Chapter IV

Results and Discussion

Results

Samples (Fig. 1) of the feces of twenty-seven captive Asian elephants at Maesa Elephant Camp, Maerim District, Chiang Mai, Thailand were collected for this study fromMarch-April 2010 between 8.30 – 9.30 A.M. Samples were collected in a sterile close fitting container and transported to the laboratory within 1 - 2 h.In total, 558 strains of microorganisms were isolated using the following three different media: (i) plate count agar (PCA) - 193 strains; (ii) potato dextrose agar (PDA) - 147 strains; and (iii) MRS agar - 218 strains. Colonies were tested to identify the probiotic properties of each strain (Fig. 2).

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Figure 8 Sampling of direct rectum feces or freshly fallen ground fecesof captive Asian elephants at Maesa Elephant Camp, Maerim District, Chiang Mai, Thailand.

Figure 9 Colonies were tested to identify the probiotic properties

4.1 Selection and characterization of probiotic properties

4.1.1 Bile salt tolerance

The abilityof the microorganisms to survive in the gastrointestinal tract depends on the properties of resistance to bile salts (Ruja, 2011). To act as a helpful probiotic, a strain must be able to survive the conditions of the elephant gastrointestinal tract. Bile salts are critical to microorganisms since their cell membranes are composed of lipids and fatty acids. Ruja (2011) considered 0.3% bile salt as the critical concentration to screen for resistant strains. From the 558 isolates chosen, 304 (65.09 %) viable bacterial isolates, and 26 (28.57 %) viable fungal isolates showed bile salt tolerance abilityon MRS agar (Fig. 3) (Table 1).

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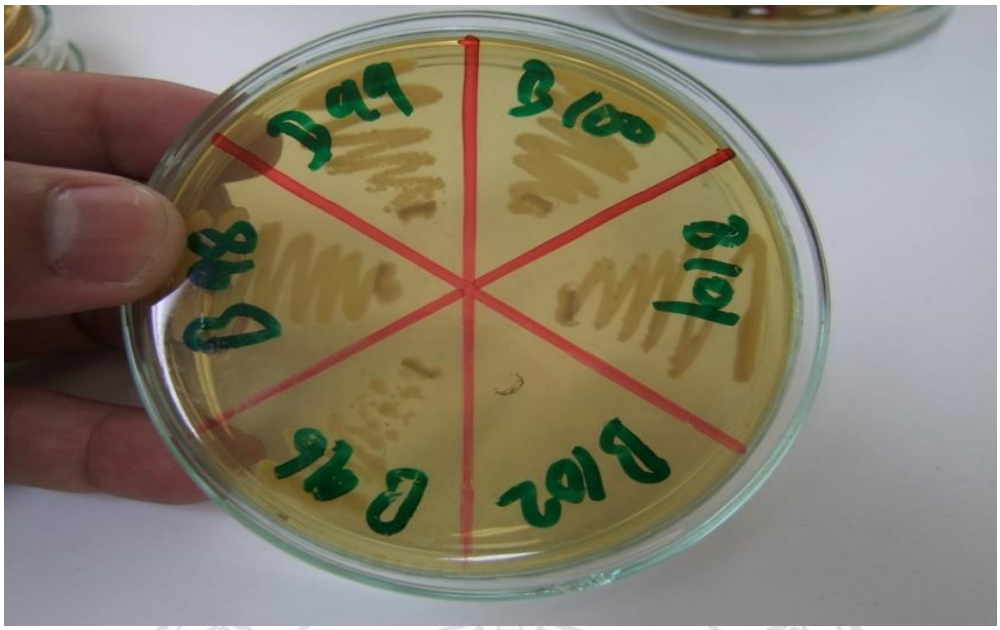

Figure 10 Results of bile acid tolerance test

Figure 11 pH tolerance test

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4.1.2 pH tolerance test (pH 2-9)

Since very little is known aboutthe pH of the elephant gastrointestinal tract, this study used comparisons with the horse gastrointestinal tract. The pH of the horse gastrointestinal tract from esophagus to duodenal is acidic (pH 1.5-2.0); theileum is almost neutral; and the caecum, the most important part for food fermentation in horses, to the large intestine varies in pH, ranging from acidic to basic.

Table 2 Total microbial strains isolated from fecal samples of 27 captive Asian elephants in various bile and pH conditions.

Conditions	Number of tests (strain)	Number of active strains	Percentage of active strains
Bacterial tests			
Survival in 0.30%(w/v) bile salt condition	467	304	65.09
Survival in pH 3, 4, 5, 8 and 9	467	84	17.99
Survival in 0.30%(w/v) bile salt and pH 3-9	467	68	14.56
Fungal tests			
Survival in 0.30%(w/v) bile salt condition	91	26	28.57
Survival in pH 3, 4, 5, 8 and 9	91	9	9.89
Survival in 0.30%(w/v) bile salt and pH 3-9	91	9	9.89

Therefore, the strain must survive in varies pH conditions of the gastrointestinal tract to grow in the ileum and act as probiotics for animals that have caecum fermentation. From the 558 samples, bile acid resistance was tested in the acid-base pH conditions of 2, 3, 4, 5, 6, 7, 8, and 9. There were 84 (17.99%) viable isolates from bacterial tests and 9 (9.89%) viable isolatesfrom fungi tests in pH conditions of 3 or 4 (Fig.4) (Table 1).

4.1.3 Cellulolytic properties test

The target property of this study is cellulolytic activity because feed stuff of elephants contains large amounts of cellulose. If the bacteria can digest cellulose, this activity will improve the digestion of the elephant. The number of strains with cellulolytic properties was 46 (59.74%) from all 77 isolates of both pH and bile salt tolerance.

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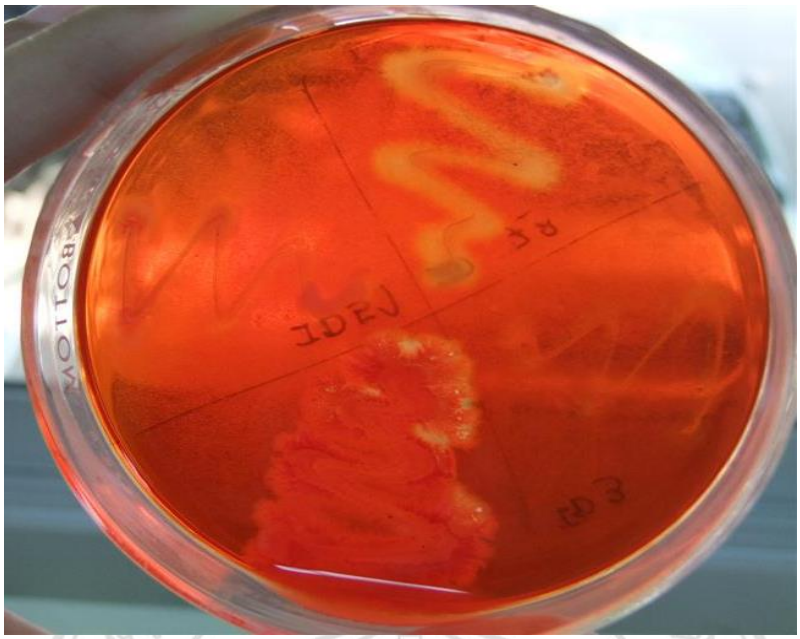

Figure 12 Cellulolytic property test using 1% (w/v) Congo red

4.1.4 Carbohydrate, protein and lipid Utilization

From the 46 strains, with cellulolytic properties, bile salt, acid and base tolerance, 12 strains (26.08%) have the carbohydrate digestion property (Fig. 6), 6 strains (13.04%) have the protein digestion property (Fig. 7), and 13 strains (28.26%) have the lipid digestion property.

Figure 13 Carbohydrate utilization test

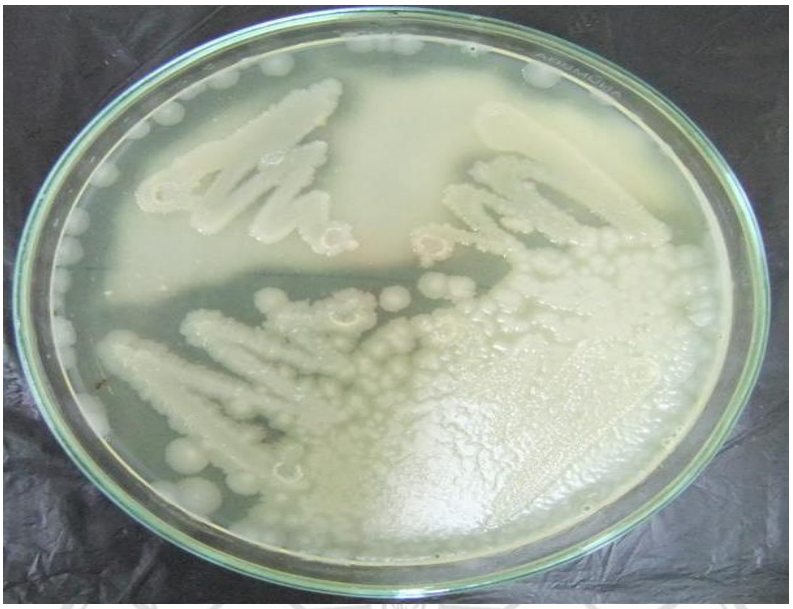

Figure 14 Protein utilization test

4.1.5 Aerobic and Anaerobic viability test

Every process was made in both aerobic and anaerobic conditions to know the character and ability to grow in both conditions. The five isolated strainscan survive and grow in both conditions.

4.1.6 Pathogen inhibiting property

After the probiotic property tests, five isolated strains were selected to test pathogen inhibiting property. These isolated strains (E1-E5) were tested by agar well diffusion technique against five pathogen strains (Table 2).

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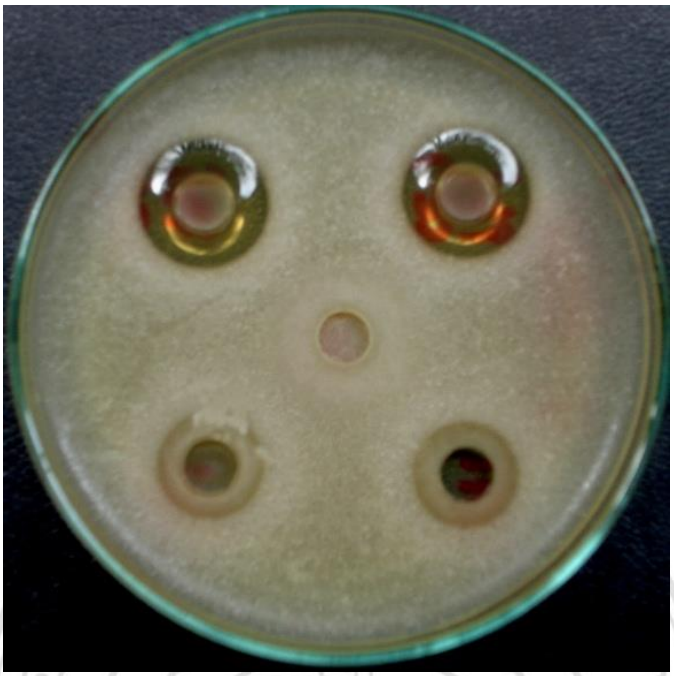

Figure 15 Pathogen inhibiting property

Table 3 Pathogen inhibiting property -*E. coli, S. aureus, B. cereus, P. aeruginosa,* and *C. albicans* activity

Sample Strain	***E. coli* ATCC 25922**	***S. aureus* ATCC 25923**	***B. cereus* ATCC 11778**	***P. aeruginosa* ATCC 27853**	***C. albicans* ATCC 90028**
E1	9	10	9	9	8
E2	9	10	8	9	8
E3	9	8	9	8	12
E4	8	8	8	7	8
E5	8	10	8	8	10

Note: The clear zone measurement includes the diameter of the aluminum rings (7 mm).

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4.1.7 Growth curve

When a microorganism is introduced into a fresh medium, it takes some time to adjust to the new environment. This phase (0-6 hours) is termed as a lag phase. In the exponential or logarithmic (log) phase (9-18 hours), the microorganisms are in a rapidly growing and dividingstate. Their metabolic activity increases.

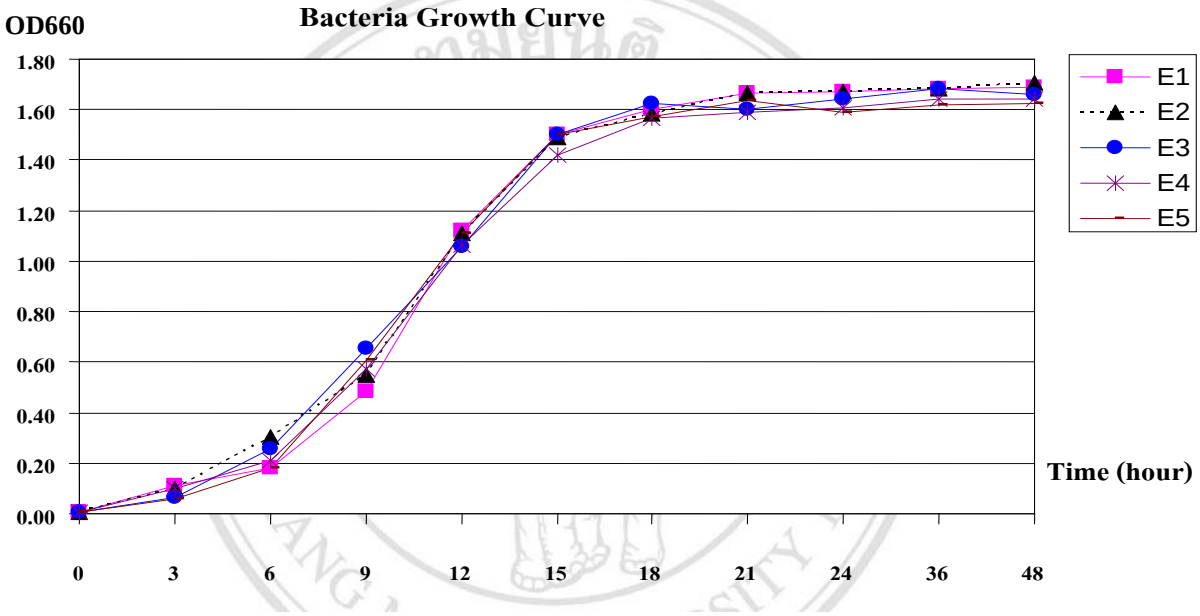

Figure 16 The plot represents the cell growth (measured as turbidity at OD_{660}) versus the incubation time from 0-48 hours.

The growth medium is exploited at the maximal rate, and the culture reaches the maximum growth rate as the number of bacteria increases logarithmically (exponentially). In the stationary phase (after 18 hours), the reproduction rate will slow down, and the number of cells undergoing division is equal to the number of cell deaths (Fig. 9).

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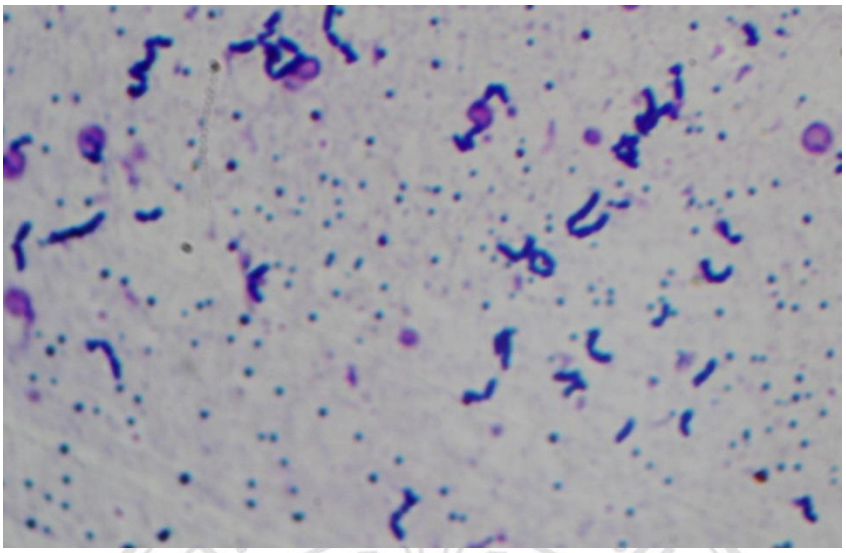

Figure 17 Gram stain shows Gram-positive (+) bacteria (purple)

4.1.8 Gram stain

The five probiotic isolates (E1-E5) were stained and identified as Gram-positive acid production bacteria (Fig. 10).

Figure 18 E1-E5 strain, the result from identification kit API 50 CHL

4.2 Sugar utilization property using identification kit API 50 CHL

All five isolates were screened for their ability to utilize sugar using the API 50 CHL identification kit (Fig. 11). Two isolates exhibited sugar utilization properties similar to those of *L. plantalum*or*L. brevis*; Two isolates like*weissella*sp.andone isolate similar to*Enterococcus* sp.as shown in Table 3**.**

Table 4 Sugar utilization property using Utilization identification kit API50CHL.

Utilization	E1	E2	E3	E4	E5
Galactose10	+	+	+	+	+
Rhamnose15	-	-	+	+	+
Sorbital19	-	-	-	-	-
N-Acetyl-D-glucosamine22	+	+	+	+	+
Amygdalin23	+	+	+	+	+
Arbutin24	+	+	+	+	+
Esculin25	+	+	+	+	+
Salicin26	+	+	+	+	+
Cellobiose27	+	+	+	+	+
Lactose29	+	+	+	+	+
Melibiose30	+	-	+	+	-
Trehalose32	+	-	+	+	+
Raffinose35	-	-	-	-	-
Gentiobiose39	+	+	+	+	+

4.3 Identification of bacteria using partial 16s RNA sequencing

The biochemical test showed that E1-E5 were Gram-positive, non-spore forming, gave a negative result for the catalase test, and had the carbohydrate utilization property. Therefore, it can be assumed that the five isolates were *Lactobacillus plantarum, Weissella cibaria*, or *Enterococcus* species. Moreover, phylogenetic identification was performed by 16S rDNA sequence analysis. The E1 and E2 strains presented 100% characteristic of the *Weissella cibaria* strain (DH8, NCBI Genbank Accession number of AB494716). The E3 and E5 strains presented 100% characteristic of *Lactobacillus plantarum* strain (LP-01, NCBI Genbank Accession number of HQ441200). The E4 strain presented 100% characteristic of *Enterococcus* sp.(SF-1, NCBI Genbank Accession number of AB470317).

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Discussion

The gastrointestinal microorganism is extremely important for human and animal health. Investigations into the composition of the microorganism and its therapeutic modification have received increasing interest in human and veterinary medicine. Probiotics are a way of modifying the microorganism and have been tested to prevent and treat diseases. Probiotics are proposed to exert their beneficial effects through various pathways. Production of antimicrobial compounds targeting intestinal pathogens, general immune stimulation, and colonization resistance are among these mechanisms. Despite widespread availability and use in human and other animal, peer-reviewed or any information in elephant is limited. Although promising *in vitro* results have been achieved, *in vivo* health benefits have been more difficult to prove. Whether the results are caused by strain selection, dosage selection or true lack of efficacy remains to be answered. Although these limitations exist, probiotics are increasingly used because of their lack of severe adverse effects, ease of administration, and low cost. This study summarizes the screening for microorganism for probiotic property use in elephant medicine. It aims to provide veterinarians and researcher with local based information on what strains of probiotics properties are indicated for prevention or treatment of gastrointestinal disease in elephants.

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The properties of three strains of this study and it further usage.

1.Weissella cibaria strain:DH8 (E1 and E2)

Weissella cibaria is Gram-positive, non-pore formulating, non-motile, hetero lactic acid fermented, and catalase negative bacillus, and cannot produce dextran from sucrose. The study of their potential as probiotics were examined in this work were related to other study. The resistance to low pHs (pH 3.0) and 0.3% bile salt were examined. Enzyme activities and various antibiotics . This strain can utilize starch, protein and lipid as well. Their potential were degrading cellulose (CMC substrate) that can use for promote ecology in digestive system of elephant or use for silage

fermentation before feeding. Antimicrobial property of Weissella cibaria were presented.

2.Lactobacillus plantarum strain LP-01 (E3 and E5)

Lactobacillus genus is a microorganism which does not form spores and is an anaerobic and facultative anaerobic gram-positive bacterium. This bacterium is not only widely dispersed in nature but is also found in human oral cavity and digestive organs. This bacterium is a beneficial microorganism that is widely used as a starter for various fermented dairy products.

Probiotic lactobacilli are also the most commonly species used as silage inoculants. However, among enterococci that belong to lactic acid bacteria (LAB) has been found bacteria with probiotic character too. It is believed that the inoculated populations of LAB genotypes become dominant in silage, thereby increasing the lactic acid concentration and decreasing pH values, gas, and protein decomposition . Probiotic LAB are also fed by livestock to improve intestinal microbial balance, including elimination or reduction of undesirable microorganisms . Viable microbial preparations, principally including lactic acid bacteria, have been proposed as a supplement in animal fodder .

3.Enterococcus sp. SF-1 (E4)

Enterococcus are also pathogens (endocarditis etc.) Some strains resistant to antibiotic and virulence factors such as adhesins, invasins pilli and haemolysin were presented. Some of *E.faecium ,E.faecalis* use as probiotics. Enterococcus can use to treat diarrhea, antibiotic associated diarrhea and prevent diarrhea in human and animal. In the agricultural industry Enteroccoccus were know as silage inoculants. In vivo health benefits have been more difficult to prove and be careful monitoring during the period of treatment.

In the present study, Enterococcus spp. was resistance to low pHs (pH 3.0) and 0.3% bile salt. Enzyme activities and antimicrobial activity were test . This strain can utilize starch, protein and lipid as well. Their potential were degrading cellulose (CMC substrate). Bacterial resistance to acid in the pH range of 2.0–3.0 is desirable for probiotic cultures. What should also be born in mind is that the combination of probiotic

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bacteria with other food ingredients present in food products may improve the viability of microorganisms during gastric transit. This is because of the protection exerted by certain food components leading to an enhanced gastric survival.

Use as silage inoculants. Silage is the feedstuff produced by fermentation of forage crops of variable but often high moisture content. Ensilaging means to preserve forage for feeding livestock when fresh material is less available. In general, silage fermentation is a natural process whereby epiphytic lactic acid bacteria ferment water soluble carbohydrates in the crop to a number of products, primarily lactic acid, thereby reducing the pH as rapidly as possible, inhibiting spoilage microbes and preserving the maximum amount of nutrients in the product.

The ensilage process is divided into the initial aerobic phase, the fermentation phase and second aerobic phase when the silo is opened. The last phase has consequences for the quality of the product fed by livestock. When the silo is opened and/or sealed inadequately, the silage is exposed to air. This may lead to aerobic deterioration. Aerobic microorganisms in silage degrade lactic acid and residual water-soluble carbohydrate to CO2 and protein and amino acids to amines, amides, and ammonia. Aerobic deterioration generates considerable heat, increases pH, and decreases digestibility . The main contaminants associated with aerobic spoilage activity in silage are yeasts, moulds, Bacillus spp. and Listeria spp.

The use of silage for animal feeding has sometimes been associated with pathological problems, including listeriosis. Because Listeria can grow at low temperatures, hay crop silage stored in large plastic bags has frequently been contaminated.

(M. Marcinakova et.al, 2004)

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