CHAPTER 4

RESULTS AND DISCUSSION

4.1 Physical characteristic and size determination of extracted polysaccharides

We found that after water extraction and let the extract stand for a short time, the gel was formed in both fresh and dried sample within 20 min and no physical difference of gel was observed. Crude polysaccharide was determined for total sugar, reducing sugar and DP. The results are shown in Table 5.

Crude polysaccharide was obtained with the total sugar yield of 5.7 and 22.35% form fresh and dried Moo-noi leaves, respectively. However, if the % yield from fresh leaves was recalculated in term of % total sugar per its dry weight, the % yield was 21.65. That is not so much different between fresh and dried leaves. From this result, we have made a conclusion to use the poly-saccharide extracted from dried Moo-noi leaves for further study according to the convenient to keep the same batch of Moo-noi leave sample for a long time period. The prepared polysaccharides from Moo-noi leaves was the high molecular weight molecules as the average DP were 19 and 21 obtained from fresh and dried Moo-noi leaves, respectively. Those of the pectin extracted from Moo-noi by Singthong *et al.* (2004) was not reported about the DP value, but the degree of esterification (DE) of extracted pectin was 41.7%.

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Table 5 Average DP and yield of polysaccharides extracted from Moo-noi leaves

Leave	%Total sugar (g/g sample)	%Total sugar (g/g dry weight)	DP
Fresh	5.7	21.65	19
Dried	22.35	22.35	21

The total uronic acid content of crude extract polysaccharides determined by carbazole assay was 85%(w/w) of total carbohydrate obtained from dried Moo-noi leaves. This is similarly with the result from Singthong *et al.* (2004) who reported that total uronic acid content of crude extract from *Cissampelos pareira* was 70.56%. The difference in uronic quantity is may be due to the difference method for determination of uronic acid as Singthong and colleque used the method of Blumenkrantz and Asboe-Hansen (1973) for uronic acid determination, while we chose the carbazole assay because of sugar composition of Moo-noi leaves was reported that having low neutral sugar content (Singthong *et al.*, 2004). Determination of uronic acid by carbazole assay has the limit that neutral sugars interfere especially interfere of hexoses. However, interference can be eliminating by using of the controls as they give significantly different absorption spectra (Chaplin, 1986).

4.2 Physical characteristic of gel formation

Characteristic of gel formation from the extract was showed in Figure17. Show gel formation when it was filtered through a thin cloth to remove the rough particle residues (A) and characteristic of gel after fine particle removal by centrifugation with 6,000 rpm at 30°C (B). However, we found that the difference in color of gel was observed as gel after centrifugation was found in orange-brown color, while the brownie-green color was observed in gel formed without the centrifugation (A). This may be because of the difference in the exposure time of gel chemical component, some compounds possible to be oxidized by light or other oxidizing agent contained in Moo-noi leave extract.

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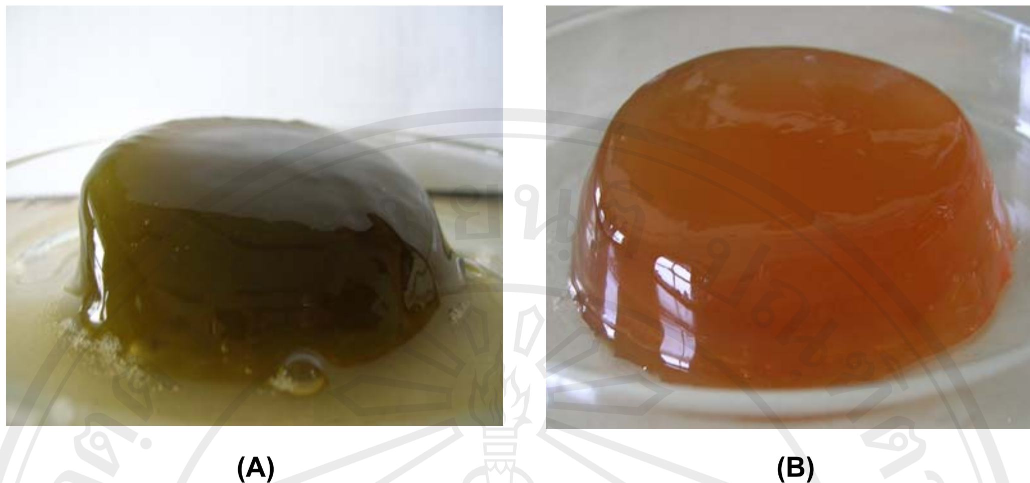

Figure 17 The gel formation of polysaccharide from Moo-noi leaves (A) Before centrifugation; (B) After centrifugation.

4.3 Analysis of polysaccharides by thin layer chromatography (TLC)

The chromatogram of Moo-noi extract obtained by TLC was shown in Figure18. It was found that sugar from Moo-noi extract (Lane8) was observed as polysaccahrides because band appeared was not separate. The results were consistent with the average calculated DP as shown in Table 5. While, hydrolyzed Moo-noi (Lane7) was showed 2 bands and seem to be mono-, disaccharide. The first band was galactose (Lane5) another band was arabinose. (Lane3). Ruangsuriya *et al.,* (2004) who hydrolyzed gel by acid and analyzed the sugar contents by thin layer chromatography and high performance liquid chromatography; simple sugar as arabinose was found. Singthong *et al.*, (2004) reported that the sugar composition of crude extract from Moo-noi leaves was contained 0.6% arabinose and 0.78% galactose. This seem to be similar with the result obtained from our result as condilation on the TLC chromatogram, we can concluded that Moo-noi extracted gel was not completely hydrolyzed as there is the big bane appearances close to the initial spot.

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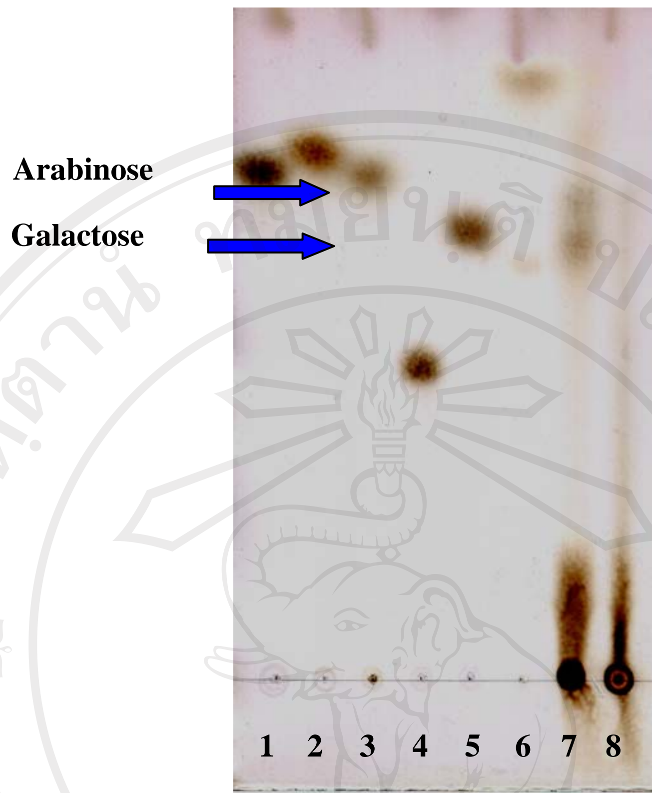

Figure 18 Sugar composition of Moo-noi by using TLC.

Note: Lane 1; glucose, Lane 2; mannose, Lane 3; arabinose, Lane 4; raffinose, Lane 5; galactose, Lane 6; gluculonic acid; Lane 7; acid hydrolyzed Moo-noi, Lane 8; crude extract of Moo-noi

4.4 Preparation of pectic oligosaccharides (POS) by enzymatic reaction

4.4.1 Effect of enzymes concentration on oligosaccharide production by pectinase

The effect of pectinase concentration on oligosaccharides production was shown in Figure 19. Polysaccharides were rapidly hydrolyzed by all tested concentration of pectinases as only within 10 minutes DP of Moo-noi polysaccharide was decreased from 21 to 3-5. The result showed that among 10, 20, 30 and 60 Units of the pectinase quantity, 10 units of enzyme concentration are the smallest and most economic quantity for obtaining the pectic oligosaccharide.

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However, the most suitable quantity of pectinases for hydrolysis of needed to be furthermore studied.

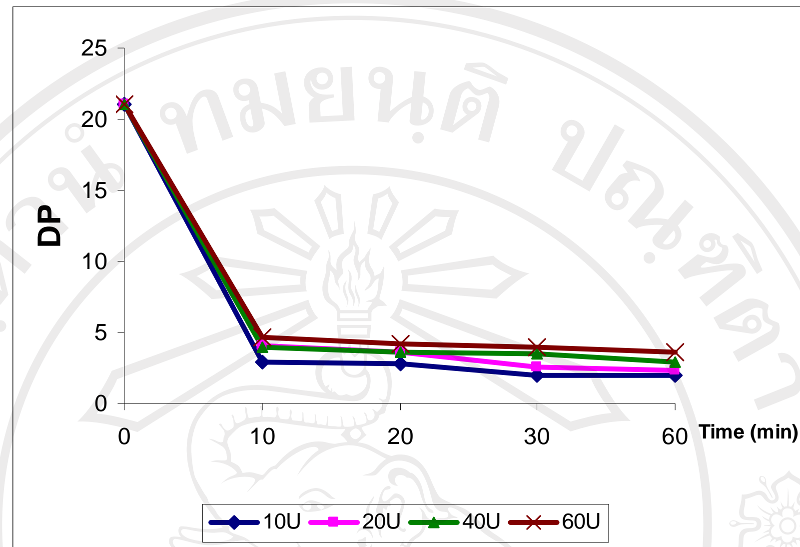

Figure 19 Effect of enzyme concentrations on enzymatic hydrolysis of 5 ml 0.2%(w/v) Moo-noi polysaccharide by commercial pectinases

4.4.2 Effect of extracted polysaccharide quantity on pectic oligosaccharide generation by commercial pectinases

The effect of various amounts of extracted polysaccharides on oligosaccharide production by pectinases was shown in Figure 20. The result showed that various amounts of extracted polysaccharide were rapidly hydrolyzed by 10 units of pectinases within 30 minutes and the DP value lower than 10 was obtained. That means those are all oligosaccharides.

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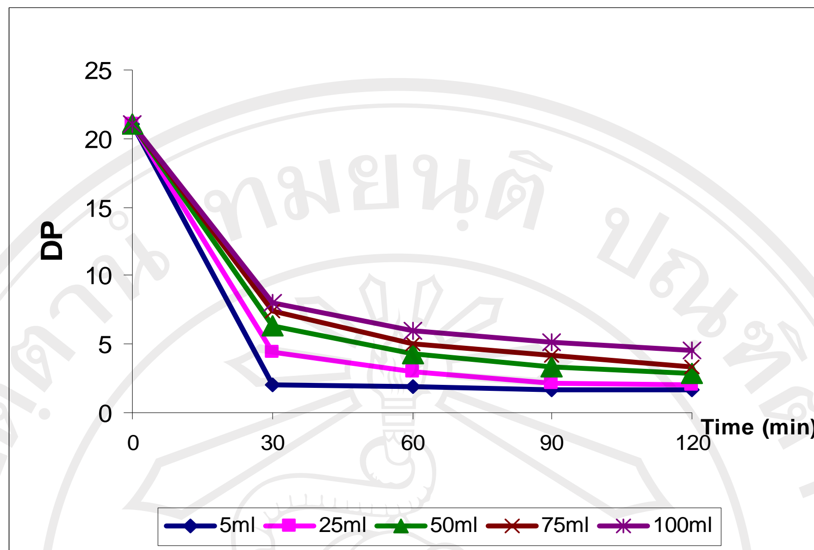

Figure 20 Effect of extracted polysaccharide quantity on enzymatic hydrolysis by 10 Units of pectinases

From our result, the condition we selected for preparing the pectic oligosaccharides was 100 ml of 0.2% extracted polysaccharide hydrolyzed by 10 units of pectinase and incubated at 37°C for 30 minutes, oligosaccharide DP 8 was obtained. Moreover, if the incubation time prolong to 120 min, the pectic oligosaccharides with average DP 5 will be obtained.

4.5 Study of prebiotic properties of prepared carbohydrates

4.5.1. *In vitro* study with defined microorganisms in pure cultures

After using extracted polysaccharide as carbon source for culturing 5 strains of probiotic bacteria and pathogens, growth of all tested strains in medium containing different carbon sources including pectin, POS, glucose and without carbon source were investigated. It was found that probiotic strains including *Ent. faecium*, LABG12 and LAB33 could be growth when cultured in the medium containing POS as showed in Figure 21 (A-E) and pH change in culture broth of each medium was showed in Figure 22 (A-E).

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However, *E. coli* and *S. havana* showed the highest growth when cultured in medium containing POS and without carbon sources, respectively, but were not able to grow on the medium containing glucose. This may be due to lower pH (Figure22A and 22B) in medium containing glucose that was decreased and generated the unsuitable condition for *E. coli* and *S. havana* to grow as the lower pH condition was not found in the medium containing pectin and POS. The growth of *S. havana* and *E. coli* in medium containing pectin and POS were suggested to be caused from peptone and yeast extract containing in the basal medium as their growths were observed when cultured in medium without carbon source, while growth of three lactic bacterial strains used in this experiment were not found.

The stimulation of *Ent. faecium* and LAB33 growth was observed in the medium containing POS, but it was lower than glucose, whereas LABG12 showed the higher growth on POS than on glucose. The population of LABG12 was increased during incubation time and still found in high number after 48 hours of incubation.

From the results, it was clearly notified that LAB displayed the higher growth on the medium containing POS as a carbon source than that of pectin from Moo-noi leaves. This might be caused from the difference in size between extracted pectin and enzymatic derived pectic oligosaccharides. Moo-noi pectin obtained from our result was 21-24 in DP as described previously, while size of the pectin extracted from Moo-noi by Singthong *et al.* (2004) was not reported about the DP value, but the molecular weight of 55 kDa. Concerning with the prebiotic property of pectin from Moo-noi leave, our results is agreed with that of Olano-Martin *et al*., (2002) who reported that pectic-oligosaccahrides are better prebiotic candidate than the pectin and low methylate pectin are a better substrate for bacterial growth than high methylate pectin. Dongowski and Lorenz (1998) showed that pectin with a DE of 95% was degraded more slowly than the low-esterified substrate with a DE of 35%. They found that highly esterified pectin

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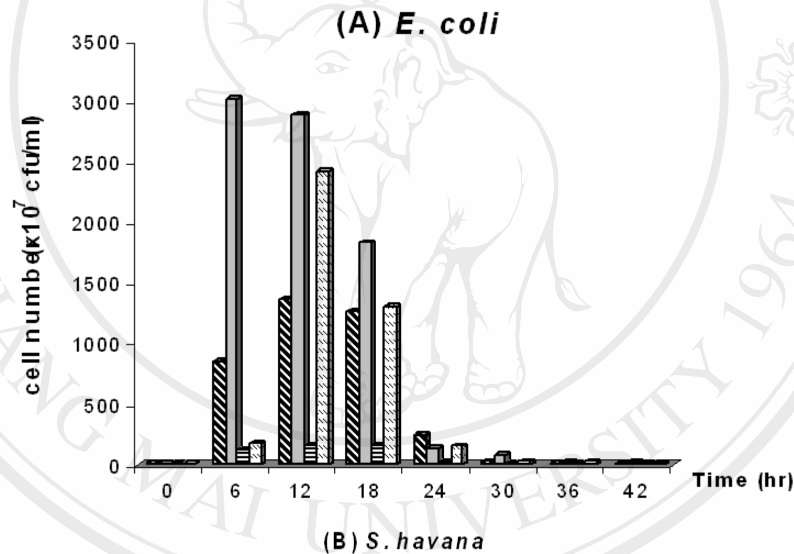
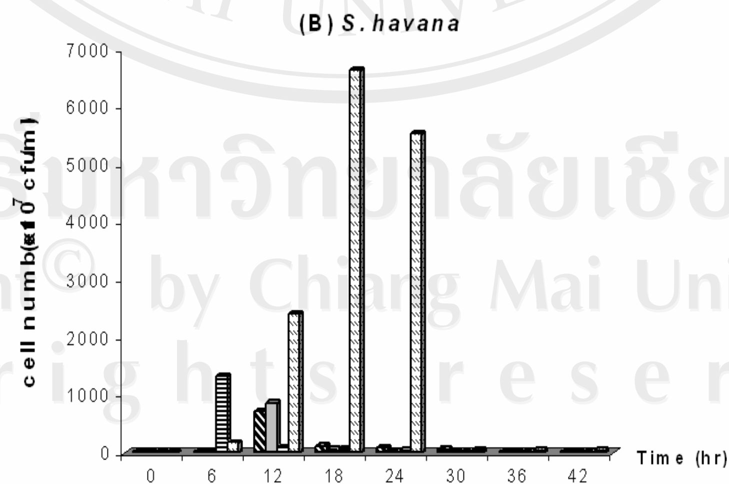
was still present after 24 hours of incubation with gut microflora whereas there was no trace of the low esterified pectins.

Oligosaccharides prepared (POS) from Moo-noi polysaccharide by enzymatic hydrolysis is possible to be used for prebiotic substances with their advantages as it can stimulate the growth of studied probiotic (LAB) and no/less chemical agent involve in preparation process. However, in pure cultures, most microorganisms capable to adapt themselves for the utilization of their carbon sources (Roberfroid, 2001). To confirm the prebiotic property of POS, the results of mixed cultures are necessary to support.





Pectin POS glucose without C-source

Figure 21 The growth of tested strains in different C-sources

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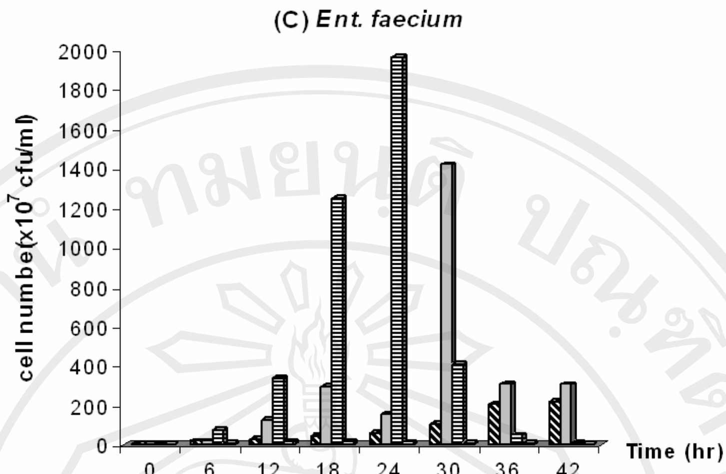

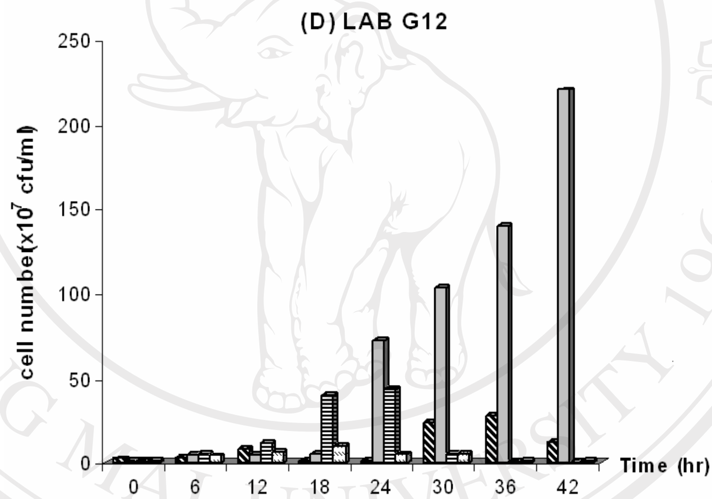

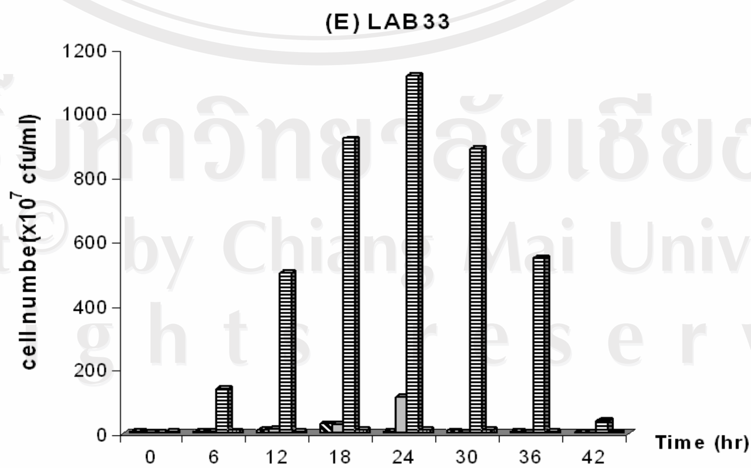

Pectin **POS** **glucose** **without C-source**

Figure 21 The growth of tested strains in different C-sources (continued).

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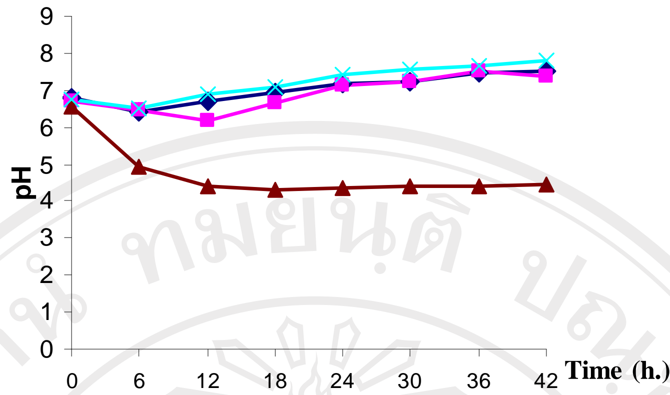

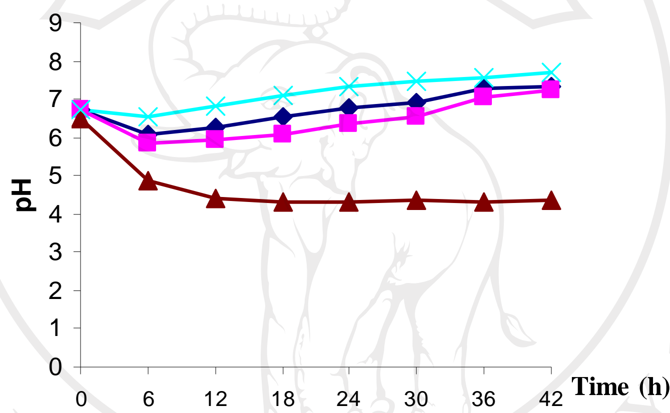

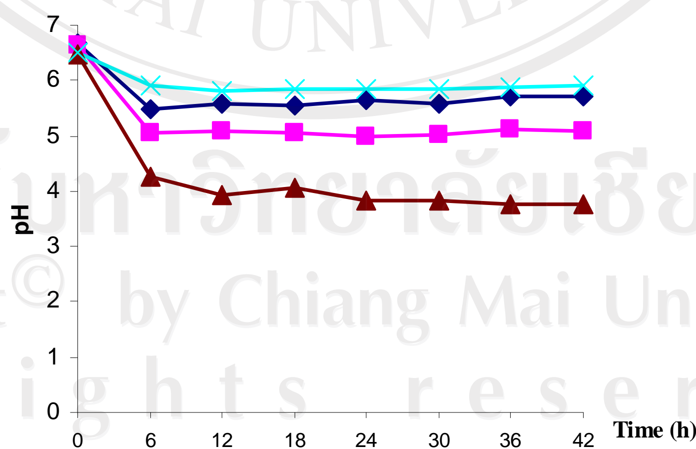

Pectin POS glucose without C

Figure 22 pH changes in culture broth during cultivation in different carbon sources

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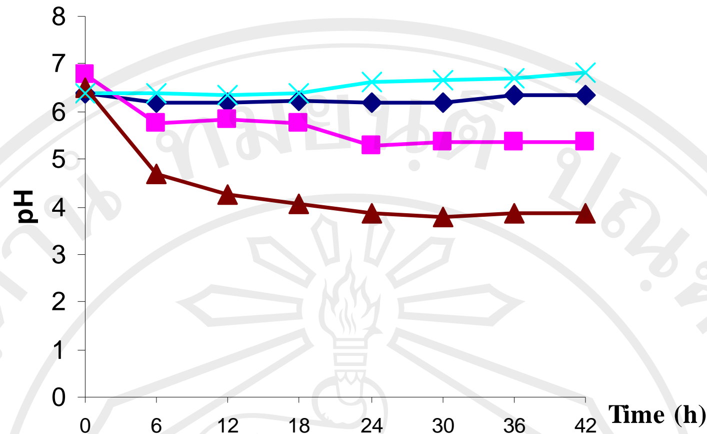
(D) LABG12



(E) LAB33

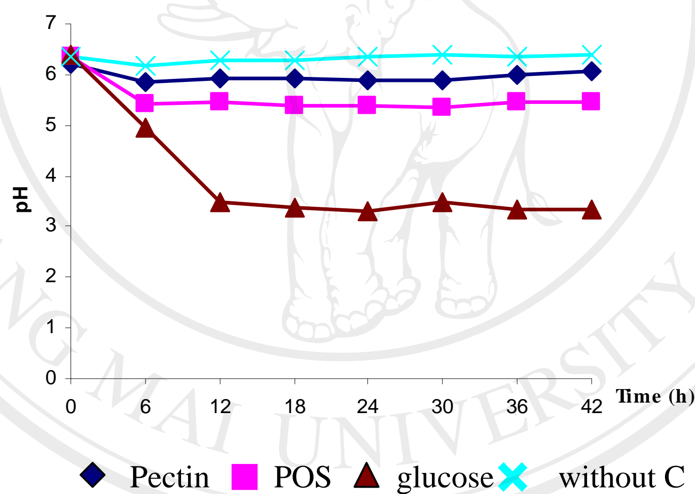


Figure 22 pH changes in culture broth during cultivation in different carbon sources (continued).

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4.5.2. Pathogen inhibition test by mixed cultures of defined microorganisms

From mixed culture test in basal medium containing different carbon sources lactic acid bacteria (*Enterococcus faecium*, LABG12 and LAB33) colonies were enumerated on MRS agar with oval shape and acid forming colony (yellow zone around colony). Black colony on SS-agar was counted as *S. havana*, while black colony with metallic sheen on EMB agar were counted as *E. coli* (see in Appendix

D). The cell number changes and pH were shown in Figure 23.1-23.3 and Figure 24 (A-C), respectively.

From Figure 23.1, the growth of LAB33 in mixed culture containing POS was lower than that of *E. coli* but the growth in glucose containing medium was increased and still high after 20 hours of incubation. Viable cell number of *S. havana* was not able to be detected since 12 hours of incubation in both POS and glucose medium, while those grew very well in the others. When comparison POS and without C-source, it was found that the growth of *E. coli* was decreased 35%, while 55.6% increasing of LAB33 growth was observed.

In case of mixed culture of LABG12, the lactic bacteria was not able to grow on pectin, but grew very well on POS. *S. havana* was not able to detect since 12 and 16 hours of incubation in glucose and POS containing medium, respectively. The result of mixed culture with *Ent. feacium* was similarly observed to that of the strain LABG12.

The viable cell number change in glucose containing medium, *S. havana* and *E. coli* growth were inhibited similarly to the result of POS containing medium. Actually, glucose exhibited to be a good carbon source for growth of all strains of LAB used in this experiment, but glucose is not considered as prebiotic because this sugar is known to be absorbed and utilized by host since the upper part of intestinal tract and will not reach the large intestine where the growth of probiotic microbes are needed for keeping the good microbial balance.

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Even LAB in mixed culture displayed the higher growth on POS as a carbon source than pectin and pectic-oligosaccahrides were reported as the better prebiotic candidate than the pectin (Olano-Martin *et al*., 2002). However, the sugar species or sugar types in oligosaccharide played an important effect on growth stimulation of lactic bacteria. Laere *et al*., (1997) produced a range of different NDOs with widely different sugar compositions and molecular sizes and tested their breakdown capability by several strains of *Bifidobacterium, Clostridium, Bacteroides*, and

Lactobacillus. They reported that the fermentabiility was differed with the oligosaccharide structure. The fructans were extensively fermented, except by clostridia, whereas few species were able to break down arabinoxylan under the conditions of the experiment. Xylooligosaccharides were well fermented. Linear oligosaccharides were catabolized to a greater degree than those with branched structures. Bifidobacteria utilized low-DP carbohydrates first and Bacteroides utilized those with a high DP (Hidaka *et al*., 1986). Metabolic collaboration among species was evident in carbohydrate breakdown. Both the structure of the carbohydrate and the bacterial species present in the ecosystem are probably important factors in controlling the fermentation of NDOs.

Consideration on our result, *S. havana* could grow in pectin containing medium, but they failed to grow in the medium supplemented with the enzymatic derived oligosaccharides. On the other hand, most of the LAB grew better in the medium containing oligosaccharides than the pectins. This indicated that the potential of oligosaccharides to be used as prebiotic as those were fail to sustain the growth of the pathogenic bacteria model (*S. havana*) and promote the growth of LAB. These findings were subsequently confirmed the mixed culture study in fecal slurry.

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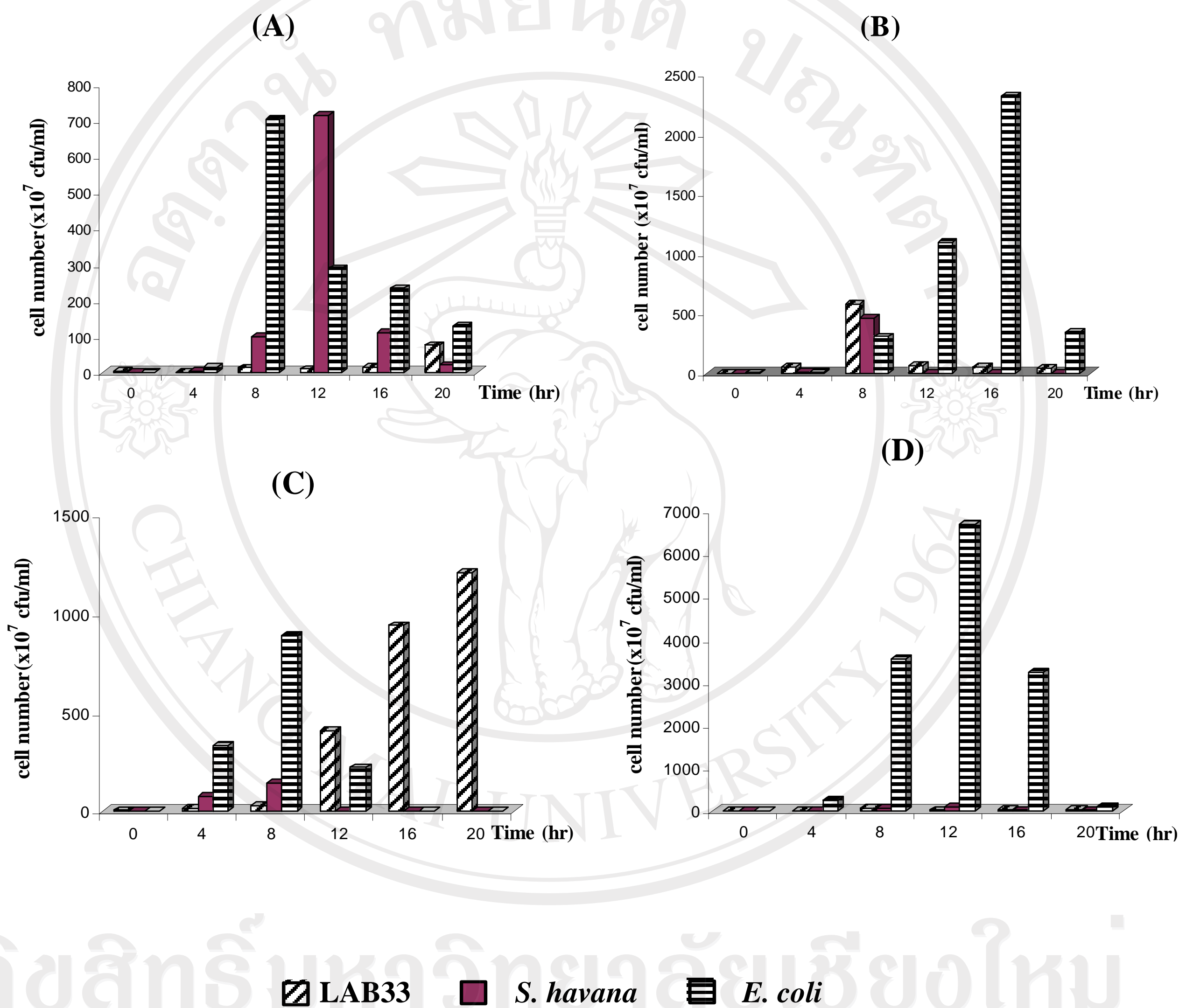

Figure 23.1 Cell number changes in different C-sources with mixed culture (LAB33 + *S. Havana* + *E. coli*)**:** (A) pectin; (B) POS; (C) glucose; (D) without C-source.

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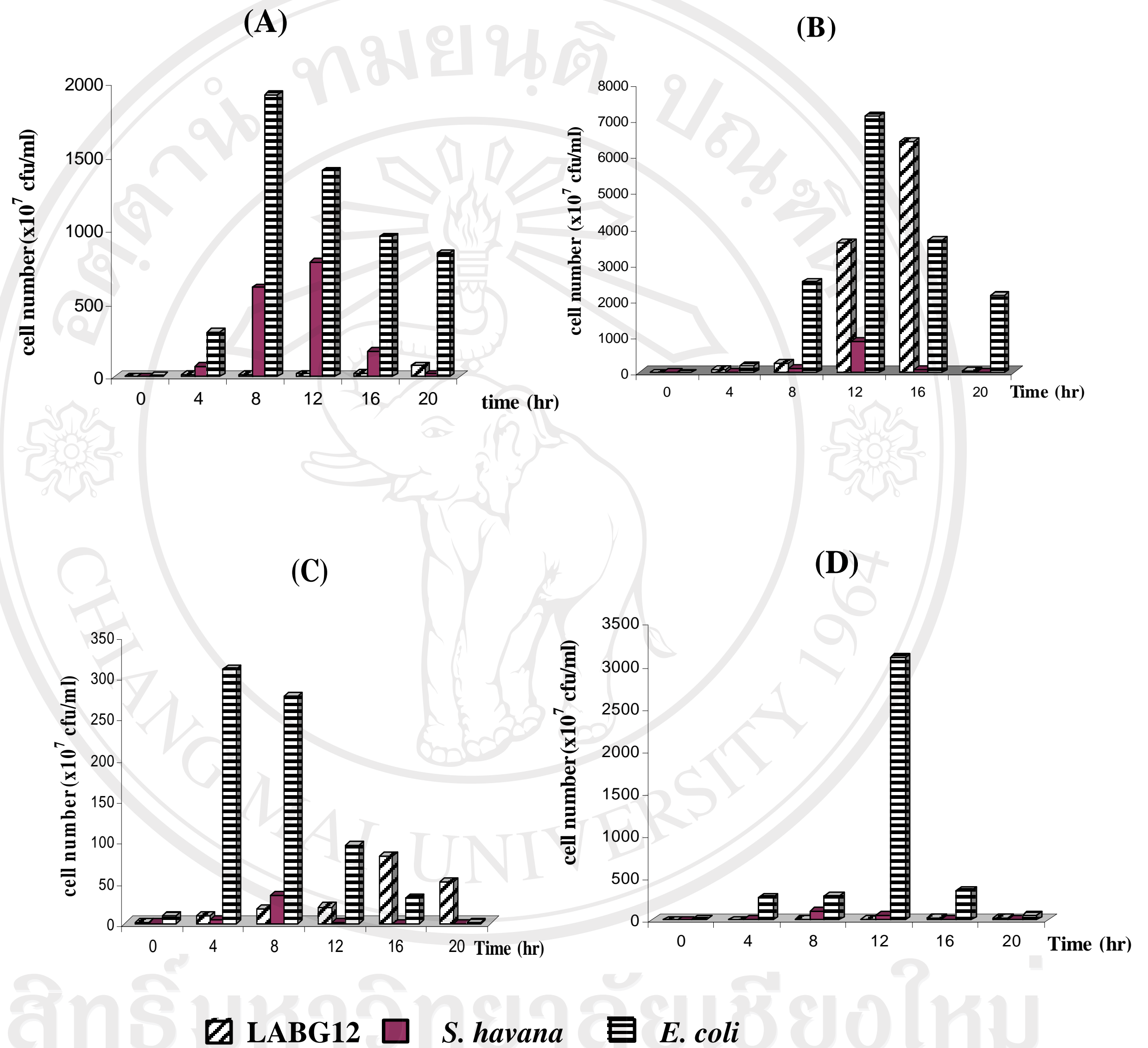

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Figure 23.2 Cell number changes in different C-sources with mixed culture (LABG12 + *S. havana* + *E. coli*): (A) pectin; (B) POS; (C) glucose; (D) without C-source.

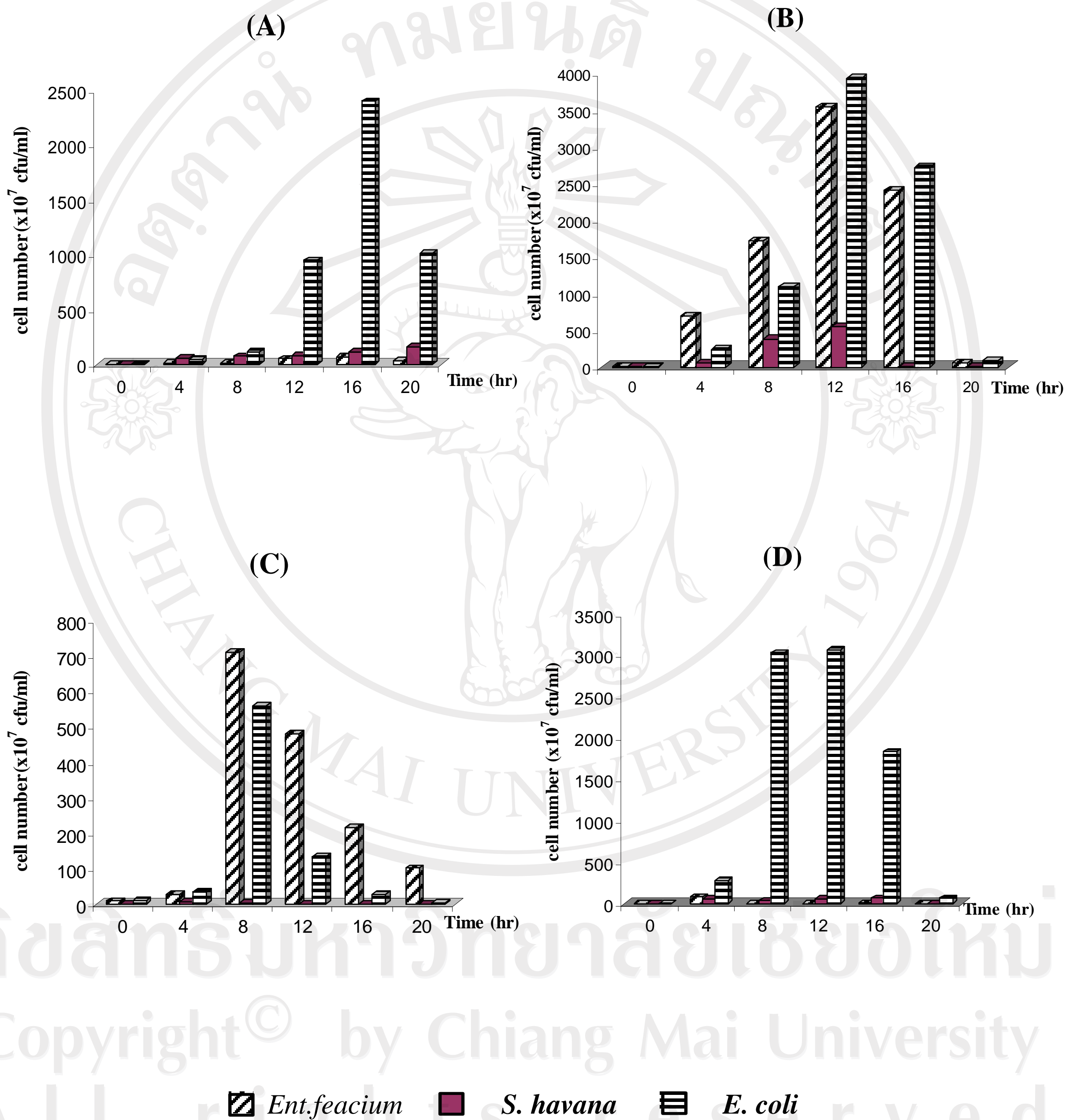

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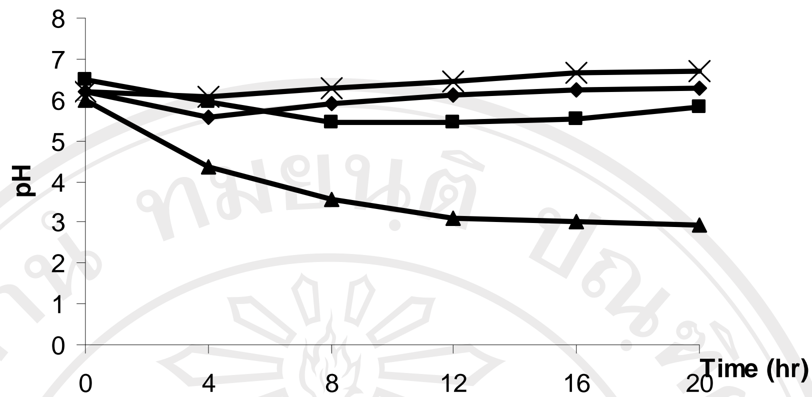
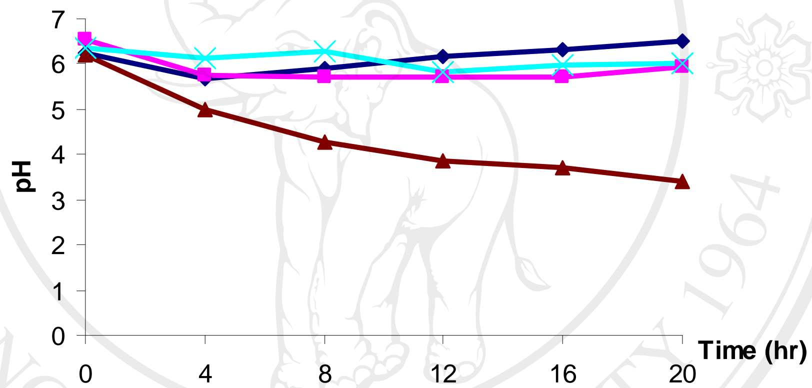
Figure 23.3 Cell number changes in different C-sources with mixed culture (*Ent. feacium* + *S. havana* + *E. coli*)**:** (A) pectin; (B) POS; (C) glucose; (D) without C-source.





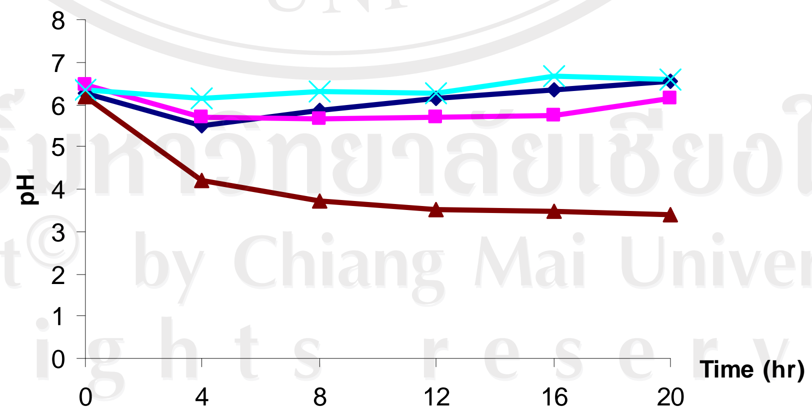


Figure 24 pH changes in different C-sources in mixed culture study

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4.5.3 Mixed culture study in fecal slurry

Fecal slurry of human was used as source of mixed microorganisms to investigate for prebiotic properties of pectin and POS. The results were shown in Table 6. The viable population of lactic acid bacterial increased during 12 hours of cultivation in all groups. In pectin, POS and glucose containing medium the growth of LAB was higher than without carbon source. *E. coli* showed the highest growth on without carbon source. While, *Salmonella* spp. was not detected in all treatments. So, the next experiment we added *S. havana* in all group. The results were shown in Table 7. *S. havana* decreased during 24 hours of cultivation in all group, however when consider in 12 hours, the viable cell of *S. havana* in pectin, POS and glucose were lower than that of without carbon source medium.

Olano-Martin *et al*., (2002) was studied pH-controlled mixed faecal bacteria batch culture fermentations. It was found that both of the pectins and the pectic-oligosaccharides significantly increased ($P<0.01$) the number of bifidobacteria and lactic acid bacteria increases during the fermentation. According to this results which the viable population of lactic acid bacterial increased during the fermentation of pentic and POS.

Pectins are metabolized by many species of the human gut microflora. Isolates reportedly obtained after pectin fermentation include *Bacteroides distasonis, Bacteroides ovatus* and *Bifidobacterium infantis* (Bayliss and Houston, 1984). These authors observed no selectivity of pectin fermentation towards bifidobacteria: the bacteria analyzed grew well with both high methylated and low methylated pectin

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The fermentation process in the gut is a sequence of different metabolic pathways carried out by bacteria growing in the colon. Each species has its own specialized ecological niche and frequently, the end products of one species are used as substrates by others. In this way, some microorganisms benefit from substrates they are not able to ferment (Gibson and Roberfroid, 1995). Therefore

mixed culture in fecal slurry was carried out, in order to evaluate the feasibility use of pectic-oligosaccharides as prebiotics.

Table 6 The result of bacterial viable cell number in fecal slurry culture

Determined microorganisms	Viable cell count (log cfu/ml)		
	0 h	12 h	24 h
Pectin			
Lactic acid bacteria	6.24±0.02	10.45±0.01	10.06±0.02
E. coli	5.91±0.04	9.76±0.04	9.42±0.01
Salmonella spp.	0	0	0
POS			
Lactic acid bacteria	6.15±0.01	10.63±0.01	10.00±0.04
E. coli	5.93±0.02	8.66±0.07	8.41±0.01
Salmonella spp.	0	0	0
Glucose			
Lactic acid bacteria	6.21±0.01	10.59±0.03	9.86±0.03
E. coli	5.77±0.02	9.82±0.04	9.11±0.03
Salmonella spp.	0	0	0
Without C-source			
Lactic acid bacteria	6.12±0.03	9.27±0.02	8.91±0.05
E. coli	5.94±0.04	10.05±0.07	9.86±0.03
Salmonella spp.	0	0	0

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Table 7 The result of bacterial viable cell number in fecal slurry culture with an addition of *S. havana*.

Determined microorganisms	Viable cell count (log cfu/ml)		
	0 h	12 h	24 h
Pectin			
Lactic acid bacteria	6.87±0.02	10.68±0.01	8.58±0.02
E. coli	5.06±0.04	9.73±0.04	9.94±0.04
Salmonella spp.	6.09±0.02	8.68±0.06	7.02±0.02
POS			
Lactic acid bacteria	6.97±0.02	10.57±0.01	8.17±0.02
E. coli	5.11±0.02	8.94±0.01	8.46±0.02
Salmonella spp.	6.06±0.05	8.80±0.08	6.48±0.03
Glucose			
Lactic acid bacteria	6.72±0.04	10.60±0.02	9.18±0.01
E. coli	5.87±0.02	9.01±0.04	9.20±0.06
Salmonella spp.	6.18±0.01	8.74±0.03	7.44±0.05
Without C-source			
Lactic acid bacteria	6.37±0.01	9.41±0.03	8.29±0.04
E. coli	5.88±0.03	9.99±0.01	8.81±0.03
Salmonella spp.	6.02±0.04	9.19±0.04	8.64±0.03

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