

Research Article

Evaluation of cholesterol-related functional traits of *Bacillus infantis* 63-11 under *in vitro* simulated small intestinal conditions and genomic analysis

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Abstract - *Bacillus infantis* 63-11 is a microorganism isolated from traditional Thai fermented fish and shows potential for probiotic development. This study evaluated cholesterol-related functional traits of strain 63-11 together with genome-based taxonomic characterization and bile salt hydrolase-associated activity. Species identification was confirmed by 16S rRNA gene sequence analysis, which showed sequence similarity with reference strains of *B. infantis* ranging from 98.13–99.35%. Whole-genome analysis also supported the classification, with an average nucleotide identity (ANIb) of 96.34% and a digital DNA–DNA hybridization (dDDH) value of 86.0%, both above the commonly accepted threshold for species identification. Phylogenomic analysis further showed that strain 63-11 clusters within the *B. infantis* group. Genome annotation identified putative BSH homologs. Phenotypic testing using a taurodeoxycholic acid (TDCA) halo assay produced precipitation halos around the colonies, with an average diameter of 1.88 ± 0.48 mm, indicating the enzyme bile salt hydrolase (BSH) activity. *In vitro* analysis also showed that cholesterol levels decreased by 78.21% after 48 h of cultivation, suggesting a possible relationship with bile salt deconjugation. Under simulated small intestinal conditions containing 0.3% bile salts, strain 63-11 remained detectable during the experiment in both vegetative cells and spores, indicating adaptation to bile stress. Metabolite analysis using ¹H NMR at

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48 h detected bile-related compounds. From this study, the persistence of strain 63-11 under simulated small intestinal conditions, together with its cholesterol reduction, suggests that BSH-related processes may contribute to the observed phenotype. However, further studies, especially *in vivo* experiments, are needed to confirm these findings and clarify their biological significance.

Keywords: *Bacillus infantis*, bile salt hydrolase, cholesterol reduction, whole-genome sequencing

1. Introduction

Probiotics are defined as live microorganisms that confer health benefits on the host when administered in adequate amounts (Food and Agriculture Organization of the United Nations & World Health Organization, 2002). In recent years, interest in probiotic microorganisms has increased significantly due to their wide range of beneficial effects on human health, including modulation of gut microbiota, enhancement of immune responses, and improvement of metabolic functions (Hill et al., 2014; Sanders et al., 2019). Among these functional properties, the ability of certain probiotic bacteria to influence cholesterol metabolism has attracted considerable attention, as this function may contribute to reducing the risk of cardiovascular diseases (Lye et al., 2010; Jones et al., 2012).

Different mechanisms have been proposed to explain how probiotic bacteria may contribute to cholesterol reduction. One mechanism that has been widely discussed in the literature is the activity of the enzyme bile salt hydrolase (BSH). This enzyme catalyzes the deconjugation of bile acids, and this reaction can affect the enterohepatic circulation of bile salts. As a result, the body may need to utilize more cholesterol in order to synthesize new bile acids, which could eventually contribute to lower circulating cholesterol levels (Begley et al., 2006; Jones et al., 2012).

Apart from BSH activity, some microorganisms have also been reported to remove cholesterol from the surrounding culture medium. This process may occur through several mechanisms, including adsorption of cholesterol onto the bacterial cell surface or assimilation into the cell membrane. However, these mechanisms do not always occur at the same level in every microorganism. Their effectiveness can vary depending on the microbial species, the specific strain involved, and the experimental conditions used in each study (Lye et al., 2010).

Most previous studies investigating cholesterol-lowering probiotics have mainly focused on lactic acid bacteria and other closely related genera. In contrast, information regarding probiotic characteristics among bacteria belonging to the genus *Bacillus* is still relatively limited. Because of this, the development of new probiotic strains intended for food applications generally requires careful and comprehensive scientific evaluation. Such evaluation usually includes accurate strain identification, genome-based characterization, safety assessment, as well as experimental confirmation of functional properties that may be supported by molecular evidence.

Bacillus infantis 63-11 is a bacterial strain that was isolated from traditional Thai fermented fish. Earlier studies have suggested that this strain possesses several characteristics associated with probiotic potential. In addition, genomic analyses have indicated that the strain

may contain genetic features related to safety and possible functional benefits (Khullar, 2019; Khullar & Karami, 2024; Khullar et al., 2024). Nevertheless, studies that combine strain stability, genomic information, and functional properties related to cholesterol reduction are still relatively limited.

Therefore, the present study aimed to evaluate the cholesterol-related functional properties of *Bacillus infantis* 63-11 under simulated intestinal conditions *in vitro*. Genomic information was also examined in order to support the functional observations obtained from the experiments. By combining genomic and experimental approaches, this study may provide additional insight into the potential of this strain as a probiotic candidate for future food applications.

2. Materials and methods

2.1 Microbial strain and cultivation

Bacillus infantis 63-11 was obtained from the strain collection of the Department of Food Technology, Faculty of Science, Chulalongkorn University. The whole-genome sequence of strain 63-11 has been deposited in the NCBI GenBank database under accession number JAJBAP02. The strain was originally isolated from traditional Thai fermented fish (plara) and stored at -80°C in nutrient broth (NB) supplemented with 20% (v/v) glycerol.

Prior to experimentation, the strain was revived on Nutrient Agar (NA) plates and incubated at 37°C for 24 h. A single colony was then inoculated into NB and cultured at 37°C with shaking at 150 rpm to prepare the starter culture for subsequent experiments.

2.2 Assessment of strain stability using 16S rRNA gene analysis

To confirm species identity and assess strain stability at the rDNA level, genomic

DNA was extracted from overnight bacterial cultures using a commercial DNA extraction kit (Vivantis, Malaysia) following the manufacturer's protocol. The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) using primers 338F and 517R. The PCR reaction was prepared in a total volume of 50 μL containing $1\times$ Taq buffer, 0.1 mM dNTPs, 0.1 μM of each primer, 1.5 mM MgCl_2 , 2 U Taq DNA polymerase (Vivantis, Malaysia), and 20 ng of template DNA. PCR amplification was carried out using a thermal cycler (T100™ Thermal Cycler, Bio-Rad, Singapore).

The PCR products were checked by electrophoresis on a 2% agarose gel and then sent for Sanger sequencing. The obtained sequences were compared with reference sequences in the NCBI GenBank database using the BLAST program to confirm species identity and evaluate strain stability. In addition, the sequences were compared with previously reported 16S rRNA sequences of strain 63-11 in order to examine rDNA-level stability (Clarridge, 2004; Chun et al., 2018).

2.3 Whole-genome sequencing and bioinformatic analysis

Whole-genome analysis (WGS) was performed to characterize genomic features and confirm the taxonomic identity of the strain. The WGS data used in this study were obtained from Whole-genome sequence data of *Bacillus infantis* 63-11, which were derived from previously published data (Khullar et al., 2022). Library preparation and sequencing were performed at the Omics Science and Bioinformatics Center, Chulalongkorn University, using the Nextera XT DNA Prep Kit and the Illumina MiSeq platform. Raw read quality was assessed using FASTQC (Galaxy Version 1.1.5). Adapter sequences and low-quality reads were trimmed using Trim Galore (Galaxy

Version 0.6.3). High-quality reads were assembled de novo using Unicycler (Galaxy Version 0.4.8.0), a genome assembly tool optimized for bacterial genomes (Wick et al., 2017).

Genome annotation was performed using Prokka version 1.13 to identify coding sequences (CDS), rRNA, tRNA, and assign functional annotations (Seemann, 2014).

No additional sequencing was conducted in the present study. The previously reported genome assembly was further analyzed bioinformatically. Average Nucleotide Identity (ANI) was calculated using the JSpeciesWS web server (Version 3.8.4), and digital DNA–DNA hybridization (dDDH) values were computed using the Genome-to-Genome Distance Calculator (GGDC version 2.1) with BLAST comparison (Goris et al., 2007; Richter et al., 2016). Species delineation thresholds of ANI >95% and dDDH >70% were applied (Kim et al., 2014).

Circular genome maps were generated using CGView Server Beta (Grant & Stothard, 2008), and additional visualization was performed using Proksee to display the distribution of bile salt hydrolase (BSH) genes on the chromosome.

Phylogenomic relationships between strain 63-11 and related *Bacillus* species were analyzed using the Type (Strain) Genome Server (TYGS v307) (Meier-Kolthoff & Göker, 2019).

2.4 Genome annotation and identification of bile salt hydrolase (BSH) genes

Genome annotation results from Prokka and BV-BRC were reviewed to identify genes associated with bile salt hydrolase (BSH). Candidate genes were screened based on functional annotation and conserved domains corresponding to cholestyglycine hydrolase (Begley et al.,

2006). The chromosomal positions of these genes were displayed using circular genome maps to assess their distribution.

2.5 Bile salt hydrolase (BSH) activity assay

Bile salt hydrolase (BSH) activity was evaluated to determine the functional capability of the strain to deconjugate bile salts. The strain was cultured on Nutrient Agar (NA; HiMedia, India) supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA) and 0.037% (w/v) CaCl₂. Plates were incubated (Memmert, India) at 37 °C for 72 h.

Following incubation, the formation of a white precipitation halo around the colonies was assessed. The halo was formed after deconjugation of bile salts, producing free bile acids that precipitated with Ca²⁺. The diameter of the halo was measured using a vernier caliper or a millimeter-scale ruler. At least four independent colonies were analyzed, and results were expressed as mean ± standard deviation (SD) (Begley et al., 2006; Shehata et al., 2016).

2.6 *In vitro* cholesterol reduction assay

The cholesterol-lowering ability of the strain was assessed using an *in vitro* assay under controlled conditions. Vegetative cells were prepared to a final concentration of approximately 7–8 log CFU/mL. Two experimental conditions were established: (1) medium supplemented with bile salts and (2) medium without bile salts. Cholesterol (Loba Chemie™ / India) was added to all tubes at a final concentration of 1% (w/v), and 1% (v/v) inoculum was introduced prior to incubation at 37 °C for 48 h.

After incubation, cultures were centrifuged to separate cells from the supernatant. Cholesterol in the supernatant was quantified using the o-phthalaldehyde (OPA) colorimetric assay.

Briefly, 0.5 mL supernatant was mixed with 0.5 mL 33% (w/v) potassium hydroxide (Ajax Finechem, New Zealand) and 1.0 mL 99% ethanol (analytical grade), vortexed for 1 min, and incubated at 37 °C for 15 min for saponification. Samples were cooled to room temperature, followed by addition of 1.5 mL hexane (RCI Labscan, Thailand) and 1.0 mL distilled water. After vortexing and phase separation, 0.5 mL of the upper hexane layer was transferred to a new tube and evaporated to dryness.

The residue was dissolved in 1.0 mL OPA solution (Loba Chemie™ / India) (50 mg/L in acetic acid), followed by addition of 0.25 mL concentrated sulfuric acid. After 20 min incubation at room temperature for color development, absorbance was measured at 545 nm using a microplate spectrophotometer, following the method described by [Lye et al. \(2010\)](#).

Cholesterol reduction (%) was calculated as:

$$\text{Cholesterol reduction (\%)} = (\text{OD}_{\text{initial}} - \text{OD}_{\text{final}}) / \text{OD}_{\text{initial}} \times 100$$

Experiments were performed in at least duplicate, and results were expressed as mean $\pm$ SD.

2.7 Simulated small intestinal conditions and growth analysis

To investigate bacterial survival and physiological responses under intestinal-like conditions, the strain was cultured in NB (HiMedia, India) supplemented with sodium bicarbonate to maintain pH 6.5–7.5 and bile salts (HiMedia, India) at a final concentration of 0.3% (w/v). The initial cell concentration was adjusted to approximately 10^8 CFU/mL.

Cultures were incubated at 37 °C with shaking at 150 rpm for 120 h. Samples were collected at 0, 24, 48, 72, 96, and 120 h for determination of total viable counts (TPC) and spore counts.

TPC were determined by serial dilution in sterile saline or Phosphate Buffered Saline (PBS), followed by spread plating on NA (HiMedia, India) and incubation at 37 °C for 24 h. Results were expressed as log CFU/mL.

Spore counts were determined by heat treatment of samples at 80 °C for 10 min to eliminate vegetative cells, followed by cooling, serial dilution, and plating under the same conditions. Results were also expressed as log CFU/mL.

Samples collected at 48 h were used for cholesterol, lactate, and metabolite analyses, as this time point corresponded to high vegetative cell counts with the onset of sporulation, enabling correlation between growth phase and functional properties.

2.8 Metabolite analysis by ^1H NMR

Metabolite profiling was performed to identify compounds associated with bile metabolism under simulated intestinal conditions. Supernatant collected at 48 h was mixed with phosphate buffer (pH 6.0) at a 1:1 (v/v) ratio. The buffer contained 300 mM KH_2PO_4 and 10% (w/w) D_2O for field-frequency locking. The internal standard 3-(trimethylsilyl) propionic-2,2,3,3- d_4 acid sodium salt (TSP) was added to a final concentration of 1 mM for chemical shift referencing. A total of 400 μL of the mixture was transferred into 5 mm NMR tubes.

Spectra were acquired using a 600 MHz ^1H NMR spectrometer. Spectral data were processed using MestReNova for phase and baseline correction and signal integration. Metabolite identification was performed using Chenomx NMR Suite by matching spectral data with reference libraries, following standard metabolomics annotation guidelines ([Wishart et al., 2018](#)).

Metabolite identification in this study was qualitative and used to support

mechanistic interpretation of bile-associated metabolism at the same functional time point.

2.9 Statistical analysis

All experiments were conducted with at least duplicate measurements as specified for each assay. Data are expressed as mean ± standard deviation (SD). Descriptive statistical analysis was performed using Microsoft Excel.

3. Results and discussion

3.1 Genetic stability and taxonomic confirmation of *Bacillus infantis* 63-11

Sequence analysis of the 16S rRNA gene showed identity values ranging from

98.13% to 99.35% relative to *Bacillus infantis* entries in GenBank, as summarized in Table 1. The highest identity (99.35%) was observed within the 338F-amplified region, while the 517R region showed an identity of 98.13%. When compared with the previously reported value of 98.14% for the same strain (Khullar et al., 2022), as presented in Table 1, the present study produced nearly identical results, differing by only 0.01% in directly comparable regions. Such minor variation is taxonomically insignificant and is most likely due to differences in amplified fragment length or primer-binding regions (Clarridge, 2004; Chun et al., 2018).

Table 1. Comparison of 16S rRNA gene sequence identity of *Bacillus infantis* 63-11 between the previous study and the present study.

Study	Target Region	Closest Species (GenBank)	Identity (%)
Khullar et al. (2022)	517R	<i>Bacillus infantis</i>	98.14
Present study	517R	<i>Bacillus infantis</i>	98.13
Present study	338F	<i>Bacillus infantis</i> (MG651268.1)*	99.35
Present study	338F	<i>Bacillus infantis</i> (OL519495.1)*	99.35

Note: * Accession numbers and percentage sequence identity of the closest relatives obtained by BLAST search (NCBI GenBank database; <https://www.ncbi.nlm.nih.gov/>).

Although 16S rRNA gene sequencing is widely used for preliminary identification, its resolution is limited among closely related *Bacillus* taxa. For this reason, whole-genome-based metrics were used to provide clear species-level confirmation.

Whole-genome comparisons showed that strain 63-11 shared an ANI_b of 96.34% and dDDH of 86.0% with *Bacillus infantis* ACN21-1, exceeding accepted species delineation thresholds (ANI ≥95–96%

and dDDH ≥70%) (Kim et al., 2014; Chun et al., 2018). Detailed genomic indices are shown in Table 2. Phylogenomic analysis using TYGS placed strain 63-11 within the *B. infantis* clade with high bootstrap support, as illustrated in Figure 1. In contrast, comparisons with *Bacillus subtilis* and *Bacillus fonticola* produced ANI values below 70% and dDDH values below 15% (Table 2), confirming clear taxonomic separation.

Table 2. Comparative genomic indices (dDDH and ANIb) of strain 63-11 against reference genomes.

Reference genome	dDDH (%)	ANIb (%)	Interpretation (based on thresholds)
<i>Bacillus infantis</i> ACN21-1	86.0	96.34	Same species (ANI ≥95% and dDDH ≥70%)
<i>Bacillus infantis</i> ACN21-9	76.1	91.30	High relatedness; meets dDDH but below ANI threshold
<i>Bacillus infantis</i> NRRL B-14911	76.4	91.28	High relatedness; meets dDDH but below ANI threshold
<i>Bacillus infantis</i> AR001	79.8	92.55	High relatedness; meets dDDH but below ANI threshold
<i>Bacillus fonticola</i> CS13	12.9	67.22	Different species
<i>Bacillus subtilis</i> subsp. <i>subtilis</i> 168	12.9	67.99	Different species

Collectively, the genomic indices presented in Table 2 and the phylogenomic clustering shown in Figure 1 provide robust species-level confirmation and support the consistent taxonomic assignment of strain 63-11 across the datasets examined. Compared with 16S rRNA gene sequencing, which relies on a single conserved locus and may lack sufficient resolution to discriminate closely related *Bacillus* species, whole-genome-based approaches such as ANI and dDDH offer significantly higher

discriminatory power by integrating genome-wide sequence similarity. These methods enable more accurate and reliable taxonomic classification, particularly among closely related taxa with highly conserved 16S rRNA sequences (Chun et al., 2018). Such taxonomic consistency is an important prerequisite for probiotic development, as functional traits are generally strain-specific and should be supported by reliable strain identification (Sanders et al., 2019)

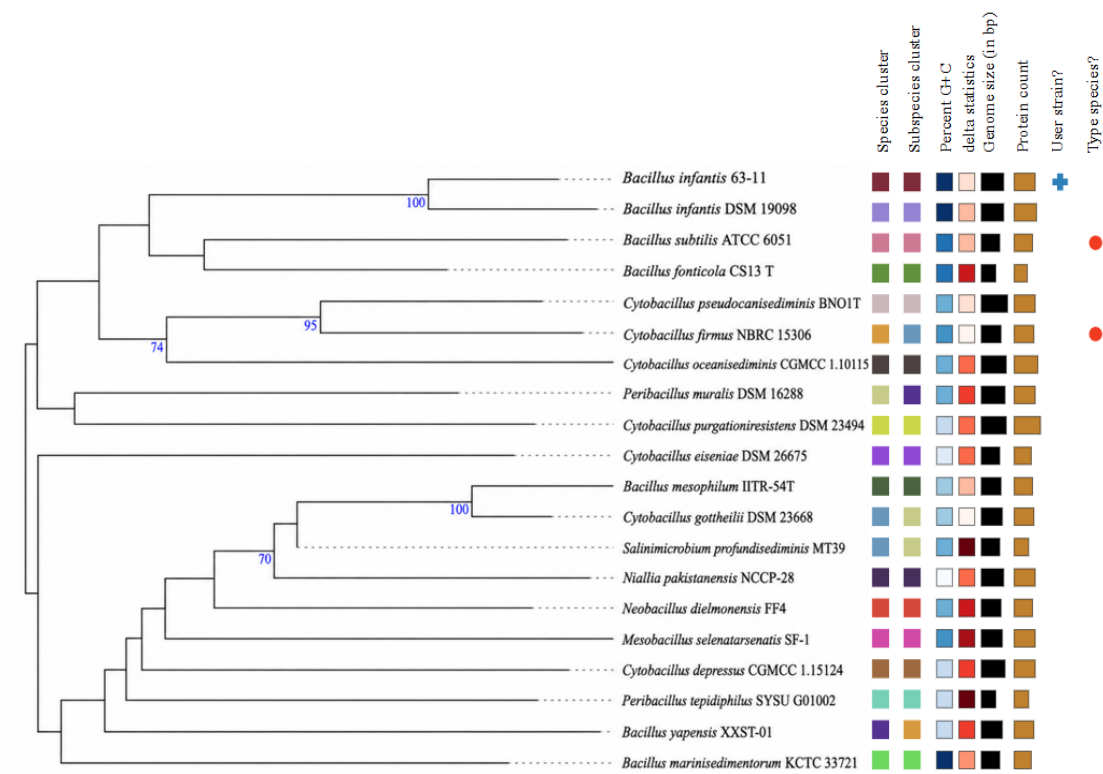


Figure 1. Phylogenomic tree inferred from whole-genome sequence comparison, illustrating the taxonomic positioning of *Bacillus infantis* 63-11 with their closely related reference strains.

3.2 Genomic identification of bile salt hydrolase (BSH) homologs

Genome annotation revealed several putative bile salt hydrolase (*bsh*) homologs, including *bshA*, *bshB1*, *bshB2*, and *bshC*. These genes were located at different

positions on the chromosome, as shown in Figure 2. The presence of multiple putative *bsh* homologs may suggest functional redundancy or differences in regulation under bile stress conditions.

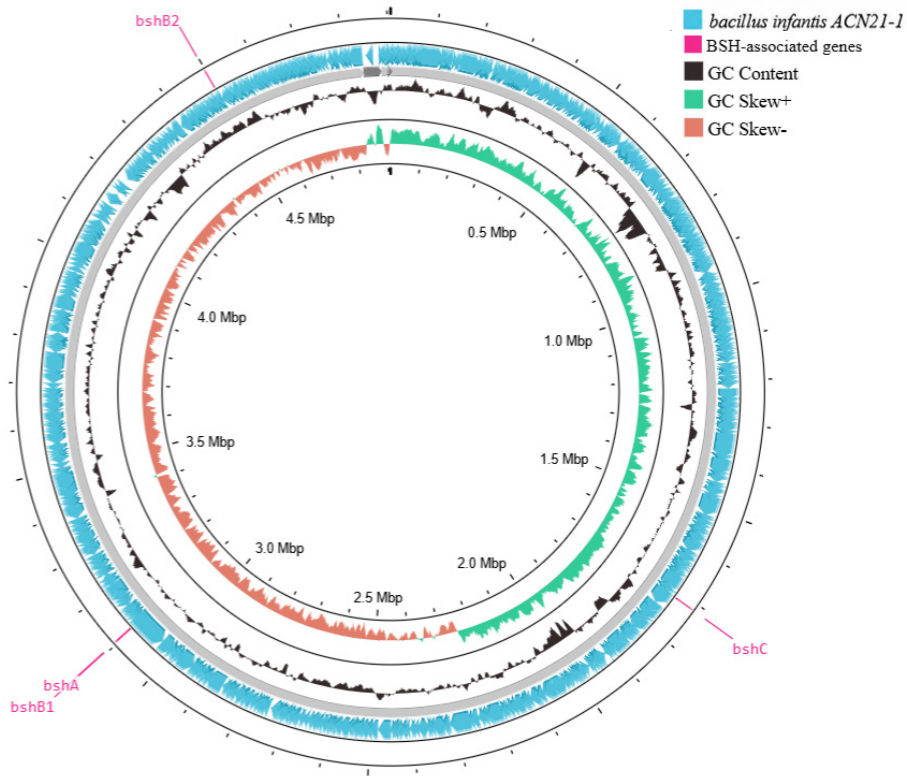


Figure 2. Circular genome map illustrating the chromosomal distribution of bile salt hydrolase (BSH) homologs in *Bacillus infantis* 63-11.

BSH enzymes belong to the N-terminal nucleophilic hydrolase (Ntn-hydrolase) superfamily and catalyze the hydrolysis of the amide bond between conjugated bile acids and their amino acid moieties (glycine or taurine) (Begley et al., 2006; Jones et al., 2012). This reaction yields unconjugated bile acids and free amino acids. Deconjugation alters bile acid physicochemical properties, including increased pKa and reduced solubility, which may decrease reabsorption efficiency in the intestine and promote bile acid loss (Begley et al., 2006). These biochemical changes have downstream implications for host cholesterol metabolism via the bile acid cholesterol regulatory axis (Ooi & Liong, 2010).

Figure 2 shows that strain 63-11 contains several putative *bsh* homologs in

its genome. These genes belong to the bile salt hydrolase family, which is involved in bile salt deconjugation. This process is associated with bile acid metabolism and may be relevant to cholesterol homeostasis in the host.

BSH activity has been widely reported in lactic acid bacteria. Similar genes have also been found in several species of the genus *Bacillus*. Genome analyses from previous studies have detected *bsh* homologs in different *Bacillus* strains. In probiotic *Bacillus*, BSH activity has been linked with bile tolerance and the ability to survive in the gastrointestinal tract. This may help bacteria remain active under bile stress conditions in the intestine (Begley et al., 2006). The presence of several putative *bsh* homologs in strain 63-11 is consistent with these observations.

3.3 Phenotypic expression of BSH activity

BSH activity was evaluated using a TDCA-supplemented agar assay. Under these conditions, strain 63-11 produced a visible precipitation halo with a mean diameter of 1.88 ± 0.48 mm ($n = 4$) (Table 3). The formation of the white precipitate is associated with BSH-mediated deconjugation of TDCA, which generates deoxycholic acid that subsequently precipitates in the presence of calcium ions (Begley et al., 2006).

Although halo size provides only a semi-quantitative indication of enzymatic activity, the consistent appearance of precipitation zones suggests that strain 63-11 expresses BSH under the experimental conditions used. This observation generally agrees with the genomic findings described above, where several *bsh* homologs were detected in the chromosome (Figure 2).

To further explore the functional implications of this activity, an *in vitro* cholesterol reduction assay was conducted. After 48 h of incubation, strain 63-11 reduced cholesterol levels in the culture system by 78.21 ± 1.29 % (Table 3). This reduction was relatively high under the conditions tested when compared with values reported for several probiotic strains in previous studies. (Lye et al., 2010; Jones et al., 2012).

One possible explanation is that BSH-associated activity may have altered bile salt chemistry in the culture system. Deconjugation of bile salts can decrease their solubility and may interfere with micelle formation, which in turn affects cholesterol solubilization in the medium. In addition, unconjugated bile acids may interact with cholesterol molecules and promote co-precipitation. These processes may have contributed to the observed decrease in cholesterol concentration.

From a physiological perspective, microbial BSH activity has also been linked to host regulatory pathways

associated with the bile acid–FXR–CYP7A1 axis. Bile acids act as signaling molecules through the farnesoid X receptor (FXR), which regulates the expression of cholesterol 7 α -hydroxylase (CYP7A1), the key enzyme involved in bile acid synthesis from cholesterol. Reduced intestinal bile acid reabsorption may weaken FXR signaling and consequently relieve repression of CYP7A1, thereby promoting the conversion of cholesterol into bile acids in the liver (Begley et al., 2006; Ooi & Liong, 2010; Chiang, 2013; Li & Chiang, 2014).

It should be noted, however, that such host-level regulatory mechanisms cannot be directly assessed in an *in vitro* system. In the present study, microbial BSH-associated activity can only be considered a possible upstream factor that may be related to this pathway.

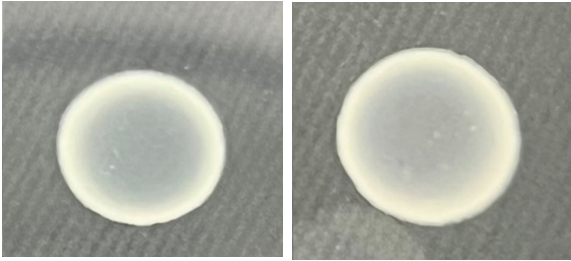
Another limitation of the experiment is that the cholesterol reduction assay was performed with limited replication ($n = 2$). The results should therefore be interpreted cautiously and are presented mainly to illustrate the functional tendency of the strain. Further experiments with a larger number of replicates would be necessary to confirm the reproducibility of this effect.

Reported cholesterol removal by probiotic microorganisms varies considerably depending on experimental design, bile salt concentration, and growth conditions. Under comparable laboratory settings, values typically fall within the range of 20–70% (Lye et al., 2010; Jones et al., 2012). For example, previously reported *Bacillus* spp., including *Bacillus coagulans* MTCC 5856, showed cholesterol removal capacities ranging from approximately 12.82–70.89%, with *B. coagulans* reducing cholesterol by 48.42% under growing conditions after 24 h. (Majeed et al., 2019; He et al., 2021). The relatively high

reduction observed for strain 63-11 under bile-supplemented conditions may suggest adaptation to bile-associated

conditions, although direct comparisons across studies should be made cautiously because of methodological differences.

Table 3. Bile salt hydrolase (BSH) activity and *in vitro* cholesterol reduction capacity of *Bacillus infantis* 63-11.

Bile salt hydrolase (BSH) activity (mm)		Cholesterol reduction (%)
 1.88 ± 0.48		78.21 ± 1.29

3.4 Simulated small intestinal conditions and growth dynamics

As illustrated in Figure 3a, under standard NB conditions, the total viable counts (TPC) gradually increased throughout the 120 h incubation period. The population eventually reached 13.54 log CFU/mL at 120 h. Spore counts followed a similar pattern and increased to 13.65 log CFU/mL at the same time point. The simultaneous increase in vegetative cells and spores under nutrient-rich conditions likely reflects normal growth behavior of *Bacillus*, where active proliferation is followed by sporulation during the later growth stages. Accordingly, the NB condition was used as a baseline reference for the growth capacity and sporulation tendency of strain 63-11 in the absence of bile-associated stress.

A different growth pattern was observed under simulated small intestinal conditions containing 0.3% bile salts (pH 6.5–7.5), as shown in Figure 3b. In this environment, TPC increased during the early incubation period and reached its highest level between 48 and 72 h, after which a gradual decline became evident

after 96 h. Spore counts increased markedly during the same period and then decreased toward the end of incubation, reaching levels close to those of vegetative cells at 120 h. When compared with the continuous increase observed in NB medium (Figure 3a), the altered growth trend in the simulated intestinal system (Figure 3b) suggests that bile salts imposed measurable physiological stress on the cells.

The contrast between the two growth profiles highlights the influence of bile salts on bacterial physiology. Bile salts are known to exert detergent-like effects on cellular membranes, which can disrupt membrane integrity and interfere with metabolic processes. Exposure to bile may also induce oxidative stress and broader metabolic imbalance. In response, *Bacillus* species typically activate adaptive strategies such as sporulation, modification of the cell envelope, and adjustments in central metabolism (Nicholson et al., 2000; Setlow, 2014; Merritt & Donaldson, 2009). Despite bile-associated stress, both vegetative cells and spores remained detectable throughout the incubation

period under simulated intestinal conditions (Figure 3b). When considered together with the baseline growth pattern observed in NB medium (Figure 3a), this persistence suggests a certain degree of physiological robustness. This persistence may be relevant to survival during gastrointestinal passage, which is often considered an important characteristic for spore-forming probiotic candidates.

The 48-h time point was selected for metabolomic analysis because it corresponded to a metabolically active growth stage. At this stage, vegetative cells were still present while stress responses associated with bile exposure were beginning to emerge. Consequently, this time point was considered suitable for capturing metabolic signatures related to bile interaction. The metabolomic results obtained at this stage are presented in Figure 4 and Table 5.

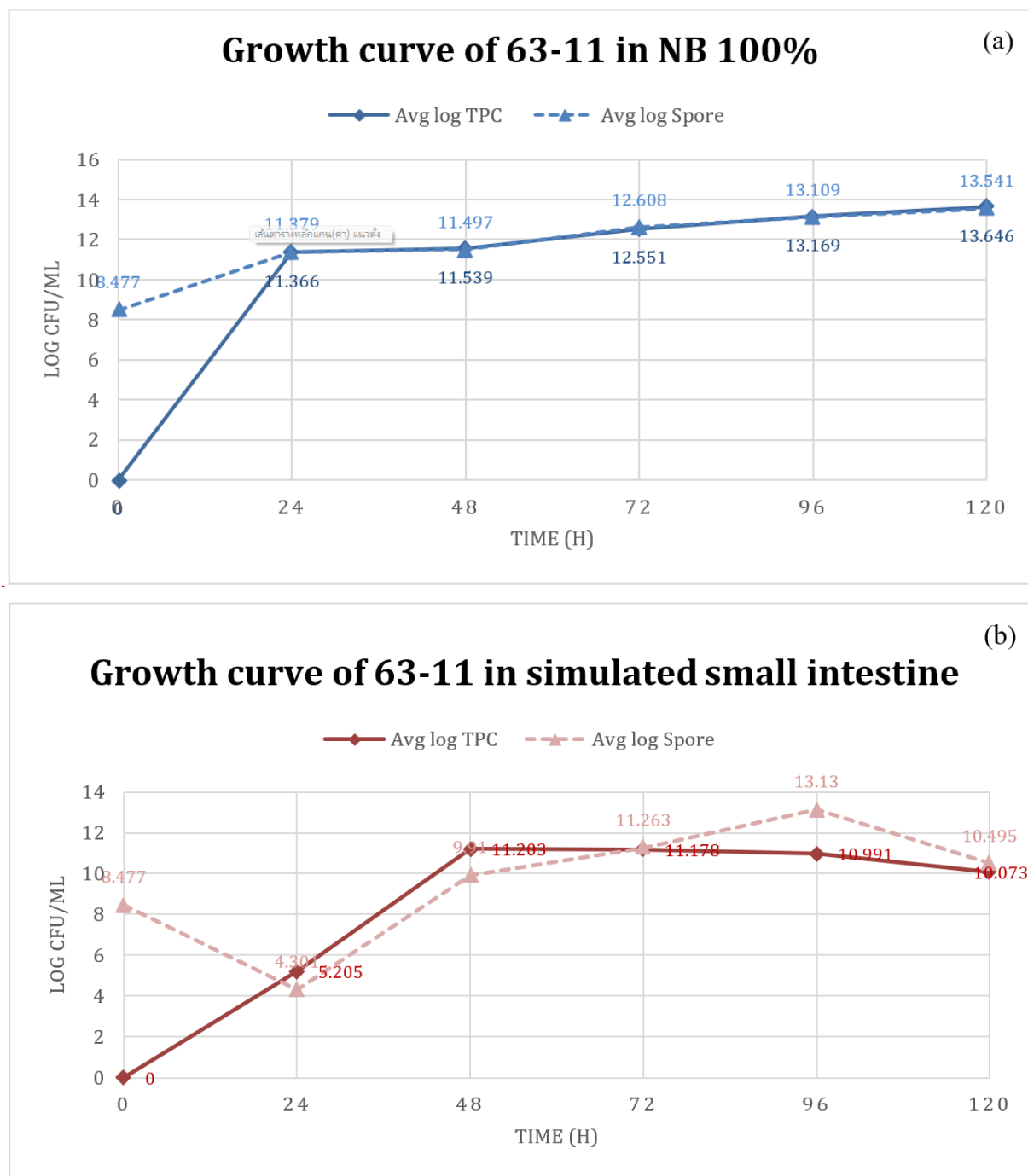


Figure 3. Growth profiles of total viable counts (TPC) and spore counts of *Bacillus infantis* 63-11 over 120 h under (a) standard nutrient broth (NB) conditions and (b) simulated small intestinal conditions. Values are expressed as mean $\pm$ standard deviation (SD).

3.5 ^1H NMR metabolite profiling as supportive mechanistic evidence

Metabolite profiling was performed using ^1H NMR spectroscopy on culture supernatants collected at 48 h under simulated intestinal conditions. The representative spectrum is presented in Figure 4, while the corresponding metabolite assignments are summarized in Table 5.

Several classes of metabolites were detected in the supernatant, including organic acids (lactate and acetate), amino acids, and bile-associated compounds such as glycocholate, cholate, and glycine.

The simultaneous detection of free glycine together with cholate is compatible with cleavage of conjugated bile acids through hydrolysis of the amide bond. This observation is therefore consistent with the proposed involvement of bile salt hydrolase (BSH)-related activity, which releases glycine or taurine from conjugated bile acids (Begley et al., 2006). The metabolite profile detected here is also consistent with the genomic detection of putative *bsh* homologs and the positive TDCA hydrolysis phenotype observed for strain 63-11.

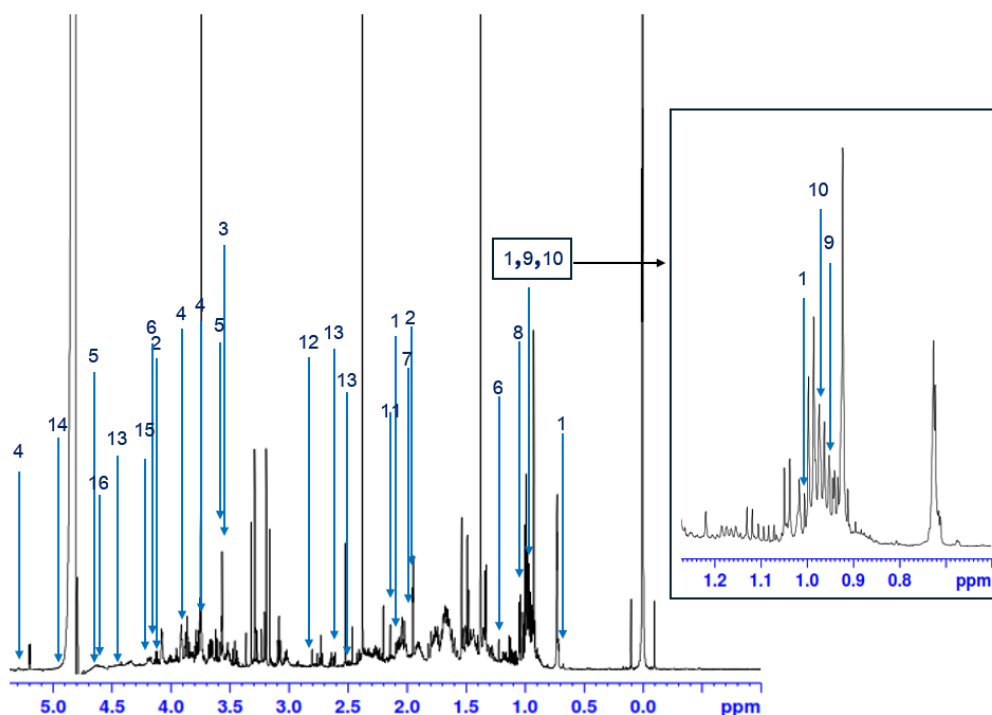


Figure 4. ^1H NMR spectrum (600 MHz) of metabolites detected in the supernatant of *Bacillus infantis* 63-11 under simulated small intestinal conditions. The numbered peaks correspond to the identified metabolites listed in Table 5.

Among the detected metabolites, lactate appeared as one of the dominant signals in the spectrum shown in Figure 4, with chemical shift values listed in Table 5. The presence of lactate generally reflects active glycolytic metabolism and

the regeneration of NAD^+ during vegetative growth. Under bile-associated stress, the production of organic acids may also contribute to intracellular redox balance and overall stress adaptation (Papadimitriou et al., 2016).

When considered together, the genomic evidence (putative *bsh* loci), halo formation in the TDCA assay, metabolomic signals, and the cholesterol assay provide multi-level support for the possible involvement of BSH-related processes in strain 63-11. In this study, ¹H NMR analysis provided qualitative insight into the metabolic environment and allowed detection of metabolites associated with bile-related transformation.

However, several limitations should be acknowledged. The present NMR analysis was qualitative rather than quantitative, and a control sample was not included in the comparison. As a result, interpretation of metabolic changes must remain cautious. According to commonly accepted metabolomics reporting standards, metabolite annotation based solely on spectral matching, as performed in this study (Table 5), corresponds to putative identification (Level 2 confidence). Definitive structural confirmation generally requires

validation using authentic standards or complementary analytical techniques (Sumner et al., 2007; Wishart et al., 2018). Therefore, the metabolomic observations reported here should be interpreted as supportive mechanistic evidence rather than definitive proof of bile acid transformation.

When considered together, the detection of *bsh* genes at the genomic level, the phenotypic expression of BSH activity observed in the TDCA halo assay, and the identification of bile-related metabolites such as glycine and cholate from metabolomic analysis demonstrate consistency across multiple levels of evidence, including genomic, phenotypic, and metabolomic data. These findings support the potential involvement of BSH-related processes in strain 63-11. However, within the scope of the present *in vitro* study, the underlying mechanism of cholesterol reduction cannot be conclusively determined.

Table 5. Chemical shift values (δ, ppm) and metabolites identified by ¹H NMR analysis in the fermentation supernatant of *Bacillus infantis* 63-11 under simulated small intestinal conditions (48 h).

Metabolite Group	No.	Metabolite	¹ H Chemical shift (δ, ppm)
Bile acids and BSH-related compounds	1	Glycocholate	2.11, 1.01, 0.73
	2	Cholate	4.13, 1.97
	3	Glycine	3.57
Carbohydrates and sugar derivatives	4	Glucose	3.75, 3.90, 5.23
	5	1,6-Anhydro-β-D-glucose	3.55, 4.65
	6	Lactate	1.33, 4.15
	7	Acetate	1.92
Amino acids	8	Valine	1.05
	9	Isoleucine	0.95
	10	Leucine	0.97
	11	Methionine	2.13
	12	Aspartate	2.83
Vitamins and cofactors	13	Riboflavin (Vitamin B2)	2.51, 2.61, 4.45
	14	1-Methylnicotinamide	4.49
	15	Nicotinic acid adenine dinucleotide	4.31
Antioxidant compounds	16	Glutathione	4.61

4. Conclusions

In this study, *Bacillus infantis* 63-11 was consistently identified at the species level, with 16S rRNA gene sequence identity ranging from 98.13% to 99.35% and whole-genome analysis showing ANiB of 96.34% and dDDH of 86.0%, confirming its taxonomic classification. Genome annotation revealed the presence of multiple putative bile salt hydrolase (BSH) homologs, including *bshA*, *bshB1*, *bshB2*, and *bshC*. Phenotypically, the strain exhibited BSH activity, producing a precipitation halo with a mean diameter of 1.88 ± 0.48 mm, and demonstrated a cholesterol reduction capacity of $78.21 \pm 1.29\%$ under *in vitro* conditions. Under simulated small intestinal conditions, the strain maintained detectable levels of both vegetative cells and spores throughout 120 h, indicating tolerance to bile-associated stress. Metabolite profiling by ^1H NMR at 48 h further detected bile-related compounds, including glycocholate, cholate, and glycine, together with dominant organic acids such as lactate, supporting active metabolism and possible bile salt transformation. Collectively, these findings provide multi-level evidence, including genomic, phenotypic, and metabolomic data, supporting the potential involvement of BSH-related processes and cholesterol-associated functional properties in strain 63-11.

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AI disclosure statement

The authors used AI-assisted tools for language editing and translation during

the preparation of this manuscript. The AI tools were used solely to improve the clarity and readability of the text. No AI tools were used to generate scientific content, data, analyses, or conclusions. All content has been reviewed and approved by the authors, who take full responsibility for the integrity and accuracy of the manuscript.

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