is then dissolved in 100 mL of Ultra pure H_2O to yield a final concentration of 20 mM Tris-HCl.

2 mM EDTA

In 30 mL of Ultra pure H_2O, 0.447 g of ethidium di tetra aceticacid (EDTA) is dissolved. The solution is then dissolved in 400 mL of Ultra pure H_2O to yield a final concentration of 2 mM EDTA. Drop by drop, the pH was adjusted to 8.0 to ensure that the EDTA was totally dissolved.

Lysis Buffer

Contents	**for 400 mL**
20 mM Tris HCl	8 mL
2 mM EDTA	20 mL
1.2% Tripton X-100	4.8 mL
20 mg/mL Lysozyme	266.7 mL

All of these components are mixed together with 100.5 mL of Ultra pure H_2O to make the final buffer volume of 400 mL.

For primer resuspensions

. **TE buffer**

3.634 g of Trizma base

30 mL of Ultra pure H_2O

The pH has been adjusted to 8.0

For Competent Cell Preparation

0.1 M $CaCl_2$

1.48 g of $CaCl_2$

100 mL of Ultra pure H_2O

The pH has been adjusted to 7.0

0.1 M $CaCl_2$ with 10% glycerol

9 mL of $CaCl_2$

1 mL of glycerol

10X TAE Buffer

Contents	**for 1 Liter**
Tris Base	48.50 g
Glacial acetic acid	11.42 g
0.5 M EDTA	20 mL

All these components are dissolved in enough of Ultra pure water to make the final buffer volume of 1Litter.

1X TAE Buffer

100 mL of 10X TAE Buffer

900 mL of Ultra pure H_2O

- **Preparation of cell extract reagents:**

1- 1 M NaH2PO4 Buffer:

In 30 ml Ultra pure water, 4.8 g monobasic sodium phosphate (NaH2PO4) is dissolved. The buffer solution is then sterilized for along 15 minutes at 121°C.

2- 1M Na_2HPO_4 Buffer

5.68 g dibasic sodium phosphate (Na2HPO4) dissolved in 30 ddH_2O; fill to 40 ml with ddH_2O. along 15 minutes, sterilize the buffer solution at 121°C.

3- 0.1M Sodium Phosphate Buffer (100 ml NaPi)

Carefully calibrate mixture of monobasic and dibasic sodium phosphate and obtain the solution pH of which is 7.8.

4- Binding buffer:

Mix 2.9g 500mM NaCl with 2mL 20mM NaPi solution and up the solution to 100 ml with H_2O, then the buffer is filtered through 0.45mm filter. PH:7.8

5- Lysozyme (1mg/ml)

6- Tween 20 (1%)
7- RNAse (5mg/ml)
8- DNAse (5mg/ml)

- **Lowry assay reagents:**

1- 0.1N NaOH

0.4 g NaOH

100 ml UpH_2O

2- 1% SDS in 0.1M NaOH

0.05 g Sodium Dodecyl Sulfate

5 ml of 0.1 M NaOH

3- Reagent A

1 g Na_2CO_3 (Sodium Carbohydrate)

50 ml of 0.1 M NaOH

4- Reagent B

5 mg $CuSO_4.5H_2O$ (Cupper II sulphate pentahydrate)

0.5 ml of UpH_2O

5- Reagent C

0.010g $C_4H_4KNaO_6.4H_2O$ (Potassium sodium tartarate)

0.5 ml of UpH_2O

6- Reagent D

500 µl Reagent B

500 µl Reagent C

7- Reagent E

50 ml Reagent A

1 ml Reagent D

- **For SDS-PAGE Analysis**

1- For separating gel:

5ml → Resolver A

5ml → Resolver B

50µl → APS

5µl → TEMED

2- For stacking gel:

2ml → Reagent A

2ml → Reagent B

20µl → APS

4µl → TEMED

3- 10% SDS

10 g → Sodium Dodecyl Sulfate

100 ml → UpH_2O

4- 10X Electrode Buffer Ph: 8.3

30g → 0,25 M Tris

144g → 1.92 M Glycine

This mixture is dissolved in UPH2O and up to 1L. The PH is adjusted by HCl

5- 1X Electrode Buffer

100 ml →10X Electrode buffer

1 g → SDS

900 ml → UpH_2O

6- Loading dye for SDS-PAGE for 1L

400µl → 10%SDS

120µl → Glycerol

100µl → Tris/HCl (stacking buffer) (Ph:6.8)

40µl → 0.5%Bromophenol Blue

340µl → Up (H_2O)

7- Comassie Stain Solution for 1L

1g → Comassie Brilliant G Blue

100ml → Glacial acetic acid

400ml → Methanol

500ml → UpH_2O

8- De-staining for comassie for 1L

100ml → Glacial acetic acid

100ml → Methanol

800ml → UPH_2O