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Review on the function, substrate affinity, and potential application of bile salt hydrolase originated from *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*

Abstract

Bile salt hydrolase (BSH: EC.3.5.1.24) has been used as a biomarker for probiotics for an extended period. It is mostly present in the gut environment of vertebrates. Additionally, it influences the viability of probiotics. This biomarker is considered a promising nutritional supplement due to its unique ability to effectively address elevated blood cholesterol levels, a common issue in modern society. However, the commercialization of BSH has been limited by an incomplete understanding of the intestinal microbiota and the function of BSH. Hence, in this review, we aim to reveal the current advancements in BSH research and outline the necessary areas of investigation for future studies. The review highlights key findings related to the substrate affinity of BSH in probiotic bacteria and its BSH gene phylogeny that have been researched until today, suggesting further research regarding the differences in multiple BSH genes and corresponding differences in BSH affinity.

Keywords: Bile salt hydrolase, Penicillin V acylase, cholesterol lowering effect, probiotics, hydrogel formation, antibiotic growth promoters

1. Introduction

Elucidating the relationship between probiotics and the deconjugation of bile salts through the activity of bile salt hydrolases (BSH; EC.3.5.1.24) remains an interesting topic. Duary et al. (2012) and Zhang et al. (2009) reported that *Lactobacillus* constitutively expressed BSH, which was slightly upregulated in the presence of bile acid. Furthermore, Begley et al. (2006) highlighted that most BSH activity is detected in gram-positive bacteria, specifically probiotic candidates. However, variation in enzyme activity across strains was significant, and the exact function or mechanism of the enzyme remains unclear (Horackova et al., 2020; Urdaneta and Casadesús, 2017; Yang et al., 2019).

Clarifying the exact mechanism of BSH activity is important. As reports of probiotics reveal blood cholesterol level reduction, potentially enabling further development of therapeutic applications (Agolino et al., 2024; Ahn et al., 2003; Begley et al., 2006; Mann and Spoerry, 1974). For modern people, Feingold (2016) reported elevated blood LDL-cholesterol levels were observed, a high-risk factor for cardiovascular disease (CVD), possibly resulting from modern diets and high-nutrient ingredients. As a result, probiotics are emerging as important health components of food for the public and patients with high cholesterol levels. Drugs that manage blood LDL-C levels include statins and their derivatives, which lower cholesterol synthesis in the liver (Feingold, 2016), and ezetimibe, which prevents the reabsorption of bile acids, promoting excretion from the body (Florentin et al., 2008; Kashani et al., 2008). However, the side effects of these medications are concerning (i.e., muscle complications, myopathy, acute pancreatitis, liver toxicity, and increased risk of diabetes) (Florentin et al., 2008; Kashani et al., 2008), which need the development of new compounds with fewer side effects. Accordingly, developing and commercializing BSH, a high-possibility reason for lower blood cholesterol (Agolino et al.,

2024; Begley et al., 2006; Guo et al., 2012), as a drug or supplement (e.g., postbiotics or genetically extracted by cloned vector) is expected.

However, BSH, a candidate for cholesterol control, remains uncommercialized because the potential risks have not been identified. For example, Sun et al. (2023) analyzed the intestinal contents of patients with colorectal cancer (CRC) and found that secondary bile acid levels increased significantly by BSH activity (Evangelakos et al., 2021; Perez and Briz, 2009; Sun et al., 2023). Secondary bile acids can cause inflammatory responses, cell membrane destruction, and DNA damage. This affects the intestinal cells, ultimately leading to CRC (Ajouz et al., 2014). Another potential risk is BSH may act as an antibiotic resistance factor. Kusada et al. (2022a) reported that *Lactobacillus paragasseri* JCM 5343^{T} has antimicrobial resistance by BSH activity, which can pose an antibiotic resistance when transformed into other bacteria (Daly et al., 2021). Because potential risks of BSH are high, it is currently difficult to use for clinical purposes.

It is still difficult to understand why probiotics synthesize BSH and deconjugate bile salt. To get enough data, it is more important to understand the exact mechanism of BSH. Furthermore, it is impossible to stop the synthesis of BSH by probiotics or gut microbiome due to various safety and ethical issues regarding genetic manipulation. Therefore, this review pointed to how differences appear by species or phylogenetic tree through the substrate specificity analysis and makes foundation for further research.

In this review, 122 published articles on BSH and probiotics were examined. These studies explained BSH activity according to taxa in the past three decades. They were sourced from electronic databases, including Public/Publisher MEDLINE (PubMed), Google Scholar, National Center for Biotechnology Information (NCBI), American Type Culture Collection (ATCC),

American Society for Microbiology (ASM) journals, SpringerLink, Food Research International, Multidisciplinary Digital Publishing Institute (MDPI), Journal of Dairy Science, Frontiers, Korea science, Proceedings of the National Academy of Sciences (PNAS), Animal Bioscience (AB), Royal Society, nature, Institute of food technologists (IFT), Wiley-online library, British Medical Journal (BMJ), Europe PMC, science direct, research gate, Talyor and Francis online, Cambridge university press, journal of lipid Research (JLR), Public Library of Science (PLOS), Tennessee university libraries, AUMA publication, Atherosclerosis journal and OXFORD Academic. The keywords used were bile salt, bile acid, bile salt hydrolase, Penicillin V acylase, blood cholesterol-lowering effect, probiotics, hydrogel formation, and antibiotic growth promoters. Main review address information regarding BSH mechanisms and activities across lactic acid bacteria (LAB) species are discussed. Additionally, the structures and functions of BSH and PVA are compared. Finally, the current challenges and possible solutions, focusing on the potential use of BSH in clinical settings, are highlighted.

2. Function and activity of bile salt hydrolase

2.1. Bile salt distribution and function

Bile salt and bile acid are distinguished if glycine or taurine is conjugated or not. If no glycine or taurine is attached, the substance is referred to as bile acid; otherwise, it is called bile salt (Daly et al., 2021). Primary bile acids are synthesized in the liver and denatured by bacteria to form secondary bile acids (Daly et al., 2021). Haslewood (1967) reported that the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are found in the bile of vertebrates. Similarly, secondary bile acids, including deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA), are found in this fluid (Haslewood, 1967). Notably, DCA is

produced by modifying CA, and LCA is derived from CDCA (Bachmann et al., 2015; Evangelakos et al., 2021; García-Cañaveras et al., 2012; Perez and Briz, 2009). Tagliacozzi et al. (2003) and Thakare et al. (2018) reported that CDCA predominated in human plasma, followed by deoxycholic acid (DCA), and glycochenodeoxycholic acid (GCDCA) was three times higher than taurochenodeoxycholic acid (TCDCA).

A wide range of bile acids are distributed across different species (Kuhajda et al., 2006; Li and Dawson, 2019). Bile salts conjugated with taurine are dominant in most cases, excluding those in humans and other animals. In addition, Karakus et al. (2024) reported that glycine-conjugated bile salts are dominant in humans, and taurine-conjugated bile salts are most common in dogs (García-Cañaveras et al., 2012; Kakimoto et al., 2017; Rabin et al., 1976; Vessey, 1978).

Bile salts primarily aid in food digestion (Maldonado-Valderrama et al., 2011). de Buy Wenniger and Beuers (2010) and Redinger (2003) reported that bile salts are synthesized in the liver, stored in the gallbladder, and secreted along with pancreatic enzymes in the duodenum upon ingestion of food. Notably, these salts have amphipathic characteristics (Daly et al., 2021). The hydrophobic part attaches to ingested lipid droplets and divides them into smaller particles. These fine lipid particles help lipolytic enzymes, such as lipase, to work better, and the bile salt is reabsorbed 95% near the ileum when the process is complete. It enters the portal vein along the capillaries and re-enters the liver thereafter. The 5% of bile salt that was not absorbed from the ileum was fermented or deconjugated by the gut microbiome. Most of the affected bile salt is excreted with feces but some of it is reabsorbed. The whole circulation and enzyme effect for bile circulation is shown in Figure 1 (de Buy Wenniger and Beuers, 2010; Redinger, 2003; Daly et al., 2021).

2.2. High blood cholesterol level and role of BSH in probiotics for low LDL-C effect

Several study reports revealed that high blood cholesterol levels (LDL-C > 190 mg/dL (Bittencourt et al., 2020)), accompanied by a sedentary lifestyle, are increasingly prevalent across generations (Evangelakos et al., 2021; Perez and Briz, 2009; Sun et al., 2023). Individuals with high plasma LDL-C levels have a high risk of developing cardiovascular disease and a shorter life expectancy. Prescription of statins or ezetimibe to patients with high-cholesterol syndrome may relieve symptoms. However, they cause side effects, such as myopathy, acute pancreatitis, and liver toxicity (Florentin et al., 2008; Kashani et al., 2008). Furthermore, patients with liver or cardiovascular diseases are particularly vulnerable to these side effects (Begley et al., 2006; Detection and Adults, 2002; Schuster, 2004).

Several studies have reported that probiotics can lower blood cholesterol levels with almost no side effects and have positive effects on various conditions, including the treatment of atopic dermatitis, colon cancer, Crohn's disease, diarrhea, and constipation (Ishimwe et al., 2015; Ooi and Liong, 2010; Sivamaruthi et al., 2019). However, their respective mechanisms remain unclear (Gill and Guarner, 2004; Mercenier et al., 2003; Reid et al., 2003; Sanders and Klaenhammer, 2001; Tuohy et al., 2003; Woo et al., 2023). Ahn et al. (2003) reported that cholesterol was reduced following 4 weeks of consuming milk containing *L. acidophilus* SNUL01, and Fuentes et al. (2013) highlighted that cholesterol was lowered by consuming the capsule form of *Lactobacillus plantarum* (CECT 7527, CECT 7528, and CECT 7529). The hypothesis supporting the reduction of blood cholesterol levels as a function of BSH revealed so far can be explained as follows: more than 95% of bile salts are reabsorbed in the human ileum (Li and Chiang, 2020; Naumann et al., 2020), and the remaining 5% passes through the ileum. Colonic bile salts are deconjugated by BSH activity, forming deconjugated bile salts. BSH has a

specific active site (Figure 3), especially the cys-2 (or 22) site, which is essential for BSH catalysis (Begley et al., 2006). In bile salt deconjugation, cys-2 attacks the carbonyl carbon of the excision amide bond in bile salt, followed by the removal of glycine or taurine by hydrolysis (Chand et al., 2018). Deconjugated bile salts are water-soluble in a colonic pH environment of 7–8 (Trivedi and Puranik, 2017; Yamamura et al., 2023). The metabolic activity of intestinal microorganisms, particularly lactic acid and short-chain fatty acid (SCFA) production, further lowers the pH, causing the precipitation of deconjugated bile salt (Begley et al., 2006). Therefore, colon enterocytes no longer absorb it, leading to its excretion in the feces, which in turn lowers blood cholesterol.

Nonetheless, confirming whether BSH is a factor remains difficult because microorganisms that reduce blood cholesterol levels exist despite the absence of the BSH gene, such as *Streptococcus thermophilus* MCC0200 (Kapse et al., 2024). In addition, Choi et al. (2015) reported that deconjugated bile salt has a stronger affinity for the farnesoid X receptor (FXR) that regulates bile synthesis, reducing hepatic bile acid synthesis; by this result, the effect of BSH does not alter the blood cholesterol concentration. Consequently, current experimental results do not identify BSH activity as a major factor for the LDL-C-lowering effect in the presence of probiotics.

2.3. Role of BSH in probiotics

Even if probiotics do not have an LDL-C-lowering effect, maintaining high survival rates in the intestine is critical to elicit other health benefits to the host. This concept originated from the study of Fuller (1995). According to Dobson et al. (2012), probiotics are resistant to acid and produce antibacterial substances, including bacteriocins, hydrogen peroxide, and organic acids.

Furthermore, probiotics are highly resistant to bile salts compared with other bacteria (da Silva et al., 2024; Gu et al., 2024; Horackova et al., 2020; Spínello et al., 2024; Urdaneta and Casadesús, 2017; Yang et al., 2019). The study by De Smet et al. (1995) suggested that BSH-positive probiotics would have stronger bile resistance than negative. However, subsequent studies showed no correlation between probiotic bile tolerance and BSH levels by enzyme knockout experiments (Begley et al., 2005; Moser and Savage, 2001). In addition, genomic analyses suggest no relationship between bile concentration and BSH gene expression (Horackova et al., 2020; Yang et al., 2019). Recently, Jarocki et al. (2014) hypothesized that deconjugated bile salt reacts with other organic substances, producing a hydrogel that can promote the colonization of intestinal microorganisms (Jarocki et al., 2014; Sobotka and Czeczowiczka, 1958). If these experiments can be replicated *in vivo*, new insights between BSH and probiotics can be processed.

3. Interspecies characteristics of BSH

The following data analysis is that which integrates the affinity between a single enzyme and bile. BSH activity has primarily been studied within the context of lactic acid bacteria (LAB) research, and many full-length genomes of the strains studied have been identified. Begley et al. (2006) analyzed BSH activity mainly in *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium*, and *Bacteroides*. However, most studies correlating enzyme activity with genomic data have used *Lactobacillus* species only (O'Flaherty et al., 2018). Considering that the taxonomy of *Lactobacillus* is newly defined, a new method for direct genetic analysis of the population is needed for comparison with other strains (Oberg et al., 2022). Furthermore, the

current knowledge regarding the substrate affinity of BSH could enhance future genetic analyses of enzymatic mechanisms.

Table 1 summarizes the measured enzyme affinities for various BSH derived from different species. Notably, the taxonomy of *Lactobacillus* was recently updated as reported by Oberg et al. (2022). The affinity results of each BSH for conjugated bile salts (cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA)) were categorized to show the differences in affinity between glycine- and taurine-conjugated bile salts for each substrate. Higher affinity differences mean that an enzyme can hydrolyze a particular substrate faster or to a greater extent in a given data analysis method.

Table 1 only indicated the affinity between glycine-conjugated bile salt and taurine-conjugated bile salt for each BSH. Generally, the BSH data of *Bifidobacterium* and *Enterococcus* showed greater affinity for glycine-conjugated bile salts. In contrast, *Lactobacillus* showed varying affinities, and several strains harbored multiple copies of BSH genes (including *Lb. acidophilus* NCK 1909, *Lb. gasseri* NCK2253, and *Lb. johnsonii* PF01). These strains exhibited a higher affinity for taurine-conjugated bile salts.

Zhao et al. (2024) conducted a gene cloning experiment to heterologously express the BSH gene, explaining the relationship between probiotic bile acid affinity and the cholesterol-lowering effect, which specifically acts on either glycine- or taurine-conjugated bile salt. In addition, they administered them in mouse models to track their activity. This study reported that blood cholesterol levels decreased in post-germ-free (PGF) mice carrying a mutant bacterium, F67A, that preferentially degrades taurocholic acid (TCA). However, the mutant bacteria, YB81, that preferentially degrades glycocholic acid (GCA), does not affect host blood cholesterol levels. In contrast, in specific pathogen-free (SPF) mice, YB81 also reduced cholesterol levels.

Therefore, the cholesterol-reducing mechanism of F67A involves altering the intestinal bile acid ratio, whereas YB81 lowers cholesterol levels by controlling the BSH activity of intestinal microorganisms. The results showed that YB81, which has a strong BSH affinity for glycine-conjugated bile salts in this case, changed the BSH activity of microorganisms in the gut in a way that does not directly lower cholesterol or have other metabolic effects. *Lb. fermentum* K73, *Lb. rhamnosus* GG, and *E. faecalis* CU30-2's high affinity for glycine-conjugated bile salts might directly change the gut ecosystem compared with lower cholesterol based on Zhao's hypothesis. In this regard, *Lb. gasseri* NCK2253 and *Lb. johnsonii* PF01 strains are important (Table 1). *Lb. gasseri* NCK2253-A and *Lb. johnsonii* PF01-A showed high affinities for the taurine-conjugated bile salt. *Lb. gasseri* NCK2253-B and *Lb. johnsonii* PF01-C BSHs showed opposite affinities (glycine-conjugated bile salts). Examining these two strains *in vivo* could provide more concrete evidence for the different substrate affinities of microbial BSH. Despite the abundance of data on BSH produced by *Lactobacillus*, additional studies on *Bifidobacterium* and *Enterococcus* are still needed to draw better comparisons of the BSH properties of different bacteria, particularly with the advent of tailored probiotics.

4. Potential BSH inhibitor for feed efficiency and probiotics

Antibiotics are widely used in farms to improve domestic animal growth and maintain animal health. This phenomenon refers to antibiotics as antibiotic growth promoters (AGPs) (Lin, 2014). However, the use of antibiotics has caused the uncontrolled development of antimicrobial resistance (AMR) in various niches. AMR is a driving force that promotes pools of resistant pathogenic bacteria and poses a serious threat to food safety and public health (Davies, 2014; Perry et al., 2014). For this reason, the incorporation of antibiotics in feed is

232 legally restricted or completely banned in the EU, UK, USA, and other countries.
233 Consequently, animal nutrition studies have focused on finding AGP alternatives and
234 improving feed efficiency (Kim and Lee, 2005). To maximize feed efficiency, a substitution
235 for antibiotics is necessary. The solution is yet to be determined, but currently, BSH control
236 has the best consequences since suppressing BSH can achieve feed efficiency similar to that of
237 using antibiotics.
238 Negga (2015) reported that BSH activity lowers blood cholesterol levels and feed efficiency.
239 Furthermore, Rani et al. (2017b) studied BSH inhibitors and found that riboflavin showed
240 almost 98% inhibition. Notably, Negga hypothesized that riboflavin could increase the growth
241 performance of domestic animals. Broiler chickens showed an increase in body weight after
242 consuming 20 mg/kg of riboflavin for 21 days. Animal experiments using chickens and pigs
243 proved that feeding vitamins, especially riboflavin, resulted in a similar level of increased
244 productivity compared to that of antibiotic treatments (Geng, 2018; Negga, 2015; Yang et al.,
245 2020). This phenomenon can be attributed to the functional inhibition of BSH by riboflavin
246 and β-lactam antibiotics (penicillin V, ampicillin), especially penicillin (Adhikari et al., 2020;
247 Daly et al., 2021; Geng and Lin, 2016; Li et al., 2022; Rani et al., 2017a; Suresh et al., 1999).
248 Given that the hypothesis is true, it is highly likely that BSH is the cause of the AGP effect. In
249 order to achieve the AGP effect, an alternative to antibiotics, such as a BSH inhibitor, is
250 required. However, the most effective treatments were limited to β-lactam antibiotics and
251 riboflavin (Lin et al., 2014; Rani et al., 2017b).
252 Therefore, to obtain sufficient feed effects from livestock, a biochemical mechanism and a
253 more effective BSH inhibitor are necessary. For improved results, it is crucial to figure out the
254 evolution of BSH and define the optimal binding site for the inhibitor. The following chapter

255 presents the results of analyses based on the BSH peptide sequence and active site identified so
256 far.
257
258 5. BSH and PVA active site and mechanism of action
259 5.1. BSH phylogeny
260 BSH and PVA may be considered moonlighting proteins because of similar structures.
261 Moonlighting proteins are defined as the same enzyme that performs more than one distinct
262 action (Jeffery, 2018). However, the results of the experiment by Kumar et al. (2006) show that
263 the enzymes presumed to be BSH or PVA from *B. sphaericus, C. perfringens,* and *B. longum*
264 have only about 30% peptide similarity (not moonlighting protein). Otherwise, BSH and PVA
265 are classified as choloylglycine hydrolases (CGH) within the N-terminal nucleophilic (Ntn)
266 hydrolase enzyme superfamily (Daly et al., 2021).
267 These two enzymes, which appear to be similar only in structure, can hydrolyze each other's
268 substrates. There are genomic analyses for this phenomenon. O'Flaherty et al. (2018) reported
269 that *Lb. gorilla, Lb. frumenti, Lb. vaginalis, Lb. panis, Lb. antri, Lb. agilis, Lb. salivarius,* and
270 *Lb. plantarum* strains are simultaneously active against bile acids and penicillin. For *in vitro*
271 tests, Lambert et al. (2008) reported that *Lactobacillus plantarum* WCFS1 has four *bsh* genes,
272 including *bsh*-1, bsh-3, and *bsh*-4, which possess BSH activity. In contrast, *bsh*-2, *bsh*-3, and
273 *bsh*-4 showed PVA activity, with *bsh*-3 showing the strongest activity. Furthermore, Kusada et
274 al. (2022a) reported that *Lactobacillus paragasseri* JCM 5343 *bsh*-A showed common substrate
275 specificity for PVA.
276 However, because *Lactobacillus* was the primary focus of these results, it was necessary to
277 compare strains belonging to *Bifidobacterium* or *Enterococcus*. For advanced data, phylogenetic

analysis based on BSH peptide sequences obtained from the NCBI or ATCC databases was performed to determine the conserved domains of BSH. A phylogenetic tree was constructed using the data described in Table 2. The bacteria information source is based on Table 1 and searched against ATCC and NCBI databases. A Neighbor-Joining (NJ) tree was constructed using the Jukes-Cantor model with uniform rates and bootstrap replications of 1,000 datasets using MEGA-11 software. Nodes farther apart are genetically distant, while genes on the same bridge are phylogenetically closer (Figure 2).

To explain Figure 2, genes from *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacteroides*, and *Clostridium* were represented using orange and blue lines. BSH genes that simultaneously or preferentially exhibit PVA activity were indicated using orange boxes, and BSH genes from strains that are highly likely to exhibit PVA activity were denoted using black boxes because they share the same bridge.

The reconstructed phylogeny suggests that *Lactobacillus* may have originated the BSH genes of *Enterococcus* and *Bifidobacterium*, in line with reports from Morinaga et al. (2022). Several clusters showed highly similar genetic distances from each other: Cluster 1 (*Lb. casei* ASCC 1521, *Lb. rhamnosus* ASCC 290, and *Lb. rhamnosus* GG), and Cluster 2 (*Lb. plantarum* WCFS1-3, *Lpb. plantarum* LP91, and *Lb. plantarum* 299V), and Cluster 3 (*Lb. acidophilus* ATCC 4356-A, *Lb. acidophilus* ATCC 4357-A, and *Lb. acidophilus* LA1-A), and Cluster 4 (*Lb. acidophilus* ATCC 4356-B, *Lb. acidophilus* ATCC 4357-B, and *Lb. acidophilus* LA1-B). The sequence identity percentage of the BSH gene was identical in each group. There is likely no affinity difference and the enzymes function similarly.

Table 1 shows differences in bile acid affinity within the same cluster, as revealed by the analysis. Within cluster 1, *Lb. rhamnosus* ASCC 290 and *Lb. rhamnosus* GG exhibited a strong

affinity for GCA, whereas *Lb. casei* ASCC 1521 demonstrates a strong affinity for TCA. *Lpb. plantarum* LP91, *Lpb. plantarum* 299V and *Lpb. plantarum* WCFS1-3 exhibited a strong affinity for GCA within cluster 2. However, *Lpb. plantarum* 299V, and *Lpb. plantarum* WCFS1-3 exhibited a strong affinity for GDCA, whereas *Lpb. plantarum* LP91 demonstrates a strong affinity for TDCA. The BSH genes of *Lb. acidophilus* ATCC 4356, *Lb. acidophilus* ATCC 4357, and *Lb. acidophilus* LA1 in Cluster 3 exhibited a completely identical gene sequence with 100% similarity. Cluster 4 exhibited the same characteristics. Therefore, in either Cluster 3 or Cluster 4, the BSH should display identical affinities. However, comparing clusters 3 and 4 was challenging because an established BSH affinity for each substrate was lacking, as indicated in Table 1.

We conducted a comprehensive analysis to draw more conclusive interpretations of BSH and PVA activities from various LAB. Particularly with *bsh* genes that encode enzymes with PVA, such as *Lb. paragasseri* JCM 5343T-A and *Lpb. plantarum* WCFS1-3, are important (Kusada et al., 2022a; Lambert et al., 2008). In addition, in Cluster 2, *Lpb. plantarum* WCFS1-3, *Lpb. plantarum* LP91, and *Lpb. plantarum* 299V had highly similar nucleotide sequences. Therefore, it was necessary to determine whether LP91 and 299V can also metabolize penicillin.

5.2. Comparative analysis of BSH and PVA active site

The phylogenetic tree significantly correlated the BSH across the same species, as demonstrated in the previous chapter. It is important to analyze genetically connected, but the active site of these enzymes is also important. The active site is predicted using point mutations as explained in Chand et al. (2018). Most active sites reported for BSH appear to be highly conserved.

To determine whether this fact appears not only in *Lactobacillus* but also in *Bifidobacterium* or *Enterococcus*, sequence alignment was performed based on the active site. Chand et al. (2018) confirmed the active site of BSH by using point mutations in a predicted region. Most active sites reported for BSH appear to be highly conserved. In *B. bifidum*, Cys-2, Arg-18, Asp-21, Asn-72, Asn-173, and Arg-226 are predicted to be the residues involved in active sites (Kim et al., 2004a; Song et al., 2019). Regarding this, the BSH gene sequence was aligned and compared with the peptides described above using MEGA. Except for *B. fragilis* ATCC 25285-B, which changed Asp-21 to Glu-43 as shown in Figure 3, all samples shared the same active site.

These results confirm the homogeneity of almost all active sites, making it difficult to distinguish between PVA and BSH based on this aspect. Finding the difference between the two is very important for understanding the identity and mechanism of BSH in the future. Meanwhile, Avinash et al. (2016) reported that two Trp residues (at positions 23 and 87, based on *B. bifidum*) of PVA were important for interactions having the benzene ring of penicillin. In addition to Trp, Phe, and Tyr (benzene ring amino acids) were discovered in PVA's peptide sequence of PVA (Chand et al., 2018; Daly et al., 2021; Suresh et al., 1999).

Based on this theory, Figure 3 is analyzed additionally. The bsh-A gene of *Lb. paragasseri* strain JCM 5343^{T} and the second, third, and fourth *bsh* genes of *Lb. plantarum* WCFS1 showed experimentally verified PVA activity. The 2nd, 3rd, and 4th *bsh* genes in *Lb. plantarum* WCFS1 have conserved Phe-23 and Tyr-87 residues. In contrast, the first *bsh* gene in *Lb. plantarum* WCFS1 did not exhibit PVA activity and contained only one benzene ring amino acid (Tyr-22 and Asn-87). However, the *bsh*-A gene of *Lb. paragasseri* strain JCM 5343^{T} contains Val-23 and Asn-87 except for the benzene ring amino acid, with an affinity for ampicillin (Kusada et al., 2022a). These results make it difficult to conclude that the amino acids at positions 23 and 87 of

346 the benzene ring are critical for PVA activity. Therefore, further research is vital and required to
347 determine which amino acid sequence produces PVA activity.
348
349 6. Challenges and proposed solutions in BSH research
350 If BSH is intended for use in medication, postbiotics (cell-free supernatants and soluble factors
351 secreted by live bacteria) (Martyniak et al., 2021) or an overexpression protocol can be used
352 without the genetic manipulation of probiotics. However, postbiotics safety has not yet been
353 verified (Zhong et al., 2023). Also, BSH has unknown risks proved by *in vitro* and *in vivo*
354 analyses.
355 Several studies reported that DCA, in reaction to BSH, can be a causal factor for colorectal
356 cancer (CRC). Analysis revealed that patients with CRC had high levels of secondary bile acids
357 in their large intestine (Aguirre et al., 2022; Choi et al., 2015; Sun et al., 2023). Ajouz et al.
358 (2014) reported that excessive concentration of secondary bile acids that pass into the large
359 intestine may cause inflammatory responses, cell membrane destruction, and DNA damage. This
360 affects the intestinal cells, ultimately leading to CRC.
361 Another potential risk is that BSH may act as an antibiotic resistance factor, given that both
362 BSH and PVA belong to the CGH family. In this regard, investigation of the active site or
363 peptide sequence holds little relevance in distinguishing BSH with PVA activity. The PVA
364 enzyme inhibits penicillin activity, allowing bacteria to survive in the presence of the said
365 antibiotics (Lambert et al., 2008; Sunder et al., 2017). Kusada et al. (2022a) reported that
366 *Lactobacillus paragasseri* JCM 5343^{T} *bsh*-A has antimicrobial resistance by BSH activity.
367 Furthermore, Lambert et al. (2008) reported that *Lactobacillus plantarum* WCFS1 *bsh*-2, *bsh*-3,
368 and *bsh*-4 showed PVA activity, with *bsh*-3 showing the strongest activity. However, these
369 phenomena were observed mostly *in vitro*.

Until now, only the negative effects of secondary bile acids produced by BSH have been highlighted. Studies have indicated that certain intestinal diseases are caused by an imbalance of secondary bile acids. Diversity of intestinal microorganisms is needed for a healthy BSH pool, which therefore balances the secondary bile acids. In several studies, patients with inflammatory bowel disease (IBD) had significantly reduced amounts of secondary bile acids, DCA and LCA (Fiorucci et al., 2021; Heinken et al., 2019; Larabi et al., 2023). Ultimately, the key is to prevent excessive formation of secondary bile acids.

Complete inhibition of BSH activity would eliminate its cholesterol-lowering effect in the blood, which would be a disadvantage in various aspects. Instead, preventing the conversion of primary deconjugated bile salt to secondary deconjugated bile salt could effectively maintain an appropriate amount of primary and secondary bile acids. Bustos et al. (2018) reported that the 7α-dehydroxylase of gut bacteria removes the 7α-hydroxy group and converts primary deconjugated bile salts to secondary deconjugated bile salts (Figure 1). However, lactic acid bacteria do not have this function. According to Takahashi and Morotomi (1994), bacterial genera used as probiotics (bifidobacteria and lactobacilli) cannot dehydrogenate primary deconjugated bile salts. Thus, if we can control the 7α-dehydroxylation pathway, we will also be able to suppress the formation of excessive secondary bile acids.

To summarize, the effects of BSH need to be studied further for safe use. While the suppression of BSH generally has positive effects, it can also lead to various side effects, underscoring the importance of mitigating methods to control the 7-dehydroxylation pathway.

7. Future research

7.1. Necessity to differentiate between BSH and PVA

Multiple bile salt hydrolase (BSH) genes and their surrounding regions showed minimal sequence similarity, indicating that BSH is the product of horizontal gene transfer. Furthermore, insertion into similar regions and the existence of mobile genetic markers support this theory (Daly et al., 2021). The PVA enzyme inactivates penicillin activity, allowing bacteria to survive in the presence of antibiotics (Lambert et al., 2008; Sunder et al., 2017). BSH and PVA are difficult to distinguish based on structural or peptide sequence differences because BSH and PVA share about 30% sequence similarity with each other, but in fact, about 30% similarity is also found among BSHs across different species (Kumar et al., 2006). Also show different substrate specificities for each strain (Lambert et al., 2008). Understanding the causes of these characteristics is important for future gut microbiome research.

7.2. Correlation between the microbiome and the toxicity of deconjugated bile salt

Many studies have examined the correlation between IBD and CRC, the amount of BSH, and the proportion of bile acid (Evangelakos et al., 2021; Fiorucci et al., 2021; Heinken et al., 2019; Larabi et al., 2023; Perez and Briz, 2009; Sun et al., 2023). However, it was only found that representative microorganisms produced active BSH and did not measure the colonic pH of healthy people and patients or sufficiently investigate the composition of lactic acid bacteria. A recent study measured the real-time colonic pH and showed that the normal cecal pH is approximately 5.5, and the large intestine is approximately pH 5.5 to pH 7 (31, 32). If the production of deconjugated bile salt occurs at the beginning of the large intestine, the pH at which it precipitates sufficiently matches this. Therefore, further research is needed on changes in the ratio of intestinal lactic acid bacteria, colonic pH, and the toxicity of deconjugated bile salt.

7.3. Correlation between substrate specificity of BSH and microbiome

Recently, a published paper showed that BSH with different substrate specificities towards glycine- and taurine-bile acids has differences in how the microorganism regulates cholesterol (Zhao et al., 2024). This study explains the reason for the difference in bile salt affinity, which was previously difficult to interpret solely based on the BSH sequence. However, the lack of relevant papers necessitates sufficient verification, allowing us to directly design cholesterol control mechanisms in patients or healthy individuals using probiotics.

7.4. BSH specificity of lactic acid bacteria other than *Lactobacillus*

To date, most studies on BSH have focused on those involving lactic acid bacteria, especially *Lactobacillus*. However, understanding the relationship between BSH and PVA, the evolutionary history of BSH and PVA, and the biological flow of genes requires a deeper understanding of the relationship between BSH and various microorganisms. Therefore, in addition to *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, research on the BSH of *Listeria, Clostridium*, and other microorganisms is required.

8. Conclusions

Compared to other blood cholesterol-reducing drugs, the body naturally uses BSH as part of food consumption, which makes it commercially valuable. Understanding the risks, functions, and characteristics of BSH can further ensure the safety of probiotics, which can directly impact the intestinal survival rate. Therefore, it is important to clarify the cause of the characteristic strains with substrate specificity and measure the pH of the patient's colon who suffered from IBD or CRC.

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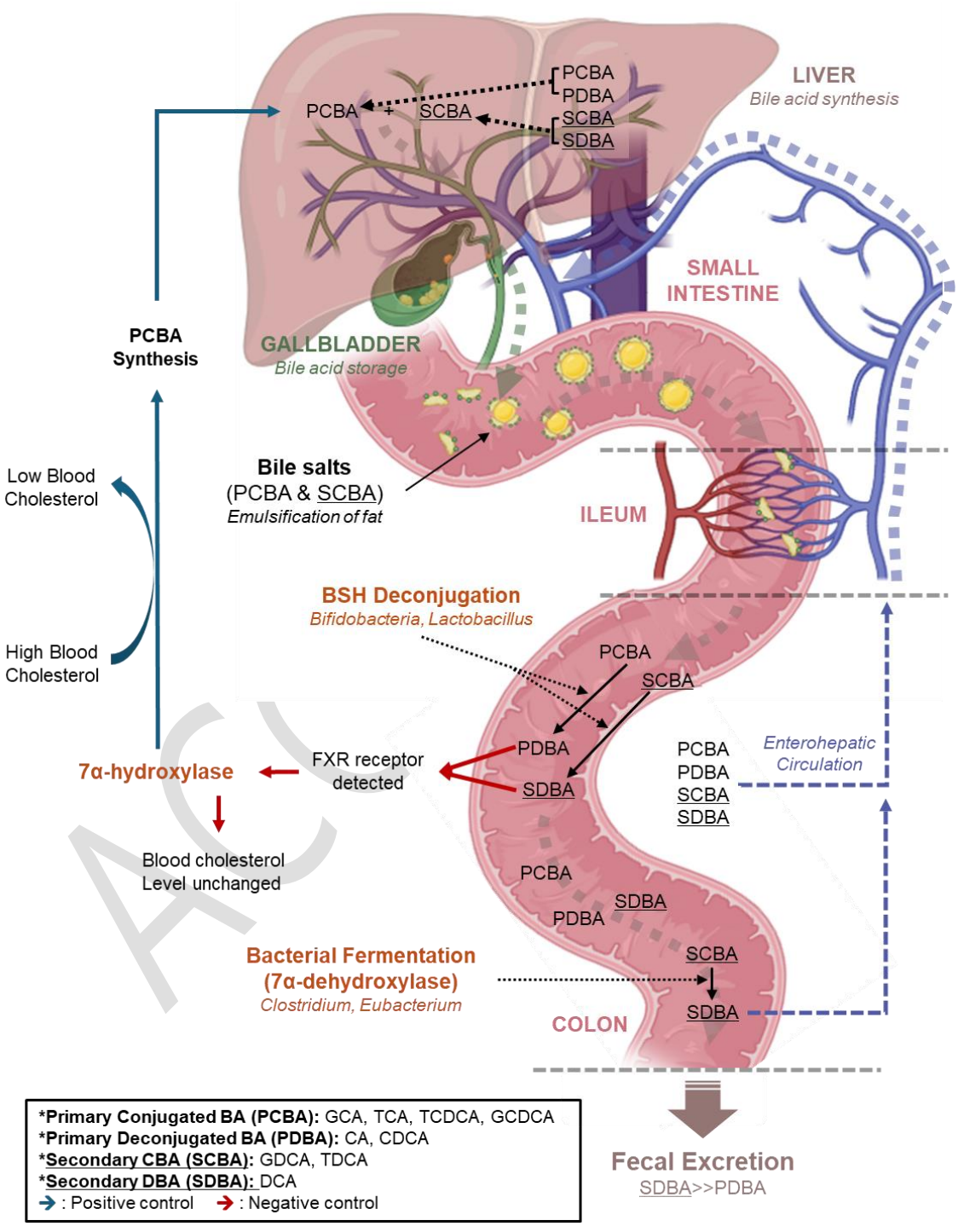
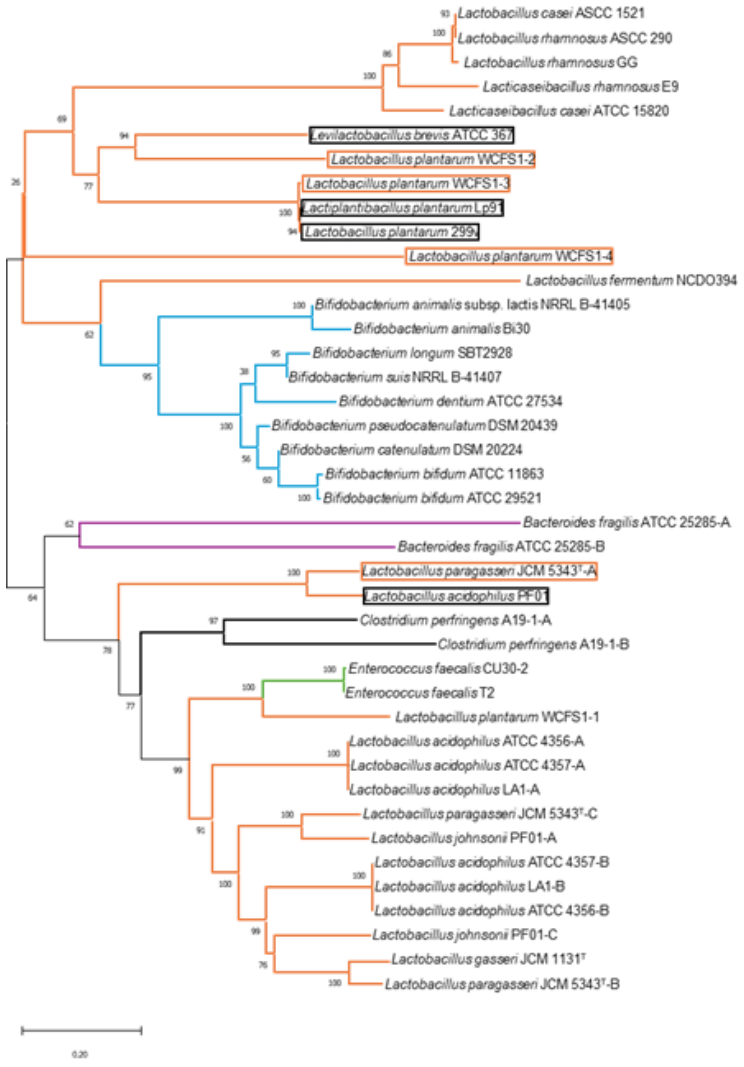
735 Tables and Figures

736 **Figure 1**. Bile salt circulation (copyright by Bio-Render[@]).





737

738 **Figure 2.** BSH phylogenetic tree of *Lactobacillus* and 29 other species based on the BSH gene.

739 The orange line represents the gene of *Lactobacillus*, the blue line represents *Bifidobacterium*,

740 the green line represents *Enterococcus*, the purple line represents *Bacteroides*, and the black line

741 represents *Closteridium*. The orange boxes indicate genes that simultaneously or preferentially

742 have PVA activity, and black boxes indicate genes from strains that are judged to be highly

743 likely also to exhibit PVA activity.

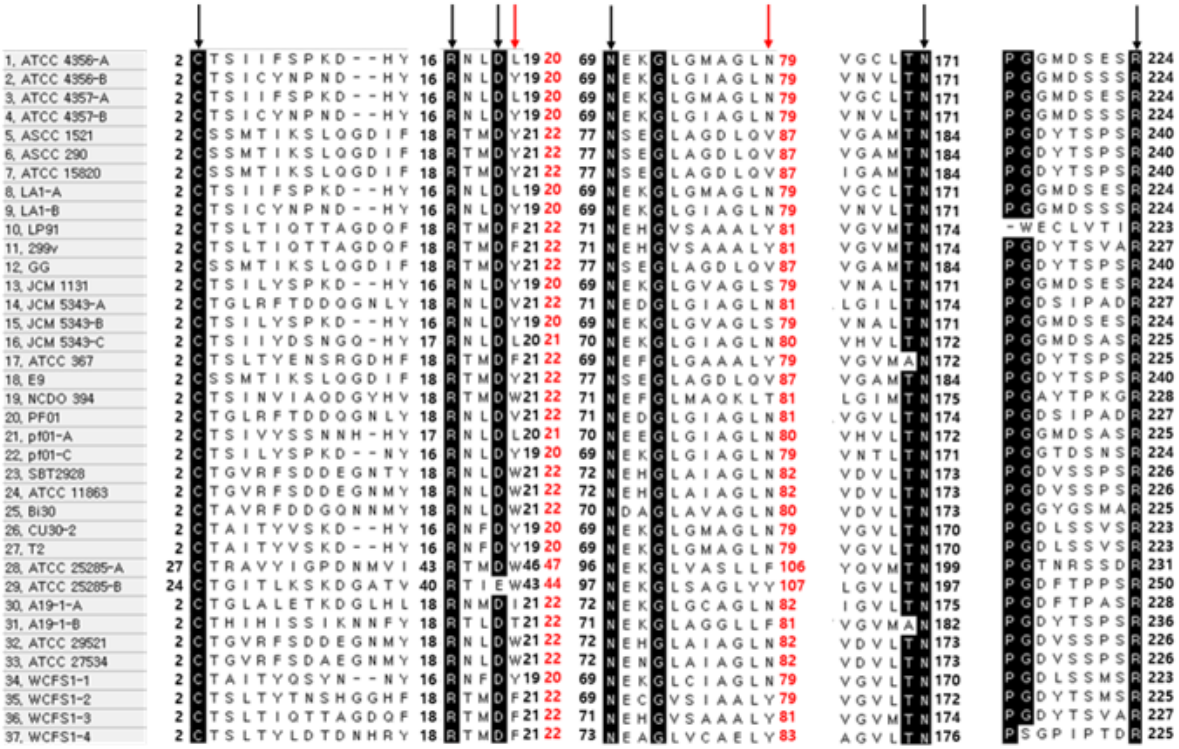


Figure 3. Comparative analysis of BSH and PVA active site.

The genes used for the phylogenetic tree were translated and aligned using the MEGA 11software. The black arrows indicate the amino acid residues involved in the active site for BSH while red arrows pertain to PVA. The black and red numbers next to each amino acid indicate the location of the residue along the length of the peptide sequence.

Table 1. Interspecies characteristics of BSH**:** *Lactobacillus, Bifidobacterium, Enterococcus, and* *Bacteroides* bile salt hydrolase information about G/T ratio by cholic acid, deoxycholic acid, and chenodeoxycholic acid affinity of BSH[1].

Strain	Q[2]	CA	DCA	CDCA	Reference
Lactobacillus					
Lb. acidophilus ATCC 4356	2	-			(Liong and Shah, 2005)
ATCC 4356-A					
ATCC 4356-B					
Lb. acidophilus ATCC 4357	2	+			(Liong and Shah, 2005)
ATCC 4357-A					
ATCC 4357-B					
Lb. casei ASCC 1521	1	-			(Liong and Shah, 2005)
Lb. rhamnosus strain ASCC 290	1	+			(Liong and Shah, 2005)
Lcb. casei ATCC 15820	1	+			(Liong and Shah, 2005)
Lb. acidophilus LA1	2	+	+		(Kumar et al., 2012)
LA1-A					
LA1-B					

754 **(continued)**

Strain	Q[2]	CA	DCA	CDCA	Reference
Lactobacillus					
Lb. fermentum K73		+	+++		(Hernández-Gómez et al., 2021)
Lb. plantarum 299v	1	+	++		(Hernández-Gómez et al., 2021)
Lb. rhamnosus GG	1	+	+++		(Hernández-Gómez et al., 2021)
Lb. johnsonii YB334		+	-		(Zhu et al., 2022)
Lpb. plantarum Y14			+ +		(Liu et al., 2021)
Lb. paracasei subsp. X11			-		(Zhang et al., 2021)
Lb. acidophilus NCK 1909					(Foley et al., 2021)
NCK 1909-A					
NCK 1909-B					
Lb. gasseri NCK2253					(Foley et al., 2021)
NCK2253-A		- - -	- - -	- - -	
NCK2253-B		+ +	+ +	+ + +	
		+	+		
Lb. paragasseri strain JCM 5343[T]	3				
JCM 5343[T]-A		- -	+	-	(Kusada et al., 2022a)

JCM 5343^{T}-B					
JCM 5343^{T}-C		-	+	-	(Kusada et al., 2022b)
Lb. salivarius NRRL B-30514		+	-	+	(Wang et al., 2012)
Lb. gasseri strain FR4		+	+		(Rani et al., 2017a)
Lev. brevis ATCC 367	1	+ +	+ +	+ + +	(Ru et al., 2019)

755

756 **(continued)**

Strain	Q[2]	CA	DCA	CDCA	Reference
Lactobacillus					
Lb. acidophilus NCDC291		+ +	+		(Kumar et al., 2012)
Lpb. plantarum Lp91	1	+	-		(Kumar et al., 2012)
Lb. plantarum WCFS1	4				(Lambert et al., 2008)
WCFS1-1		+ + +	+ + +	+ + +	
WCFS1-2		NA	NA	NA	
WCFS1-3		+	+	+	
WCFS1-4		+ + +	+ + +	+ + +	
Lcb. rhamnosus strain E9	1	+	+	+	(Kaya et al., 2017)
Lb. fermentum NCDO394	1	+	+	+	(Kumar et al., 2013)
Lb. acidophilus PF01	1	- - -	- - -	- - -	(Oh et al., 2008)
Lb. johnsonii PF01	2				(Chae et al., 2013)
PF01-A		- - -	- -	- -	
PF01-C		++	++	++	

757

758 **(continued)**

Strain	Q[2]	CA	DCA	CDCA	Reference
Bifidobacterium					
B. longum ATCC 15708		+	+	+	(Kim et al., 2004b)
B. infantis KL 412		+ +	+ +	+ +	(Kim et al., 2004b)
B. suis NRRL B-41407	1	+	+	+	(Jarocki et al., 2014)
B. pseudocatenulatum DSM 20439	1	+	+	+	(Jarocki et al., 2014)
B. animalis subsp. lactis NRRL B-41405	1	+ +			(Jarocki et al., 2014)
B. catenulatum DSM 20224	1	+			(Jarocki et al., 2014)
B. longum SBT2928		+	+ +	+	(Tanaka et al., 2000)
B. bifidum ATCC 11863	1	+	+	+	(Kim et al., 2004a)
B. animalis Bi30	1	+ +	+ +	+ +	(Jarocki, 2011)
B. longum BB536		-	+	+	(Grill et al., 1995; Li et al., 2021)
Enterococcus					
E. faecalis CU30-2	1	+ + +	+ + +	+ + +	(Eom and Kim, 2011)
E. faecalis T2	1	+	+	+ +	(Chand et al., 2016)
E. faecium CRL183			-		(Taranto et al., 2000)
Bacteroides					

B. fragilis ATCC 25285	2	+	+	+	(Li et al., 2021; Stellwag and Hylemon, 1976)
ATCC 25285-A					
ATCC 25285-B					

[1] The affinity of glycine-conjugated bile salt is greater, and the difference is less than 3 times is (+), between 3 and 10 times is (++), and more than 10 times is (+++). The same applies to taurine-conjugated bile salt, denoted with (-). Inactive BSH is denoted by NA.

[2] In addition, the number of multiple BSH genes present in the strain was reported as "Q".

764 **Table 2.** BSH gene location in *Lactobacillus, Bifidobacterium, Enterococcus, Bacteroides,*

765 *Clostridium*

Strain	Source ID[1]	Gene location	Protein ID or locus tag[2]
Lactobacillus			
Lb. acidophilus ATCC 4356	ATCC® 4356™		
Lb. acidophilus ATCC 4356-A		856359..857336	HPHOBLBD_00910
Lb. acidophilus ATCC 4356-B		1045312..1046289	HPHOBLBD_01086
Lb. acidophilus ATCC 4357	ATCC® 4357™		
Lb. acidophilus ATCC 4357-A		831210..832187	GCNKGLDF_00859
Lb. acidophilus ATCC 4357-B		1059984..1060961	GCNKGLDF_01066
Lb. casei ASCC 1521	MLKA01000007.1	129107..130123	OHF11431.1
Lb. rhamnosus ASCC 290	CP014645.1	294027..295043	AMQ02171.1
Lcb. casei ATCC 15820	ATCC® 15820™	1580898..1581914	LHLEJOBK_01553

766

(continued)

Strain	Source ID[1]	Gene location	Protein ID or locus tag[2]
Lactobacillus			
Lpb. plantarum Lp91	NZ_AXDQ00000000.1		NZ_AXDQ00000000.1
Lb. plantarum 299v	NZ_LEAV00000000.1	NZ_LEAV00000000.1	NZ_LEAV00000000.1
Lb. rhamnosus GG	FM179322.1	510599..511615	LGG_00501
Lb. gasseri JCM 1131^T	NZ_WBMG00000000.1		NZ_WBMG00000000.1
Lb. paragasseri JCM 5343^T	NZ_BEXH00000000.1		
Lb. paragasseri JCM 5343^T-A		44935..45885	GBA85885.1
Lb. paragasseri JCM 5343^T-B		328042..329019	GBA84956.1
Lb. paragasseri JCM 5343^T-C		539487..540467	GBA85403.1
Lev. brevis ATCC 367	ATCC® 367™	1918238..1919221	NFLFJFFJ_01948
Lcb. rhamnosus E9			ANQ47241.1
Lb. fermentum NCDO394	JQ293998.1		AEZ06356.1
Lb. acidophilus PF01	DI175191.1		DI175191.1
Lb. johnsonii PF01	CP024781.1		
Lb. johnsonii PF01-A		905340..906320	
Lb. johnsonii PF01-C		1090170..1091147	
Lb. acidophilus LA1	NZ_CP017062.1		
Lb. acidophilus LA1-A		869305..870282	WP_013086210.1
Lb. acidophilus LA1-B		1058266..1059243	WP_013437974.1

(continued)

Strain	Source ID[1]	Gene location	Protein ID or locus tag[2]
Lactobacillus			
Lb. plantarum WCFS1	AL935263.2		
Lb. plantarum WCFS1-1		3154512..3155486	CCC80500.1
Lb. plantarum WCFS1-2		64823..65839	CCC77632.1
Lb. plantarum WCFS1-3		2987554..2988540	CCC80350.1
Lb. plantarum WCFS1-4		2290117..2291070	CCC79725.1
Bifidobacterium			
B. suis NRRL B-41407	JQ696822.1		AFK13062.1
B. pseudocatenulatum DSM 20439	JQ696820.1		AFK13060.1
B. animalis subsp. lactis NRRL B-41405	JQ696813.1		AFK13053.1
B. catenulatum DSM 20224	JQ696817.1		AFK13057.1
B. longum SBT2928			
B. bifidum ATCC 11863	ATCC® 11863™	1014541..1015491	MKIGBIAF_00835
B. animalis Bi30		HQ845206.1	AEK27050.1
B. bifidum ATCC 29521	Orla-Jensen 29521 ™	1056525..1057475	HGNFBAPB_00881
B. dentium ATCC 27534	Scardovi and Crociani 27534 ™	1251641..1252591	FMGAMNED_01090

(continued)

Strain	Source ID[1]	Gene location	Protein ID or locus tag[2]
Enterococcus			
E. faecalis CU30-2	Lab source		
E. faecalis T2		GG692840.1	EET97240.1
Bacteroides			
B. fragilis ATCC 25285	NZ_MTGH00000000.1		
B. fragilis ATCC 25285-A		118929..119984	OOD28746.1
B. fragilis ATCC 25285-B		125353..126432	OOD24735.1
Clostridium			
C. perfringens A19-1	AP024982.1		
C. perfringens A19-1-A		634265..635254	BDA33526.1
C. perfringens A19-1-B		1315101..1316084	BDA34151.1

[1] The bacteria information source is based on Table 1 and searched against ATCC and NCBI databases. The *Source ID* starts with ATCC® is searched ATCC strain name using a search engine:(https://www.atcc.org/?matchtype=&network=x&device=c&adposition=&keyword=&gad_source=1). The *Source ID* starts with the other searched strain name using a search engine: (https://www.ncbi.nlm.nih.gov/).

[2] *Protein ID or locus tag* is the code written in ATCC and NCBI BSH protein fasta-format information.