Research Article

Evaluation of Anti-Foodborne Bacterial Activity, Digestive Enzyme Secretion, and Antimicrobial Resistant Genes as Probiotic Strains Selection for Industrial Interest

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Abstract
Beneficial microbes, such as probiotic bacteria, are increasingly in demand in the food and feed industry. Lactic acid bacteria and bifidobacteria are commonly used as commercial probiotics, only a few species have been isolated from Southeast Asia areas. This study employed criteria including antimicrobial activity, the release of digestive enzymes, and the absence of antibiotic-resistant (AMR) genes to screen potential local isolates. The results revealed that 4 out of 16 isolates met these criteria, displaying anti-foodborne bacterial activities and a lack of fifty-one tested AMR genes. Furthermore, the four selected isolates demonstrated the production of extracellular digestive enzymes, including amylase, lipase, protease, β-glucanase, and cellulase, with enzyme indices ranging from 1.09–1.31. Among these isolates, two potential probiotics were identified as *Bifidobacterium animalis* subsp. *lactis* (strain H9-01) and *Lactobacillus reuteri* (strain P4-S03). Importantly, both species are approved for use as food and feed supplements in accordance with Thai regulations. This research outlines an approach for screening potential probiotics for industrial-scale applications.

Keywords: Antimicrobial activity, Antimicrobial resistant gene, Bifidobacteria, Digestive enzyme, Lactobacilli, Probiotics

1 Introduction

Probiotics, which are beneficial microorganisms, have gained popularity as food supplements and pharmaceutical products. The global probiotic product market has experienced significant growth, more than 8% per annum. Its value reached 58.1$ billion in 2020 and escalated to 68.56$ billion in 2022 [1]. This value

was estimated to reach 84.5$ billion in 2026, with the largest share anticipated in the Asia Pacific region [1]. In the case of Thailand, the probiotics market is predicted to reach 6.4฿ billion in 2026, with the animal sector accounting for the majority of this share [2]. Because probiotics are recognized as alternatives to antibiotic growth promoters. When supplementing probiotics in animal feed, the main priorities are maintaining and enhancing productivity and growth, as well as preventing and balancing enteric pathogens [3]. Most probiotic products originate from European, Korean, or Japanese companies. Thus, there is a need to develop locally isolated strains to suit the ASEAN region's population. Despite the growing number of probiotic products being developed for animal nutrition to reduce antibiotic use in farm animals, only a limited number of local isolates are available. These genera include *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Streptococcus* [4]–[6] The investigation of novel strains with probiotic properties should adhere to the guidelines for evaluating probiotics in food by FAO/WHO [7]. However, in order to commercialize the product, the properties need to meet the requirements of the standard regulations. For instance, the regulation for the use of probiotics as food announced by the Ministry of Public Health, Thailand acquired the basic criteria of strain identification, resistance to gastric acidity and bile salt, and adherence to mucus [8]. In addition, the safety criteria that need to be performed are non-haemolytic activity and antibiotic-resistant testing [8]. The latter one is usually conducted by using antibiotic discs placed on the lawn of test probiotic bacteria [4], [9]–[11]. Another literature investigated further the auto-aggregation, coaggregation with the pathogen, cell surface hydrophobicity, and heat resistance [10], [12]. Furthermore, *in vitro* analyses for gamma amino butyric acid, bile salt hydrolase, and beta-galactosidase were also reported to express functional benefit for human health [13]. For an industrial aspect, technological criteria must be tested for the benefits of food processing and large-scale production, such as O_2 and heat tolerance. While various probiotic bacteria are well-regarded for thriving in the GI tract and producing health-promoting metabolites, they may not be conducive to industrial-scale development, leading to limited applications [14].

Antimicrobial activity is a crucial criterion in *in vitro* testing for selecting potential probiotics. Some probiotics can produce antimicrobial compounds that can suppress the growth of pathogenic bacteria within the intestine. Notably, probiotic bacteria such as lactic acid bacteria (LAB), *Bifidobacterium*, and *Bacillus* can produce bacteriocins [15]–[17], which have an inhibitory effect on various foodborne pathogens, including *Bacillus*, *Escherichia*, *Enterococcus*, *Listeria*, *Salmonella*, and *Staphylococcus*. Furthermore, many probiotics within the LAB and *Bifidobacterium* groups produce organic acids, such as lactic and acetic acids, which lower pH levels and employ an inhibitory mechanism against certain pathogens [9], [18]. Another concern is the presence of antibiotic-resistant genes, as the transfer of such genes from probiotics to other potentially pathogenic microorganisms is a theoretical risk associated with using probiotics in animal feed. Several species of *Lactobacillus*, *Bacillus*, and *Enterococcus* possess antibiotic-resistance genes that can be transferred, whereas *Bifidobacterium* carries non-transferable antibiotic-resistance genes [19]–[21]. Additionally, the production of digestive enzymes is another important criterion for promoting animal health [22], [23] and fewer studies have focused on this criterion for probiotics selection in *Lactobacillus* specifically in *Bifidobacterium* genus.

Therefore, this study aims to select probiotics in alignment with the industry's most interests. The sixteen potential probiotic strains were evaluated for basic probiotic properties required by Thai regulations [8], [24] and O_2 and heat tolerance in previous research [25]. However, there is still a lack of evaluation for antibacterial activity, screening of extracellular enzymes, and detection of antimicrobial resistance genes. This information will provide advanced insights for developing new probiotic products with industrial applications.

2 Materials and Methods

2.1 *Bacterial strains and culture conditions*

This study employed sixteen potential probiotic strains sourced from infants and pigs. These strains underwent primary assessments for their resistance to acid and bile salts, mucus adherence, and tolerance to oxygen [25]. *Bifidobacterium animalis* subsp. *animalis* ATCC 25527 was used as a reference strain. All isolates were provided by K.M.P. Biotech Co., Ltd and were

maintained in 20% (v/v) glycerol at −80 °C as part of the stock culture collection. To cultivate these strains, agar plates containing de Man-Rogosa-Sharpe (MRS) (Difco™) supplemented with 0.05% (w/v) L-cysteine HCl were inoculated with cultures from the stock collection. The plates were then placed in an anaerobic jar containing AnaeroPackTM (MGC, Japan) and incubated at 37 °C for 72 h.

The pathogenic strains, including, *Escherichia* coli ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter aerogenes* ATCC 13048 were obtained from Thai Can Biotech Co., Ltd. *Salmonella* Enteritidis DMST 15676 and *Salmonella* Typhimurium DMST 15674 were obtained from the Department of Medical Sciences. These six strains underwent sub-culturing in Tryptone Soya Broth (TSB) and were incubated at 37 °C for 24 h before being used in the experiments.

2.2 *Screening for antimicrobial activity*

To assess antimicrobial substance production, we employed two types of culture agar media, MRS agar (containing sugar) and tryptic soy agar (TSA, no sugar). Both agar media were supplemented with 0.5% g/L L-cysteine HCl and 0.17 g/L bromocresol purple (a pH indicator). The procedure began by inoculating a single colony of each isolate onto both test agar media. These plates were then incubated at 37 °C for 24 h under anaerobic conditions until the colonies developed. Subsequently, we prepared the test pathogens by diluting them with 0.85% (w/v) normal saline to achieve an initial concentration of 10^5–10^6 CFU/mL. This pathogen mixture was then combined with melted TSA at a ratio of 1:15 mL. The resulting mixture was poured over the previously grown bacteria agar plates and incubated at 37 °C for 24 h. The inhibition zone around the colonies and the color changes in the pH indicator were observed.

To measure the inhibition percentage in the broth, we followed a modification of the method [26]. The sixteen isolates were inoculated in tryptic soy broth (without sugar) supplemented with 2% (w/v) yeast extract (TSBY) and incubated under anaerobic conditions at 37 °C for 24 h. Afterward, the cultured broth was filtered through a 0.45 µm membrane to collect cell-free filtrate (CFF). Simultaneously, we cultured the six strains of test pathogens in TSB at 37 °C for 24 h, adjusting the turbidity (OD600) to 0.5. Thereafter, an equal volume of the CFF and pathogen suspension were mixed and incubated at 37 °C for 24 h. As a control, we used TSBY mixed with pathogen suspension. To calculate the inhibition percentage, we measured the absorbance at 600 nm (OD600) and applied the following Equation (1):

$$\%\ inhibition = \frac{(ODc\ -\ ODs)}{ODc} \times 100 \quad (1)$$

Where *ODc* represents the absorbance at 600 nm of the control, and *ODs* is the absorbance of the sample.

2.3 *Antimicrobial-resistant genes screening*

The examination of antimicrobial-resistant (AMR) genes was detected as previously published [27]. Briefly, all isolates used in this study were cultured, and their collected pellets served as the starting material. Genomic DNA extraction was performed by resuspending the pellet with the sterile distilled water, then heating it at 80 °C for 20 min. The supernated DNA was collected after being centrifuged at 13,500 rpm for 5 min. The DNA was quantified using a Nanodrop 2000c spectrophotometer (Thermo Scientific, MA, USA). The real-time PCR with specific probes contained QuantiNova Probe Master Mix (Qiagen, Germany), 0.125 µM probe, 0.25 µM primer, and 50 ng of DNA template. A total of fifty-one AMR genes were targeted for DNA detection. Nuclease-free water and synthetic positive control plasmid (104 copies number) were used as negative and positive controls, respectively. The reaction was carried out in a CFX96 Real-Time system instrument (Bio-Rad, Hercules, CA, USA), at 95 °C for 10 min, then followed by 40 cycles of 95 °C for 15 s, and 60 °C for 15 s.

2.4 *Detection of extracellular enzyme*

Extracellular enzyme production of protease, amylase, lipase, xylanase, cellulase, and β-glucanase were screened in this study. Detecting protease, amylase, and lipase enzymes involved using nutrient agar (NA) mixed with 1% of the respective substrate. For protease screening, we employed either UHT skim milk or soy milk. To assess amylase activity, we added 1% (w/v) of various starch sources, including wheat

flour, corn starch, rice flour, and cassava starch. Lipase determination, meanwhile, utilized 1% (v/v) of tributyrin, rice bran oil, olive oil, soybean oil, palm oil, and lard, with the addition of 0.02% bromocresol purple to enhance the visualization of the digestive zone. The procedure involved spotting a single colony of selected isolates on the agar, followed by anaerobic incubation at 37 °C for 48 h. A clear zone of milk around the inoculated area indicated protease production. For NA mixed with starch, we flooded the agar with 1% Lugol iodine solution and measured the transparent zone around the colony. Meanwhile, lipase activity manifested as a yellow halo around the colony, employing a modified method derived from references [28], [29].

Cellulase production was determined using CMC agar (Himedia) with 1% (w/v) CMC as the substrate. Following a 48 h plate inoculation, we flooded the agar with 1% Congo red for 20 min and then removed it. Subsequently, 1M NaCl was added and left for 20 min before being drained, the clear zone was then measured [30].

For the detection of β-glucanase, we followed the method outlined in [31] with some modifications. Initially, the β-glucan was dissolved (0.1% w/v) by heating in a 100 mM sodium acetate buffer (pH 5) and mixed with MRS agar. After inoculating the colony for 48 h, the plate was stained with 1% Congo red and fixed with 1M NaCl, as previously described, before examining the resulting clear zone. Xylanase detection was carried out in accordance with the method detailed in [32].

An enzyme index was employed to express each enzymatic production. The enzymatic index is represented as following Equation (2) [33]:

$$Enzyme\ index = \frac{R}{r} \quad (2)$$

Where R is the diameter of the clear zone and r is the diameter of the colony.

2.5 Sequencing of 16S rDNA and phylogenetic analysis

The PCR amplification, direct sequencing of 16S rDNA, and sequence analysis were proceeded by the Thailand Bioresource Research Center. The purified PCR products were subjected to sequencing using an ABI Prism® 3730XL DNA Sequence (Applied Biosystems, Foster City, California, USA). For single-strand 16S rDNA sequencing, two pairs of primers, namely 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') or 800R (5'-TAC CAG GGT ATC TAA TCC-3') and 518F (5'-CCA GCA GCC GCG GTA ATA CG-3') or 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') [34], were employed. The full-length sequences of the selected isolates (approximately ≈1,500 bases) were then compared with 12 rDNA sequences of *Bifidobacterium* spp., 10 rDNA sequences of *Lactobacillus* spp., and 1 rDNA sequences of *E. coli* and *Vibrio owensii*, which served as an outgroup. These sequences ranged from 1,400 to 1,500 bases and were sourced from the National Center for Biotechnology Information (NCBI) [35] and the European Nucleotide Archive databases [36]. To construct the phylogenetic tree, we utilized the Molecular Evolutionary Genetic Analysis software version 10.1.7 with the maximum likelihood method. The sequences of the selected isolates were submitted to the GenBank database on the NCBI webpage to obtain accession numbers.

3 Results and Discussion

3.1 *Antimicrobial activity*

3.1.1 Agar spot assay

Sixteen isolates of potential probiotic strains, along with a reference strain, underwent evaluation for their antimicrobial activity against six pathogens using two types of media, MRS agar (containing sugar) and TSA (no sugar). This was to assess the antibacterial capabilities and investigate whether this ability stemmed from producing organic acid or other antimicrobial substances. Bromocresol purple, which is violet above pH 6.8 and yellow below pH 5.2, was added as a pH indicator. In MRS agar, the presence of acid production was indicated by a yellow color change, while on TSA agar, the indicator remained purple. The inhibition results against six foodborne bacteria are summarised in Table 1. All sixteen isolates exhibited the ability to produce acid from glucose, forming a clear suppressive zone on MRS agar (with a zone diameter of approximately 15 mm). Conversely, no inhibition zone was observed on TSA agar. The reference strain demonstrated similar results

however there are no inhibitory properties against both *Salmonella* strains even on MRS agar. The antibacterial activities of probiotic bacteria, especially LAB and *Bifidobacterium*, primarily stem from releasing organic acids. This inhibitory mechanism is associated with the undissociated form of organic acids penetrating the cytoplasmic membrane, thereby reducing intracellular pH and disrupting transmembrane processes, which inhibited the activities of susceptible bacteria [17], [37]. Furthermore, organic acids can act as permeabilizers for the outer membrane of gram-negative bacteria and may enhance the effects of other antimicrobial substances [18].

3.1.2 Broth assay

However, to ensure that the antimicrobial effects were not solely due to organic acids, additional testing of antagonistic activity was carried out in a broth medium using tryptic soy broth (no sugar) supplemented with 2% (w/v) yeast extract (TSBY). Surprisingly, this testing in TSBY revealed the presence of other inhibiting substances. The inhibition percentage ranged from 0 to nearly 60%, and these variations were attributed to the varying sensitivity of the tested pathogens (Table 2). This finding explained why the inhibitory activity was not evident on TSA agar, as achieving 100% complete suppression is necessary to visualize the inhibition zone on agar. Apart from nonspecific antimicrobial agents like organic acids, more specific molecules such as bacteriocins and bacteriocin-like inhibitory substances were considered. Such compounds include nisin, pediocin, sakacin, reuterin, reutericyclin, lacticin, and enterocin [38]. Bacteriocins are biologically active proteins that exert antimicrobial effects and target specific binding sites on sensitive bacteria, typically within a narrow range of sensitive organisms [39]. Mostly bacteriocins would inhibit the microorganism within close proximity. For example, *Enterococcus* hirae HM02-04 produced hiracin, which inhibits *Enterococcus faecalis*, vancomycin-resistant enterococci, *Bacillus coagulans* JCM2257, and three strains of *Lactobacilli* [40]. In other studies, genera like *Lactobacillus* and *Bifidobacterium* have been reported to produce bacteriocins that inhibit foodborne pathogens including *Staphylococcus aureus*, *Escherichia coli*, *Bacillus* cereus, *Listeria monocytogenes*, and *Clostridium perfringens* [16], [17]. The effects of bacteriocins can range from bactericidal to bacteriostatic and depend on both extrinsic and intrinsic factors of the susceptible microorganisms, as well as the degree of purification and concentration of the substances [41]. Given the neutral pH conditions of the large intestine, which are conducive to various microorganisms, it is

Table 1: Antimicrobial activity of sixteen isolates on MRS and TSA agar

Isolates	*E. coli* ATCC 25922		*St. aureus* ATCC 6538		*P. aeruginosa* ATCC 27853		*Ent. aerogenes* ATCC 13048		*S.* Enteritidis DMST 15676		*S.* Typhimurium DMST 15674	
	MRS	TSA	MRS	TSA	MRS	TSA	MRS	TSA	MRS	TSA	MRS	TSA
H1-05	+	-	+	-	+	-	+	-	+	-	+	-
H9-01	+	-	+	-	+	-	+	-	+	-	+	-
H9-02	+	-	+	-	+	-	+	-	+	-	+	-
H9-03	+	-	+	-	+	-	+	-	+	-	+	-
H9-04	+	-	+	-	+	-	+	-	+	-	+	-
H9-05	+	-	+	-	+	-	+	-	+	-	+	-
H9-06	+	-	+	-	+	-	+	-	+	-	+	-
H10-01	+	-	+	-	+	-	+	-	+	-	+	-
H10-03	+	-	+	-	+	-	+	-	+	-	+	-
H10-05	+	-	+	-	+	-	+	-	+	-	+	-
P1-P01	+	-	+	-	+	-	+	-	+	-	+	-
P4-S01	+	-	+	-	+	-	+	-	+	-	+	-
P4-S03	+	-	+	-	+	-	+	-	+	-	+	-
P8-S01	+	-	+	-	+	-	+	-	+	-	+	-
P8-S03	+	-	+	-	+	-	+	-	+	-	+	-
P9-P01	+	-	+	-	+	-	+	-	+	-	+	-
B. animalis ATCC 25527	+	-	+	-	+	-	+	-	-	-	-	-

believed that probiotic LAB strains can be beneficial in countering gram-negative pathogens in the large intestine by producing relevant concentrations of lactic acid in confined environments. This inhibition against pathogens occurs through a combination of the lactic acid's capabilities and the presence of bile salts [18]. Nevertheless, such antagonistic effects are subject to the probiotic strains themselves, and the pathogenic strains used.

Antimicrobial activity against pathogenic bacteria served as another criterion for selecting potential probiotic strains [7]. Based on the results of antagonistic activity (Table 2), strains H9-01 (infant-originated strain) and P4-S03 (pig-isolated strain) were selected due to their notable broad-spectrum inhibitory effects on all test pathogens. In addition, both strains presented excellent basic probiotic properties as described by [25]. Although the H1-05, H9-06, and P8-S01 strains demonstrated varying degrees of antibacterial activity against five pathogens, they were co-selected because of their remarkable tolerance to acid, oxygen, and heat [25], which facilitated food processing and industrial-scale production. Compared to the reference ATCC 25527 strain, which showed antagonistic activity against only four out of six pathogens, the five isolates above demonstrated superior antibacterial efficacy against the test organisms (Table 2). Therefore, these five isolates were chosen as potential probiotic bacteria for further experiments.

3.2 *Antimicrobial-resistant gene detection*

Antimicrobial resistance (AMR) is one of the most significant public health challenges in the 21st century, according to the World Health Organization (WHO). Infections resistant to antimicrobial drugs can lead to higher mortality rates than those caused by susceptible infections [42]. Bacterial antibiotic resistance can manifest through several mechanisms, including alterations to the antibiotic molecule, reduced antibiotic penetration and efflux, mutations, and enzymatic alterations of target sites. Our research used real-time PCR with specific probes to determine the presence of relevant antimicrobial-resistant genes in selected probiotic bacteria (Table 3). We observed positive signals associated with three antimicrobial classes, focusing on ß-lactams, which constitute a broad class of antibiotics. The primary mechanism behind ß-lactam resistance relies on synthesizing ß-lactamase enzymes, which destroy the antibiotic molecule. For instance, the CTX-M class, recognized as an extended-spectrum β-lactamase (ESBL), is commonly detected

Table 2: Antagonistic activity of sixteen isolates in TSBY medium against foodborne bacteria

Isolates	*E. coli* ATCC 25922		*St. aureus* ATCC 6538		*P. aeruginosa* ATCC 27853		*Ent. aerogenes* ATCC 13048		*S.* Enteritidis DMST 15676		*S.* Typhimurium DMST 15674	
	MRS	TSA	MRS	TSA	MRS	TSA	MRS	TSA	MRS	TSA	MRS	TSA
H1-05	-		+		+		+		+++		++	
H9-01	++		+		+		+		+++		++	
H9-02	+		+		+		+		+++		++	
H9-03	+		+		+		+		+++		++	
H9-04	++		+		-		+		+++		+	
H9-05	+		+		-		+		+++		++	
H9-06	+		+++		-		+		+++		++	
H10-01	++		+		-		+		+		+	
H10-03	+		+		-		+		++		+	
H10-05	++		+		-		+		+		++	
P1-P01	+		+		-		+		+		-	
P4-S01	+		+		+		+		-		-	
P4-S03	+		++		+		+		++		+	
P8-S01	+		++		+		-		++		-	
P8-S03	+		+		++		+		++		-	
P9-P01	+		++		+		+		+		-	
B. animalis ATCC 25527	+		-		+		+		++		-	

Note: - is No inhibitory activity, + is Less than 20%, ++ is Less than 40%, and +++ is More than 40%

Table 3: Antimicrobial-resistant gene detection

Antimicrobial Classes	Target Gene	Isolates				
		H1-05	H9-01	H9-06	P4-S03	P8-S01
ß-lactams (penicillin, amoxicillin, cephalosporin)	CTX-M1	N	N	N	N	N
	CTX-M2-M74	N	N	N	N	N
	CTX-M8-M25	N	N	N	N	N
	CTX-M9	N	N	N	N	N
	PER	N	N	N	N	N
	VEB	N	N	N	N	N
	CMY1-MOX	N	N	N	N	N
	CMY2-LAT	N	N	N	N	N
	DHA	N	N	N	N	N
	FOX	N	N	N	N	N
	ACT-MIR	N	N	N	N	N
	OXA-1	N	N	N	N	N
	OXA-9	N	N	N	N	N
Carbapenems	KPC	N	N	N	N	N
	GES	N	N	N	N	N
	NDM	N	N	N	N	N
	VIM	N	N	N	N	N
	IMP	N	N	N	N	N
	OXA-48	N	N	N	N	N
Folate pathway inhibitors	*sul1*	N	N	N	N	N
	sul2	N	N	N	N	N
	sul3	N	N	N	N	N
	dfrA1	N	N	N	N	N
	dfrA5-14	N	N	N	N	N
	dfrA12	N	N	N	N	N
	dfrA17	N	N	N	N	N
Polymycins	*mcr-1*	N	N	N	N	N
	mcr-2	N	N	N	N	N
Tetracyclines	*tetA*	N	N	N	N	N
	tetB	N	N	3+	N	N
Phenicols	*cmlA*	N	N	N	N	N
	floR	N	N	N	N	N
	catA1	N	N	N	N	N
	catB3	N	N	N	N	N
Aminoglycosides	*aacC1*	N	N	N	N	N
	aacC2	N	N	N	N	N
	aacC4	N	N	N	N	N
	aphA1	N	N	N	N	N
	aadA4-5	N	N	N	N	N
	aphA6	N	N	N	N	N
	aadA1-2-17	N	N	N	N	N
	aadB	N	N	N	N	N
	armA	N	N	N	N	N
	rmtB	N	N	N	N	N
Macrolides	*ermB*	N	N	N	N	N
	mphA	N	N	N	N	N
Quinolones	*qnrA*	N	N	N	N	N
	qnrS	N	N	3+	N	N
	qnrB1	N	N	N	N	N
	qnrB4	N	N	N	N	N
	QepA	N	N	N	N	N

Note: ~3–8 × 10^6 cells were tested, Positive grading criteria; 1+; ≥ 10^1 –10^2, 2+; >10^2 –10^3, 3+; >10^3 positive cells and N = Not found

in bacteria like *Klebsiella* pneumoniae and *E. coli*, among other *Enterobacter*iaceae. Girlich *et al.*, [43] reported the presence of CTX-M-1 genes in twelve *E. coli* isolates from poultry used for food production in France. This resistance gene may be prevalent in poultry due to its presence on transferable plasmids.

Tetracycline has a long history of use in the pig industry for growth promotion and treatment against common infections. Tetracycline resistance serves as an example of efflux-mediated resistance mechanisms, with the *tet* genes predominantly found in gram-negative organisms, including the *tetB* gene [37], [38]. Nevertheless, Chander, Oliveira, and Goyal [44] made a significant discovery when they identified the *tetB* gene in *Streptococcus* suis isolated from infected pigs. This finding was the first report of this resistance gene in Gram-positive bacteria. Regarding quinolone resistance, the primary mechanism involves the accumulation of mutations in genes encoding quinolone target DNA gyrase and topoisomerase IV, as well as alterations in regulatory genes that affect permeability or efflux [45]. Various *qnr* genes, especially *qnrA*, *qnrB*, and *qnrS*, are typically found on multidrug resistance plasmids and have been identified in bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella* spp. [45], [46].

The assessment of antibiotic resistance is essential for characterizing probiotic bacteria, even within the Generally Recognized as Safe group (GRAS) [7]. The basic protocol for determination is using antibiotic discs laid on the spread bacteria on the agar or growing the bacteria on the agar medium mixed with antibiotic drugs [4], [9]–[11], [47]. Such a method examines the end result of gene expression, which may be obstructed by the culture conditions, whereas the direct detection of the AMR genes in our work is to ensure that there will be no transferable gene. While natural antibiotic resistance in probiotic strains may enable them to survive certain antibiotic treatments, there is a potential risk of genetic transfer to pathogenic bacteria within the gut microbiota, posing a significant health concern for the host. Notably, reports have documented the presence of drug resistance and AMR genes, even in commonly consumed probiotics, like *Lactobacillus* and *Bifidobacterium*, through commercial products [48], [49]. European Food Safety Authority standards underscore the necessity of antibiotic susceptibility assays for probiotics intended for human and animal use, particularly evaluating transferable antibiotic resistance at the genome level to ensure safety [48]. As seen in the results, only the H9-06 detected the AMR gene for CTX-M1 and qnrS gene among the five selected isolates, it deemed necessary for exclusion due to the concerns previously described. Consequently, we selected H1-05, H9-01, P4-S03, and P8-S01 for further experimentation.

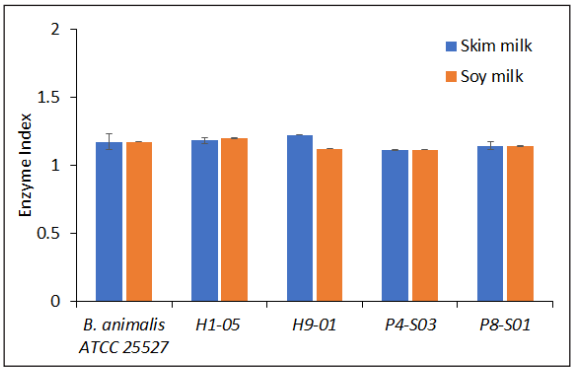

Figure 1: Enzyme index of proteolytic enzymes produced by potential probiotic isolates and the reference strain. Error bars represent the standard deviation of six replicates.

3.3 *Extracellular enzyme production*

Although digestive enzymes, namely amylase, protease, and lipase, exist in the gastrointestinal (GI) system and are secreted to digest carbohydrates, proteins, and lipids, respectively, the use of probiotic supplements in animals has been shown to support digestion and the absorption of nutrients, improving carcass quality and weight gain [3]. We conducted enzyme production screening for protease, amylase, and lipase using the substrate hydrolysis method, and the ability of each enzymatic production was expressed as an enzyme index. For skim milk digestion, H1-05, H9-01, and *B. animalis* subsp. *animalis* ATCC 25527 expressed a significantly higher enzyme index (p-value < 0.05) than P4-S03 and P8-S01 (Figure 1). Similarly, soy milk yielded comparable results, except for H9-01, which exhibited a lower proteolytic zone than skim milk. The proteolytic system functions through protease, which hydrolyzes peptide bonds, selectively targeting amino acid molecules on both sides of the peptide bonds [50]. Skim milk comprises approximately 3.7% protein,

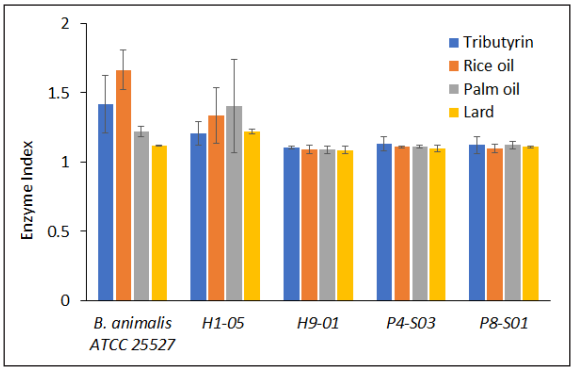

Figure 2: Enzyme index of lipolytic enzymes produced by potential probiotic isolates and the reference strain. Error bars represent the standard deviation of six replicates.

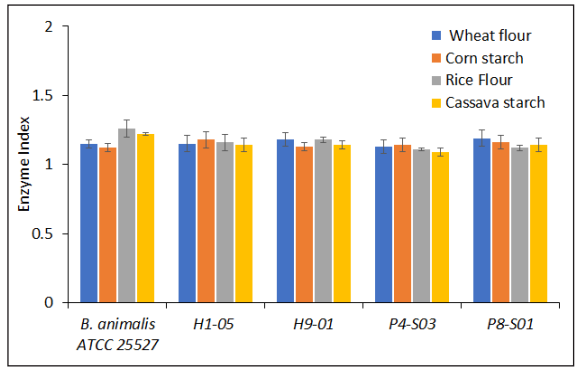

Figure 3: Enzyme index of amylolytic enzymes produced by potential probiotic isolates and the reference strain. The error bars in the graph represent the standard deviation calculated from six replicates.

76% being casein with high glutamic acid, proline and leucine [51], [52]. Extracellular proteases break down caseins into peptides; peptidases further hydrolyze peptides and transport systems facilitate the movement of breakdown products across the cytoplasmic membrane [53]. Therefore, hydrolyzing casein molecules into peptide fragments expresses the digestive zone around the culture [54]. In the case of soy milk, which contains approximately 2.4% protein and is high in glutamic acid and aspartic acid, while methionine is only half of that in casein [51], [52]. Bacterial proteases are diverse and classified by the practical location, namely, cell-associated enzyme complexes and extracellular proteases. The latter enzymes are highly specific to the substrate [55]. It seems that the protease from H9-01 may have the target site to the type of amino acid substrates that appear in skim milk more than soy milk.

Lipase production was investigated on agar medium containing four oils as substrates, namely, tributyrin, rice bran oil, palm oil, and lard, some of which may be used in animal feed. The results are displayed in Figure 2. Tributyrin, rice bran oil, and palm oil were hydrolyzed clearly on the tested agar by *B. animalis* ATCC 25527 (reference) and H1-05, while H9-01, P4-S03, and P8-S01 showed a slightly transparent zone. H1-05 also exhibited a significantly higher enzyme index in lard (p-value < 0.05). Lipase catalyzes the hydrolysis of triacylglycerol into fatty acids and glycerol. Microbial lipases are specific to stereochemical, positional, and substrate characteristics [56]. Lipases can act on substrates with long-chain (C > 10), intermediate, and short-chain fatty acids [57]. Tributyrin is an ester of glycerol and butyric acid. Soybean oil and rice bran oil contain higher proportions of unsaturated fatty acids (75%–80%) than saturated fatty acids (14.5%–25%), whereas palm oil and lard are composed primarily of saturated fatty acids (40%–45%) [58], [59]. The variation in enzyme activity depends on the cleavage site within the lipid structure.

In the case of amylase production, we conducted tests using four starch agars (corn starch, wheat flour, rice flour, and cassava starch), as illustrated in Figure 3. Notably, only the reference strain showed a slightly higher enzyme index on rice flour (1.26 ± 0.06) and cassava starch (1.22 ± 0.01) compared to the other isolates (p < 0.05). The enzyme indices of all types of starch tested in the four isolates did not show significant differences (p-value > 0.05) and ranged from 1.09 ± 0.03 to 1.19 ± 0.06. Starch is a common source of carbohydrates, providing energy to humans and animals. It comprises amylose and amylopectin [60]. Amylases are enzymes that catalyze the hydrolysis of starch by breaking down α-D 1, 4 glycosidic linkages into short oligosaccharides. Various factors may influence starch hydrolysis, including the type and origin of starch, with the proportion of amylose and amylopectin content being among the critical factors [61].

Probiotics are recognized for their influence on the intestinal environment and their ability to

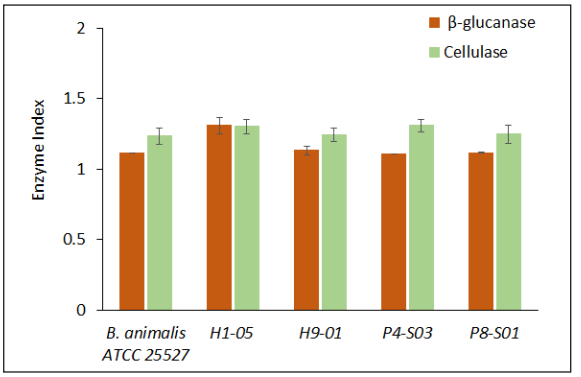

Figure 4: Enzyme index of β-glucanase and cellulolytic enzymes screened by potential probiotic isolates and the reference strain. Error bars indicate the standard deviation of six replicates.

deliver enzymes and other beneficial substances to the intestines [22]. The study of extracellular enzyme production can be essential in selecting potential probiotic strains [10], [23]. Generally, probiotic bacteria in the *Bacillus* spp. group are known as producers of extracellular hydrolytic enzymes, including amylase, protease, and lipase, which aid in nutrient digestion and feed utilization. This finding suggests that using enzyme-producing bacteria as probiotics represents a viable nutritional strategy for the livestock industry [23], [28], [62]. While there are several reports on the secretion of amylase, protease, and lipase in major probiotic groups such as *Lactobacillus*, *Lactococcus*, *Streptococcus*, and Pediococcus [10], [54], [60]–[63], there are almost no reports on *Bifidobacterium*.

For β-glucanase and cellulase enzymes, the results of the enzyme index are presented in Figure 4. β-glucanase did not exhibit distinctive activity; only H5-01 showed a significantly high enzyme index of 1.31 ± 0.06 (p-value < 0.05). Furthermore, cellulolytic enzymes displayed values in the range of 1.23–1.31, with no significant differences observed among the five tested bacteria (p-value > 0.05). However, none of the strains showed xylanase activity. Cellulose is a non-starch polysaccharide commonly found in plant cell walls, typically consisting of non-branched linearly linked d-glucose with β-D-(1-4) bond. However, β-glucan is a primary structural biopolymer present in the cell walls of cereal grains and fungi, characterized by a mixed linkage of β-(1-3)-(1-4)-D glucosyl units [60]. Consequently, the production of enzyme cellulase and β-glucanase enzymes by probiotics could be beneficial for the digestion of food in livestock and swine.

3.4 *Identification and molecular characterization of the isolates*

Three isolates were selected following initial screening for probiotic properties. The strain H9-01 was isolated from an infant source and exhibited strong antimicrobial properties, while the strains P4-S03 and P8-S01 originated from swine manure [25]. We conducted complete 16S rDNA gene sequencing to identify these isolates and compared the results with the GenBank database of the NCBI. The analysis using the Megablast program on the NCBI webpage [64] revealed that H9-01 was closely related to *Bifidobacterium animalis* subsp. *lactis* YIT4121 shares 99.73% similarity. In contrast, strains P4-S03 and P8-S01 were found to be similar to *Lactobacillus reuteri* DSM 108836, with 93.33% and 100% identity, respectively. The phylogenetic tree in Figure 5 shows two different genotypic groups of the genus *Bifidobacterium* and *Lactobacillus*. H9-01 was clustered to the same topology of *Bifidobacterium animalis* subsp. *lactis*, which shared the same node with the strain YIT4121 with less than 95% of the bootstrap value. While P4-S03 and P8-S01 fell in the same branch of *Lactobacillus reuteri* DSM 108836 with strong bootstrap value support (>95%) which is considered accurate [65]. Finally, sequence data for H9-01, P4-S03, and P8-S01 were deposited in the GenBank, NCBI publicly collection under the accession numbers MT355433, MT355434, and MT35544. The three strains have also been registered with the Thai food and drug administration and re-numbering as KMP-602, KMP-215, and KMP-126, respectively.

Lactobacillus reuteri has found a commercial application in combination with *Lactobacillus rhamnosus* and is being commercialized by Chr. Hansen, Denmark in food and capsule form [66]. Whereas *Bifidobacterium animalis* subsp. lactis has been launched as a commercial products namely, lactis BB-12 by Chr. Hansen and DN-173 010 by Danone, the strains contribute to gastrointestinal health and immune system [67]. However, these strains are European isolates. To our knowledge, there are no *Bifidobacterium animalis subsp. lactis* and

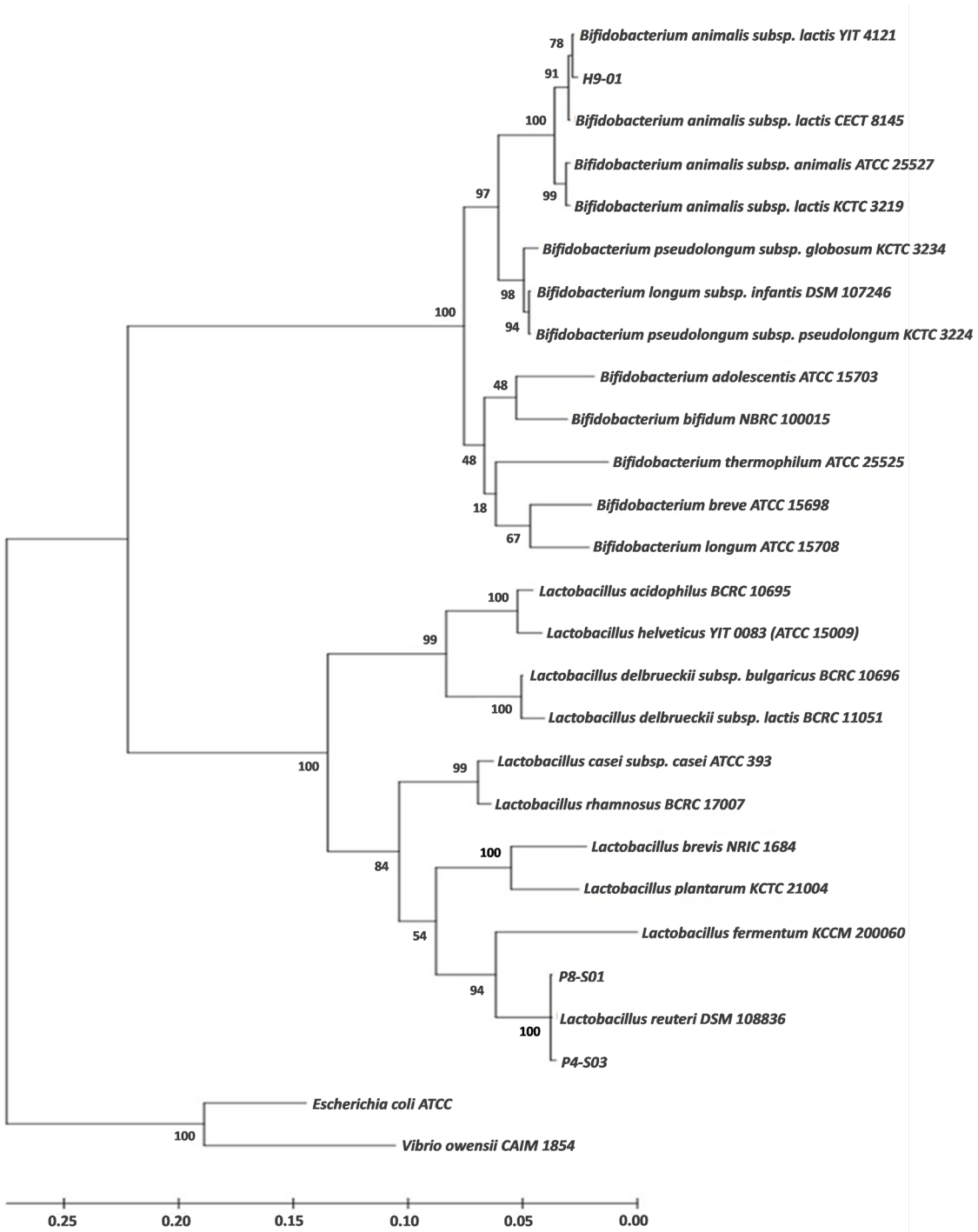

Figure 5: The phylogenetic tree relationship of H9-01, P4-S03, and P8-S01 with other strains using the maximum likelihood method. The numbers at the nodes correspond to the percentage that occurred from a total of 1,000 bootstrap replications. The scale bar indicates the number of base substitutions per site.

Lactobacillus reuteri isolated from the southeast Asian in the market. Therefore, these new strains have the potential for further research and development to be alternative strains in the food and feed industry for this region.

4 Conclusions

Three main criteria, in addition to standard regulations, were employed to evaluate potential local probiotics. These criteria included assessing inhibitory activity against six foodborne bacteria, the absence of 52 AMR genes, and the secretion of specific digestive enzymes. As a result, two isolates were of interest: H9-01 and P4-S03, which have been identified as *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus reuteri*, respectively. Both of these species are listed for use as food and feed supplements [8], [24], [68]. Previous research has demonstrated their outstanding *in vitro* probiotic properties, including tolerance to acid and bile salts, adhesion to mucosal surfaces, resistance to oxygen, and high-temperature resilience. This research also unveiled the ability to produce extracellular enzymes in *Bifidobacterium animalis*, particularly β-glucanase and cellulase, which have never been reported. Additionally, these strains will be conducted *in vivo* investigation for future studies. Consequently, the selection of probiotic strains in our research will be ensure that the selected strains is appropriate for scaling up on an industrial level.

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Author Contributions

S.V.: funding acquisition, project administration, reviewing and editing; C.B.: research design, methodology, data analysis, writing an original draft; C.P. and V.D.: supervision, validation; R.C.: genes analysis; P.T.: conceptualization, data curation, partial funding; S.H.: assisted in some experimental sections. All authors have read and approved the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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