JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

THE EFFECTS OF COPRA-MANNO-OLIGOSACCHARIDE ON BACTERIUM AGGREGATION ACTIVITY AND MICROBIOLOGICAL CHANGES IN A SIMULATED DIGESTIVE TRACT

*Nawapan Pongsapipatana[1,2,3], Wireeya Chawjiraphan[1], Suttipun Keawsompong [1,2,3], and Sunee Nitisinprasert[1,2]**

Address(es):
[1] Specialized Research Unit: Prebiotics and Probiotics for Health, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.
[2] Center for Advanced Studies for Agriculture and Food, KU Institute for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand (CASAF, NRU-KU, Thailand).
[3] Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand.

*Corresponding author: fagisnn@ku.ac.th

https://doi.org/10.55251/jmbfs.4407

ARTICLE INFO

Received 26. 2. 2021
Revised 5. 11. 2022
Accepted 22. 11. 2022
Published 1. 2. 2023

Regular article

OPEN ACCESS

ABSTRACT

This study aimed to present the interaction of Mannooligosaccharide (MOS) produced by optimum hydrolytic reaction of the recombinant mannanase KMAN treated defatted copra meal (DCM) to bacterial aggregation activity and to microbial changes in simulated gastro-intestinal tract which were two main functions of prebiotics as a decoy receptor and a substrate for bacterial fermentation, respectively. The suitable ratio of KMAN and DCM (0.8U:1mg) achieved high DCM hydrolysate-MOS production of 2.13-2.38 g/l during 6-24 h. It enhanced *Limosilactobacillus reuteri* KUB-AC5 growth for 0.32-0.33 h^{-1} and accelerated autoaggregation activity by 95.86% and 62.89% at 37 and 42°C, respectively. It decreased autoaggregation activities of *Salmonella* Enteritidis DMST17368 by 75.03-81.66%, and *Lactobacillus johnsonii* KUNN19-2 by 38.68-55.63% at 24 h. By simulated human GI model, COPRA-MOS significantly enhanced only *Clostridium coccoides-Eubacterium rectale* at 24 h with high acetic acid concentration of 87.43 mM. Whereas the chicken simulation model led to low abundance of Enterobacteriaceae but a higher *Lactobacillus* population with acetic acid concentration of 22.94 mM. These results suggested that COPRA-MOS could potentially apply for poultry industry as it exhibited beneficial effect on cecal fermentation and could be used as a decoy to prevent colonization of a pathogen while to increase the autoaggregation leading to adherence of a probiotic.

Keywords: Aggregation activity, Copra meal, Mannanase KMAN, Mannooligosaccharides

INTRODUCTION

Copra meal (CM) is an agricultural waste from the coconut cream and oil industries which is widely used in feedstock. However, 60-70% of carbohydrate content in CM contains high amounts of mannan (26%), galactomannan (61%) and cellulose (13%) which are considered as an anti-nutritional substance **(Ariandi *et al*., 2015; Sundu & Dingle, 2003)**. To overcome this problem, β-mannanase treatment has been actively studied to obtain more value-added compounds as mannooligosaccharides (MOS) **(Chauhan *et al*., 2012; Srivastava & Kapoor, 2017)**. In our laboratory, **Pongsapipatana *et al.* (2016)** successfully cloned and expressed a novel mannanase *kman-2* from *Klebsiella oxytoca* KUB-CW2-3 which had a broad range of substrate specificity of homopolymers and heteropolymers. Mannanase hydrolysis of defatted CM (H-DCM) yielded mannobiose (M2), mannotriose (M3) and mannotetraose (M4) which enhanced the growth of lactic acid bacteria (LAB). The gene coding for mannanase KMAN was subcloned into pFlag-CTS system to obtain the recombinant *E. coli* KMAN-3 exhibiting activity up to 400 times higher than the activity of native enzyme produced from *K. oxytoca* KUB-CW2-3. The deduced amino acid of KMAN-2 displayed two starting codons of methionine at the N-terminal. Gene coding for the second starting codon was subcloned and expressed by the same system as KMAN-2 resulting in the recombinant clone KMAN-3 which was used in this study. The highest activities were obtained from periplasmic fraction which was higher than KMAN-2 for 10.7 folds.

In recent years, numerous studies have investigated gut microbiota and their relationship with dietary carbohydrates such as prebiotics and human health. MOS, one of the potential prebiotic, has also been reported as feed supplement in the form of yeast cell walls for pig and poultry **(Markowiak & Śliżewska, 2018; Ricke *et al*., 2020)**. Enzymatic treatment of agricultural waste such as CM treated with β-mannanase, resulted in MOS as well including a small amount of mannose and could improve feed digestibility and provide an energy source, while also promoted the growth of beneficial microorganisms in the gastrointestinal tract **(Sundu & Dingle, 2003)**. MOS from other plant-based sources such as coffee mannan, konjac glucomannan, guar gum and ivory nut mannan as well as their prebiotic roles have also been reported **(Asano *et al*., 2003; Srivastava & Kapoor, 2017)**; however, scant information exists regarding MOS from CM. **Pangsri *et al.* (2015)** reported that enzymatic hydrolysis of defatted copra meal with mannanase M1 from *Bacillus circulans* NT 6.7 promoted growth of the *Lactobacillus* group and inhibited pathogenic bacteria such as *Shigella dysenteria* DMST 1511, *Staphylococcus aureus* TISTR 029, and *Salmonella enterica* serovar Enteritidis DMST 17368. In a previous study, we presented a potential prebiotic property of COPRA-MOS produced by KMAN treated CM with the optimal condition of enzyme and substrate ratio at 4 :1 (units per gram) for up to 24 h at 40°C and resulted in enhancing the growth of two potential probiotic strains such as *L. reuteri* KUB-AC5 and *L. johnsonii* KUNN19-2 **(Pongsapipatana *et al*., 2016)**. Usually, the half-life of mesophilic enzyme activity is about 10-90 min **(Srivastava & Kapoor, 2017)**, and it is important to know the suitable reaction time of each typical enzyme. Here, suitable reaction time to produce COPRA-MOS by enzymatic hydrolysis of mannanase KMAN from *E. coli* KMAN-3 was presented. These certain prebiotics, and some bacteria in GI-tract, can shape the composition of the gut microbiota and its metabolic activities to promote host health and/or prevent diseases. Some prebiotics are not only substrates for selective fermentation for probiotics, but also play a role in protection against pathogens by acting as a decoy and directly interaction with pathogenic bacteria **(Monteagudo-Mera *et al*., 2019)** in the form of autoaggregation which is a common phenomenon of bacteria from the same strain forming multi cellular bacterial clumps and *in vitro* settling at the bottom of culture tubes due to chemicals or electrostatic interaction between cell surface molecules **(Trunk *et al*., 2018; Nwoko & Okeke, 2021).** Autoaggregation of bacterial cells may also involve with some molecules that act as aggregation promoting factors such as cell-surface proteins, exopolysaccharides, carbohydrates, glycoproteins, teichoic and lipoteichoic acid secreted proteins **(Isenring *et al*., 2021)**. It could contribute to the adherence of beneficial bacteria to host's GI tract and forms a protective barrier against colonization and infection of pathogens **(Lukic *et al*., 2014).** Therefore, its hydrolysate, COPRA-MOS, characters affecting gut microbiota including COPRA-MOS participation in bacterial cell aggregation for potential probiotic adhesion and pathogen prevention were also proposed.

MATERIAL AND METHODS

Preparation of defatted copra meal

CM obtained from the commercial coconut milk industry was ground and sieved (Retch, Germany) to obtain a particle size of 40 mesh (less than 400 µm according to Ecologix environment system). Then, soxhlet extraction was performed following the procedure of the Association of Official Analytical, Helrich **(Association of Official Analytical & Helrich, 1990)** to remove fat contents to increase hydrolysis efficiency **(Pongsapipatana *et al.*, 2016)**. The defatted copra meal (DCM) was used as a substrate for COPRA-MOS production.

Bacterial strains and culture conditions

The recombinant *E. coli* KMAN-3, harboring pFlag-CTS vector fused with *kman-3* coding for mannanase KMAN from *K. oxytoca* KUB-CW2-3 **(Titapoka *et al.*, 2007)** and used *E. coli* TOP10 as an expression host, was used as a source of mannanase production and cultured in Luria-Bertani broth (1% tryptone, 0.5% yeast extract and 1% NaCl) containing 100 µg/ml ampicillin and incubated at 37°C on a rotary shaker at 200 rpm for 18 h **(Pongsapipatana *et al.*, 2016)**. A pathogenic bacteria *Salmonella* Enteritidis DMST 17368, a reference strain obtained from the National Institute of Health, Department of Medical Sciences Thailand (DMST) (http://nih.dmsc.moph.go.th/), and probiotics strain *Limosilactobacillus reuteri* KUB-AC5 (*Lactobacillus reuteri* KUB-AC5) **(Nitisinprasert *et al.*, 2000)**, as well as *L. johnsonii* KUNN19-2 **(Prommadee *et al.*, 2012)** used for COPRA-MOS quality evaluation were cultured at 37°C for 18 h in NB (Nutrient broth, Merck, Germany) on a rotary shaker at 200 rpm and MRS (de Man, Rogosa and Sharpe, Difco, France), respectively.

Enzyme production

Mannanase KMAN was produced following the procedure of **Pongsapipatana *et al.* (2016)**. Briefly, one percent of overnight culture of *E. coli* KMAN-3 was transferred to 1 liter of fresh Luria-Bertani (LB) broth containing 100 µg/ml ampicillin and cultured at 37°C with shaking at 200 rpm until OD_{600} reached 0.6. Then, IPTG was added to a final concentration of 0.25 mM and subsequently incubated at 26°C for 8 h. The culture solution obtained was further incubated on ice for 5 min. Cell pellets were collected after centrifugation at 4°C, 8000×*g* for 10 min and resuspended in 50 ml of spheroplast buffer (0.5 mM sucrose, 100 mM Tris-HCl pH 8, 0.5 mM EDTA pH 8 and 20 µg/ml phenylmethane-sulfonyl fluoride, PMSF) on ice for 5 min. The supernatant was discarded by centrifugation at 8000×*g* for 10 min. Cell pellets were resuspended in 100 ml of ice-cold sterile deionized water supplemented with 1 mM $MgCl_2$ and shaken frequently for 5 min. The supernatant, obtained by centrifugation at 4°C and 8000×*g* for 10 min, was stored at 4°C as the periplasmic mannanase.

Enzyme assay

Mannanase activity was assayed using 1% (w/v) locust bean gum as a substrate according to the procedure of **Pongsapipatana *et al.* (2016)**. Reducing sugars released were determined using the 3,5-dinitrosalicylic acid (DNS) method **(Miller, 1959)**. One unit of enzyme was defined as the amount that liberated 1 µmole of mannose per minute under assay conditions.

Production of COPRA-MOS by enzymatic reaction

COPRA-MOS was prepared by enzymatic hydrolysis of DCM according to the modified method of **Pongsapipatana *et al.* (2016)**. Due to the optimum pH and temperature of 4 and 40°C and the stability in the range of 4-10 and 10-40°C, respectively, the reaction containing 1% (w/v) of DCM dissolved in 50 mM citrate buffer (pH 4) and mannanase KMAN from *E. coli* KMAN-3 at final concentration of 8 units/ml was carried out at 40°C for 0, 1, 3, 6, 12 and 24 h, and stopped by boiling for 10 min. After centrifugation at 9,100 ×*g* for 10 min, the supernatant containing MOS was stored at 4°C until required for further study.
Large scale preparation of COPRA-MOS at 1 liter under optimal conditions was carried out and freeze-dried using CoolSafe 110, Scanvac, Denmark. The sample was poured into 4.5 × 4.5 cm aluminum trays to a depth of 0.5 cm. Each tray was covered by a parafilm film with small holes and pre-frozen at -80°C overnight (18-24 h). The freeze-drying process was carried out at -110°C for 48 h and the product was stored at -20°C until required for further study.

Analysis of mannan hydrolysate by High performance liquid chromatography (HPLC) and Thin layer chromatography (TLC)

HPLC analysis of H-DCM was performed using Aminex® HPX-42C Column (300 × 7.8 mm) (BioRad, USA) at 75 °C of external temperature and 40 °C of internal temperature using deionized water as mobile phase. Each sample was run for 60 min at the flow rate of 0.4 ml/min. Mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5) and mannohexaose (M6) were used as standards at the concentration range of 0.125-2 mg/ml. For TLC analysis of H-DCM was performed by TLC according to the procedure of **Pongsapipatana *et al.* (2016)**. Glucose, galactose and mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5) and mannohexaose (M6) were used as standards at concentrations of 10 mg/ml.

Determination of bacterial growth

Two probiotic strains of *L. reuteri* KUB-AC5 **(Nitisinprasert *et al.*, 2000)** and *L. johnsonii* KUNN19-2 **(Prommadee *et al.*, 2012)** including *S.* Enteritidis DMST 17368 were used for growth evaluation against COPRA-MOS. Culture reactions and growth determinations were performed according to the procedure of **Pongsapipatana *et al.* (2016)**.

An *in vitro* model of batch intestinal fermentation of mannooligosaccharide (MOS)

Batch culture fermentation was set up using 100 ml-vessels (70 ml working volume) for human and chicken gastrointestinal tract simulation. A 1% of COPRA-MOS solution (w/v) was used as the sole carbon source for treatment condition compared to control (without COPRA-MOS).
For human condition, basal medium (BM) ingredients (per liter) contained 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.6H_2O$, 2 g $NaHCO_3$, 2 ml Tween 80, 0.05 g hemin, 10 µl vitamin K1, 0.5 g L-cysteine-HCl, 0.5 g bile salt and 4 ml resazurin (0.05 g/L) **(Onumpai *et al.*, 2011)** and was adjusted to pH 7.4. The medium was sterilized at 121°C for 15 min. A fecal sample was obtained from a healthy volunteer who had not taken probiotics or antibiotics for three months. One gram of fecal sample was dissolved in 9 ml of PBS (phosphate buffer saline, pH 8.0) and homogenized in a stomacher (Stomacher®80 Biomaster, Seward Medical, UK) at maximum speed for 2 min. One percent of fecal slurries were added into the medium of each vessel. Fermentation was carried out at 37°C under anaerobic condition for 24 h and maintained at pH 6.65-6.95 by 0.5M NaOH and 0.5M HCl. Four milliliters of culture solution were withdrawn at 0, 6, 12 and 24 h. and kept at -20°C for further evaluation of microbiota and short chain fatty acid contents. Each treatment was carried out with two replications.
For chicken condition, Viande Levure (VL) medium was used as BM according to the modified method of **Nisbet (2000)** containing 5 g yeast extract, 5 g NaCl, 0.6 g cysteine-HCl, 2.4 g beef extract, 10 g tryptose and 1 ml resazurin (0.025 g/L) and adjusted to pH 5.5. The medium was sterilized at 121°C for 15 min. A cecal sample obtained from a healthy male 21-d Arbor Acres Plus Broiler fed with commercial feed without antibiotic was used as a microbial community source. The fermentation experiment was similar to the human model except that incubation temperature and pH of 42°C and 5.6-6.0, respectively, were applied.

Real-Time PCR analysis of gut microbiota

Genomic DNA from fecal and cecal culture solutions was extracted using a combination technique of QIAamp DNA Stool Mini Kit (QIAGEN, Germany) and zirconia bead **(Sakamoto *et al.*, 2011)**. Primers targeting bacterial strains for human and chicken gut microbiota are listed in Table 1. Real-Time PCR analysis was performed according to the modified method of **La-ongkham *et al.* (2015)** using LightCycler®480 Real-Time PCR (Roche, Germany). Each reaction comprised 10 µl of 2X FastStart Essential DNA Green Master (Roche, Germany), 0.8 µl of each primer (0.5 µM/µl), 2 µl of DNA template (10-50 ng), with adjustment to a final volume of 20 µl by deionized water. The amplification program consisted of one cycle of pre-incubation at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at optimal temperature shown in the Table 1 for 10 sec and extension at 72°C for 10 sec. A melting curve was generated during post-extension at 72°C.

Table 1 Primers targeting bacterial genera in human and chicken gut model

Species	Primer name	Nucleotide (5'-3')	Tm (°C)	Size (bp)	Reference
Prevotella spp.	g-Prevo-F g-Prevo-R	CACRGTAAACGATGGATGCC GGTCGGGTTGCAGACC	62	513	**(Matsuki *et al*., 2002)**
Bacteroides fragilis	Bfra-F2 Bfra-R	AYAGCCTTTCGAAAGRAAGAT CCAGTATCAACTGCAATTTTA	53	495	**(Matsuki *et al*., 2002)**
Clostridium leptum	sg-Clept-F sg-Clept-R3	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	239	55	**(Matsuki *et al*., 2004)**
Bifidobacterium spp.	BifF BifR	TCGCGTC(C/T)GGTGTGAAAG CCACATCCAGC(A/G)TCCAC	62	243	**(Rinttilä *et al*., 2004)**
C. coccoides-E. rectale group	ClosF ClosR	CGGTACCTGACTAAGAAGC AGTTT(C/T)ATTCTTGCGAACG	51	429	**(Rinttilä *et al*., 2004)**
Enterobacteriaceae	EnF EnR	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	57	195	**(Bartosch *et al*., 2004)**
Lactobacillus spp.	LbF LbR	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	53	341	**(Walter *et al*., 2001)**
Campyrobacter sp.	CamF CamR	GGATGACACTTTTCGGAG AATTCCATCTGCCTCTCC	54	246	**(Rinttilä *et al*., 2004)**
Acinetobacter	AcF AcR	TTTAAGCGAGGAGGAGG ATTCTACCATCCTCTCCC	52	240	**(Vanbroekhoven *et al*., 2004)**
Pseudomonas	PseF PseR	GGCGACGATCCGTAAC CCTTCCTCCCAACTT	57	180	**(Khan & Yadav, 2004)**
Bacteroides-Prevotella-Porphyromonas	BacPF BacPR	GGTGTCGGCTTAAGTGCCAT CGGA(C/T)GTAAGGGCCGTGC	57	140	**(Rinttilä *et al*., 2004)**
C. perfringens group	PerfF PerfR	ATGCAAGTCGAGCGA(G/T)G TATGCGGTATTAATCT(C/T)CCTTT	51	120	**(Rinttilä *et al*., 2004)**

The standard curve of each bacterial group was performed according to the method of **La-ongkham *et al*. (2015)**. For human gut microbiota, *Prevotella* spp., *Bacteriodes fragilis*, *Clostridium leptum*, *Bifidobacterium* spp., *Clostridium coccoides-E.rectale*, Enterobacteriaceae and *Lactobacillus* spp. were constructed using specific primers to amplify the genomic DNA of *Prevotella anigrescens* JCM 12250, *Bacteriodes fragilis* ATCC 25285, *Clostridium leptum* DSM 753, *Bifidobacterium bifidum* JCM 1255, *Blutia productus* JGD 07421, *Salmonella* Typhimurium TISTR 292 and *Lactobacillus salivarius* AC21, respectively. Chicken gut microbiota standard curves were constructed by *Campylobacter jejuni* ATCC 33291, *Acinetobacter calcoaceticus* TISTR 360, *Pseudomonas* spp. TISTR 1249, *Bifidobacterium bifidum* JCM 1255, *Blutia productus* JGD 07421, *Salmonella* Typhimurium TISTR 292, *Lactobacillus salivarius* AC21, *Bacteriodes fragilis* ATCC 25285 and *Clostridium perfringens* ATCC 13124 to determine the bacterial groups of *Campylobacter* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Bifidobacterium* spp., *Clostridium coccoides-E. rectale*, Enterobacteriaceae, *Lactobacillus* spp., *Bacteriodes-Prevotella-Porphyromonas* and *Clostridium perfringens*, respectively according to the method of **Nakphaichit *et al*. (2014)**.

Short chain fatty acid (SCFA) analysis

SCFA analysis was performed according to the modified method of **Onumpai *et al*. (2011)**. Briefly, 1 ml of culture solution from each vessel was centrifuged at 15,300 ×*g* for 5 min to collect the supernatant and kept at -20°C. Each sample was filtered using 0.2 µm membrane prior to analysis. Organic acids were analyzed by HPLC using Aminex® HPX-87H Column (Bio-Rad, USA). HPLC was performed at 55°C using sulfuric acid at a concentration of 0.005 M as mobile phase at a flow rate of 0.6 ml/min for 40 min. Tartaric acid at a concentration of 0.2% (w/v) was used as internal standard and mixed with samples at a ratio of 2:1 prior to analysis. Concentration of organic acids was calculated from the standard curves of lactic acid, acetic acid, propionic acid and butyric acid at concentrations of 0.1-0.5% (w/v).

Determination of autoaggregation activity

Aggregation of bacterial cells was assayed according to the modified method of **Kos *et al*. (2003)**. Target strains, *L. reuteri* KUB-AC5, as well as *L. johnsonii* KUNN19-2 and *S.* Enteritidis DMST 17368, were cultured in MRS and Nutrient Broth, respectively, for 18 h. Cell pellets were collected by centrifugation of overnight cultures at 4°C, 8,000 ×*g* for 10 min and further washed with PBS buffer (pH 7.4). The cell pellets obtained were resuspended in PBS buffer and adjusted to the absorbance of 0.5 at 600 nm. The pH of COPRA-MOS solution (1.7 mg/ml) was also adjusted to neutral pH by 1 N NaOH. To determine the effect of COPRA-MOS on cell aggregation, three treatments of bacterial cells alone, COPRA-MOS alone and a combination of cells and COPRA-MOS were performed. A combination reaction composed of solutions of cells and COPRA-MOS at the ratio 1:1 (2 ml:2 ml) provided final COPRA-MOS concentration of 0.085% (w/v), while another two treatments were performed using PBS buffer instead of either of them at the same ratio. The reactions were incubated at 37 and 42°C. Two hundred microliters of upper phase of solution were withdrawn at 0, 5 and 24 h to measure the absorbance at 600 nm. The results expressed as % autoaggregation were shown in the equation below:

Percentage of autoaggregation = $(A_0 - A_t)/A_0 \times 100$

where A_0 is the initial absorbance at 600 nm and A_t is the absorbance at 600 nm at time t.

Statistical analysis

All results were analyzed using one-way ANOVA with post hoc tests, LSD (the Least Significant Difference) and Duncan with the statistically significant at $P \leq 0.05$.

RESULTS

Time course of COPRA-MOS production in a scale-up of 1 liter

In a previous study by **Pongsapipatana *et al*., (2016)**, 24 h defatted copra meal hydrolysate (H-DCM) containing COPRA-MOS which mainly consisted of M2, M3 and M4 was produced at laboratory scale of 200 µl for 24 h under optimal conditions. However, reaction time can impact on mannanase stability. Therefore, various reaction times of 1, 3, 6, 12 and 24 h were performed to obtain a suitable hydrolytic time for H-DCM production. The results are shown in the figure 1. Productivities of H-DCM expressed as reducing sugar rapidly increased within 1 h for 1.74 g/l/h and then decreased during 3-24 h.

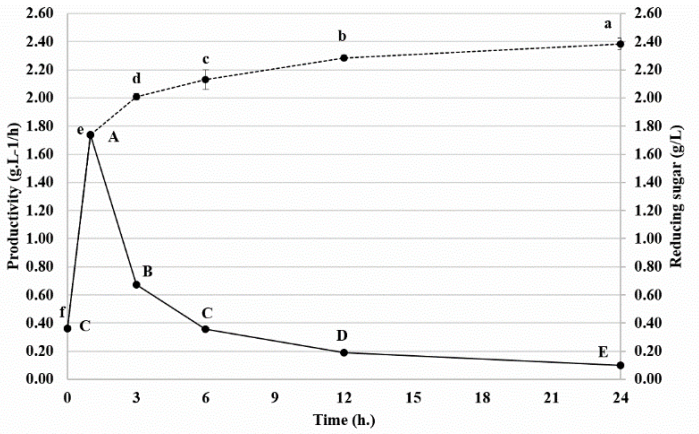

Figure 1 Time course hydrolysis of defatted copra meal (DCM). Productivity ( ) and reducing sugar contents (—●—). [a-f, A-E] Mean data showing different common superscripts are significantly different ($P \leq 0.05$)

Maximum H-DCM concentration occurred at 24 h for 2.38 ± 0.042 g/l. Oligosaccharide composition of H-DCM named COPRA-MOS analyzed by HPLC (Fig 2A) was M1, M2 and M3. However, the remaining products could not be analyzed because of the interference with the peak of citrate buffer (pH4) used in

the hydrolysis reaction which covered the peak area of M4 and higher molecular weight. The amount of M1, M2 and M3 calculated from peak area were significantly increased to 1.36, 4.48 and 5.96 mg/ml, respectively after 24 h digestion (Fig 2B). The remaining end-products were confirmed by TLC (Fig 2C). The products consisted of M2, M3, M4 and higher molecular weight than M4 (M4-H) which showed similar oligosaccharide chromatograms for all 1, 3, 6, 12 and 24 h enzyme reactions. The product M1 could not be detected in TLC due to its low amount and the sensitivity was lower than HPLC. Different intensities of each product were shown with treatment 6-24 h trending to be higher than 1-3 h. The increase in the amount of products from TLC was corresponded to the amount of hydrolysis product analyzed by HPLC. Therefore, H-DCM from 6, 12 and 24 h reaction time, named treatments COPRA-MOS-6, COPRA-MOS-12 and COPRA-MOS-24, respectively, were further tested for their bacterial interaction to observe their effect on the growth of two probiotics, *L. johnsonii* KUNN19-2 and *L. reuteri* KUB-AC5 comparing to a positive control (glucose and commercial MOS Actigen™) and negative one (BM) for choosing better condition for COPRA-MOS production used in further studies.

Growth of the two probiotics, *L. johnsonii* KUNN19-2 and *L. reuteri* KUB-AC5 in the presence of 0.05% (w/v) of treatment outputs for COPRA-MOS-6, COPRA-MOS-12 and COPRA-MOS-24 were evaluated by comparing with control treatments. Results showed that all COPRA-MOS treatments enhanced growth of *L. johnsonii* KUNN19-2, with specific growth rates of 0.60-0.64 h^{-1} which were statistically equal to glucose treatment (Table 2). Concentrations of 2.13-2.38 g/l were sufficient to enhance the growth of *L. johnsonii* KUNN19-2. While specific growth rates of *L. reuteri* KUB-AC5 were significantly lower than glucose treatment ($P < 0.05$) with no significant difference to either BM or Actigen™ treatment. However, the COPRA-MOS-24 treatment exhibited significantly higher specific growth rate of *L. reuteri* KUB-AC5 than BM and the commercial prebiotic Actigen™ for about 1.5 times. It seemed that COPRA-MOS trended to enhance the growth of *L. reuteri* KUB-AC5 compared to the control ($P \leq 0.2$). Therefore, higher contents of MOS supported the growth of *L. reuteri* KUB-AC5. Since all COPRA-MOS conditions tended to support the growth of tested probiotics, and COPRA-MOS-6 treatment exhibited higher productivity than COPRA-MOS-12 and COPRA-MOS-24 while all showed similar oligosaccharide composition. Therefore 6 h hydrolytic reaction was selected to prepare large scale production of 1 liter to obtain enough COPRA-MOS quantity for further study on its bacterial interaction effect.

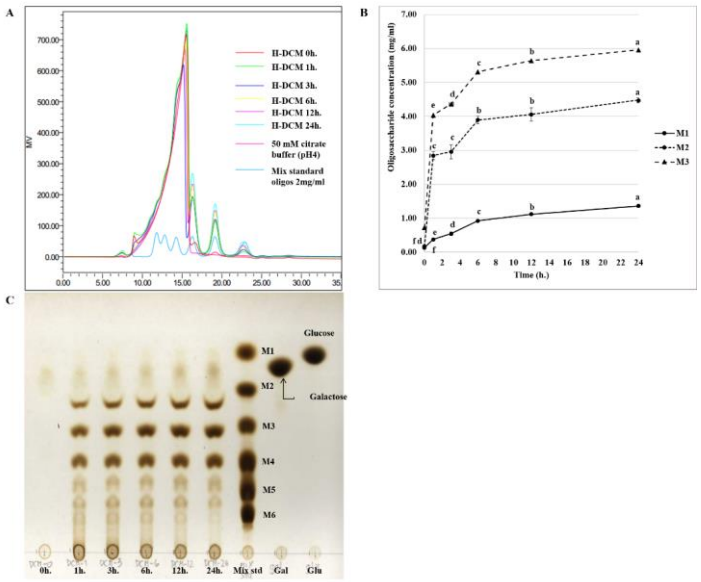

Figure 2 Mannooligosaccharides in defatted copra meal hydrolysates analyzed by high performance liquid chromatography (HPLC) and Thin layer chromatography (TLC). (a) chromatogram of oligosaccharides by HPLC, (b) time course production of oligosaccharides obtained by HPLC analysis, (c) chromatogram of oligosaccharides by TLC. Standard contains M1, mannose; M2, mannobiose; M3, mannotriose; M4, mannotetraose; M5, mannopentaose; M6, mannohexaose; Glu, glucose; Gal, galactose. $^{a-f}$ Mean data showing different common superscripts are significantly different ($P \leq 0.05$)

Table 2 Specific growth rate (h^{-1}) of two probiotic strains grown in medium containing COPRA-MOS at various times.

	Specific growth rate (h^{-1})[1]					
Strain	**BM**	**Glucose**	**Actigen™**	COPRA-MOS **6 h**	COPRA-MOS **12 h**	COPRA-MOS **24 h**
L. johnsonii KUNN19-2	0.52 ± 0.06^{b}	0.60 ± 0.004^{a}	$0.58 \pm 0.03^{a,b}$	0.64 ± 0.03^{a}	0.60 ± 0.03^{a}	0.62 ± 0.004^{a}
L. reuteri KUB-AC5	$0.27 \pm 0.04^{b,c,C,D}$	$0.44 \pm 0.03^{a,A}$	$0.22 \pm 0.05^{c,D}$	$0.32 \pm 0.05^{b,B,C}$	$0.30 \pm 0.04^{b,c,B,C}$	$0.33 \pm 0.05^{b,B}$

[1] Data are mean from 2 replicates ± standard deviations; ^{a,b,c} means within a row with a common superscript were found to differ significantly ($P \leq 0.05$) while ^{A,B,C} means within a row of *L. reuteri* KUB-AC5 with a common superscript were also found to differ significantly ($P \leq 0.2$); Basal medium, control (MRS without sugar); Glucose, control; Actigen™, Commercial mannooligosaccharide; COPRA-MOS, mannooligosaccharide from defatted copra meal hydrolysate.

Effect of COPRA-MOS on autoaggregation of bacterial cells

Most prebiotics properties involved in modulation of gut microbiota by selectively fermentation of prebiotics and production of SCFA, modulation of immune systems, improvement of bowel function and defense against pathogenic bacteria by acting as a decoy receptor **(Monteagudo-Mera *et al*., 2019; Sanders *et al*., 2019)**. Especially, MOS from yeast *Saccharomyces cerevisiae* which was used to reduce cecal colonization of *E. coli* and *Salmonella* spp. in chicken by agglutination between Type 1 fimbriae of pathogenic strains and D-mannose in MOS **(Walker *et al*., 2017)**. One of COPRA-MOS functionality produced by KMAN treated copra meal was previously studied and known as probiotic growth promotion **(Pongsapipatana *et al*., 2016)** as well. The ability of lactobacilli to adhere to epithelial and mucus cells is considered to be an important property of probiotics. Recently, adherence of lactobacilli is proposed to relate to the aggregation ability of each strain which involves interaction between the same strain of microorganisms (autoaggregation) or between different strains (coaggregation) **(Nishiyama *et al*., 2016; Trunk *et al*., 2018)**. In previous study, probiotic properties and mucin adhesion ability of *L. reuteri* and *L. johnsonii* have been presented by **Nitisinprasert (2006) and Prommadee *et al.* (2012)**. The adhesion mechanisms of lactobacilli vary among strains and species and involve carbohydrate-protein interactions between oligosaccharides on mucin chains and adhesins on bacterial cell surfaces.

Table 3 Autoaggregation of bacterial cells incubated at 37 and 42°C during 24 h.

Microorganism	**Temperature (°C)**	**Time (h.)**	**% Autoaggregation**		**% Change***
			Cells	**Cells + COPRA-MOS**	
L. reuteri KUB-AC5	37	5	10.16 ± 0.97^{b}	19.91 ± 0.91^{a}	95.86
		24	81.10 ± 9.30^{a}	76.89 ± 1.69^{a}	
	42	5	16.97 ± 3.28^{b}	27.64 ± 1.04^{a}	62.89
		24	81.01 ± 4.01^{a}	78.80 ± 2.36^{a}	
S. Enteritidis DMST 17368	37	5	18.49 ± 3.15^{a}	3.39 ± 0.77^{b}	-81.66
		24	35.26 ± 0.46^{a}	8.80 ± 0.20^{b}	-75.03
	42	5	9.94 ± 7.43^{a}	2.07 ± 0.57^{a}	
		24	32.22 ± 5.05^{a}	15.31 ± 3.38^{a}	
L. johnsonii KUNN19-2	37	5	18.40 ± 1.45^{a}	8.16 ± 0.0^{b}	-55.63
		24	23.21 ± 1.66^{a}	14.23 ± 0.66^{b}	-38.68

^{a,b} Means within a row identified with a common superscript were significantly different ($P \leq 0.05$). * Change of cell aggregation in the presence of COPRA-MOS (Cells + COPRA-MOS) compared to cells alone. Minus values indicate reduction of cell aggregation while plus values indicate enhancement of cell aggregation.

While pathogens use lectin for mucin adhesion **(Nishiyama *et al*., 2016)**. However, the effect of prebiotics, COPRA-MOS to cell aggregation activity is not known yet. Here, the autoaggregation of two probiotic strain, *L. reuteri* and *L. johnsonii* and a pathogen *S.* Enteritidis DMST17368 in the presence of MOS from COPRA-MOS treatment were investigated to explore more on its function as a decoy receptor and its influence on the autoaggregation of probiotic strains. Three

treatments were conducted as bacterial cells alone, COPRA-MOS alone and a combination of bacterial cells and COPRA-MOS at 37 and 42°C, except that *L. johnsonii* was performed only at 37°C due to its growth efficacy in human as shown in the Table 3. Results showed that no autoaggregation of the treatment COPRA-MOS alone at both 5 and 24 h performed at 37 and 42°C was observed. Considering to *L. reuteri* KUB-AC5, its % autoaggregation at 24 h of both treatments rapidly increased and displayed higher activities than the one at 5 h for 8- and 4-times of cell alone and the combination treatment, respectively at 37°C. Autoaggregation activity of *L. reuteri* KUB-AC5 from combination treatment at 37 and 42°C increased significantly by 95.86% and 62.89% at 5 h, respectively comparing to the cell alone treatment. However, the autoaggregation activities of cell alone and the combination treatment at 24 h showed no significant difference. This implied that COPRA-MOS enhance autoaggregation activity of the strain KUB-AC5 at shorter time of 5 h for both temperatures.

An investigation on autoaggregation of both *L. johnsonii* KUNN19-2 and *S.* Enteritidis DMST17368 alone showed lower aggregation activity at 37°C for 24 h than *L. reuteri* KUB-AC5 did for 3.5-fold. However, in the presence of COPRA-MOS at 37°C, % autoaggregation of both *L. johnsonii* and *S.* Enteritidis significantly decreased with reductions of 38.68-55.63% and 75.03-81.66%, respectively due to high aggregate cells suspension, while the one of *S.* Enteritidis at 42°C showed no significant difference. Results implied that the presence of COPRA-MOS reduced autoaggregation activity of both *L. johnsonii* KUNN19-2 and *S.* Enteritidis at 37°C but had no effect at high temperatures of 42°C.

Effect of COPRA-MOS on bacterial community and SCFA production in simulated human and chicken gastrointestinal tract

Both human and chicken are classified as the monogastric animals, their relative intensity of bacterial fermentation in compartments of the gastrointestinal tract varies. For humans, the primary fermentation site in the lower digestive tract is the colon. However, in chickens, bacterial fermentation occurs mainly in the cecum. Even, both humans and chickens are omnivores, the diversity of the microbial communities in the gastroenterological tracts of different animal species is related to the host life-style. **Lei *et al*. (2012)** found that chicken cecal microbiotas required simple sugars and peptides to maintain balanced growth *in vitro* but that human fecal microbiotas preferred polysaccharides and proteins. Chicken microbiotas also produced higher concentrations of volatile fatty acids than did human microbiotas. The availability of different fermentable substrates in the chicken cecum, which exist due to the unique anatomical structure of the cecum, may provide an environment favorable to the nourishment of microbiotas suited to the production of the higher-energy metabolites required by the bird. Therefore, it was interesting to know whether COPRA-MOS, which is short chain carbohydrate, can affect microbiota and the metabolites of these two different species.

Human simulation model

Two treatments of control (without COPRA-MOS) and COPRA-MOS treatment were carried out to determine the abundance of seven dominant bacterial groups of *Prevotella* spp., *Bacteroides fragilis*, *Clostridium leptum*, *Bifidobacterium* spp., *C. coccoides-E. rectale* group, Enterobacteriaceae and *Lactobacillus* spp. existing in human and short chain fatty acid (SCFA) production during 24 h anaerobic fermentation. These bacterial groups were selected because of the beneficial human health effects of *Bifidobacterium* spp. and *Lactobacillus* spp., potential pathogens of Enterobacteriaceae and Thai diet correlation of *Prevotella* spp., *Bacteroides fragilis*, *Clostridium leptum* and *C. coccoides-E. rectale* group **(Laongkham *et al*., 2015; Ruengsomwong *et al*., 2014)**. Based on standard curve analysis, detectable minimum concentration of *Prevotella* spp., *Bacteroides fragilis*, *Clostridium leptum*, *Bifidobacterium* spp., *C. coccoides-E. rectale* group, Enterobacteriaceae and *Lactobacillus* spp., were 1.84, 5.40, 1.97, 2.89, 1.82, 2.04 and 1.86 log copy number/ml, respectively. In this study, only five groups of bacteria as *Clostridium leptum*, *Bifidobacterium* spp., *C. coccoides-E.rectale*, Enterobacteriaceae and *Lactobacillus* spp., were found in the simulated human gut condition, with initial concentrations of 6.61-6.80, 5.77-6.60, 9.60-9.85, 7.94-8.15 and 5.90-6.08 log copy number/ml, respectively, as shown in the figure 3. Abundances of the five bacterial groups studied from the control did not significantly change during fermentation. However, abundances of *Bifidobacterium* spp. and Enterobacteriaceae from the COPRA-MOS treatment increased significantly ($P \leq 0.05$) at 6 h fermentation and remained stable for 24 h, whereas *Clostridium leptum*, *Clostridium coccoides-E.rectale* and *Lactobacillus* spp. remained stable.

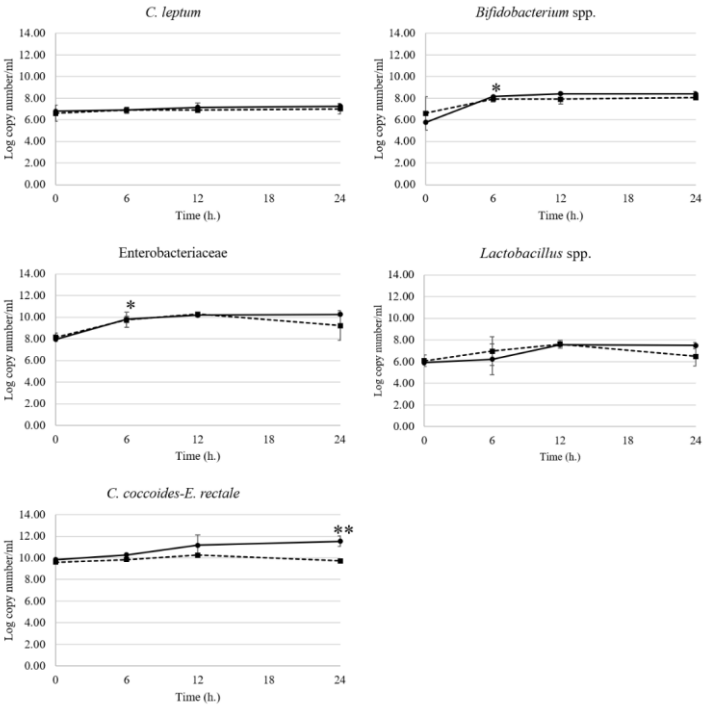

Figure 3 Fecal bacteria found in simulated human gastrointestinal tract. Control or basal medium alone (-■-); Basal medium supplemented with 1% (w/v) COPRA-MOS (—●—). Time course in the graph was indicated by single star (*) with significant differences ($P \leq 0.05$). Treatment in the graphs was indicated by double star (**) with significant differences ($P \leq 0.05$)

When abundances of each bacterial group from the control and COPRA-MOS treatment were compared at each fermentation time. *Clostridium leptum*, *Bifidobacterium* spp., Enterobacteriaceae and *Lactobacillus* spp. of both control and COPRA-MOS treatment were not significantly different ($P > 0.05$), while abundance of *Clostridium coccoides-E. rectale* at 24 h from COPRA-MOS treatment was significantly higher than the control by approximately 1.2 times.

Four SCFA of lactic, acetic, propionic and butyric acids were determined as shown in figure 4. Low propionic acid concentration of 14.81-15.78 mM and 13.55-23.17 mM were detected from the control and COPRA-MOS treatment, respectively, while only acetic acid in the COPRA-MOS treatment significantly increased during 6-24 h fermentation with maximum concentration of 87.43 mM at 24 h.

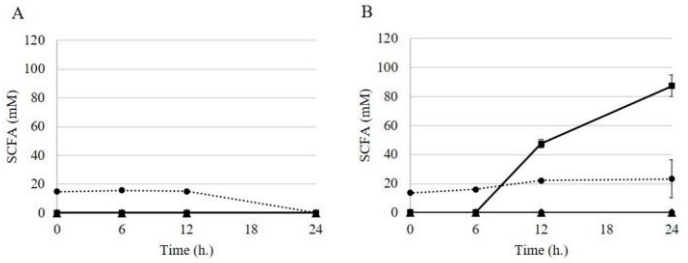

Figure 4 Short chain fatty acid (SCFA) concentrations of cultures fermentation in simulated human gastrointestinal tract (A) Control or basal medium alone and (B) Basal medium supplemented with 1% COPRA-MOS (w/v). (—●—) Lactic acid; (—■—) Acetic acid; (···●··) Propionic acid; (-▲-) Butyric acid

Chicken simulation model

Two treatments of control and COPRA-MOS were carried out to determine the abundance of nine bacterial groups from the jejunum, ileum and cecum as *Campyrobacter* sp., *Acinetobacter* spp., *Pseudomonas* spp., *Bifidobacterium* spp., *C. coccoides-E. rectale*, Enterobacteriaceae, *Lactobacillus* spp., *Bacteroides-Prevotell-Porphyromonas* and *C. perfringens*, which were mostly found in the chicken intestine **(Nakphaichit *et al*., 2014)** as well as short chain fatty acid (SCFA) production during 24 h anaerobic fermentation. Only three groups of *C. coccoides-E. rectale*, Enterobacteriaceae and *Lactobacillus* spp. were detected in simulated chicken gut condition with initial concentrations of 9.85-10.40, 10.04-10.60 and 7.39-7.66 log copy number/ml, respectively as shown in the figure 5. While *Campyrobacter* sp., *Acinetobacter* spp., *Pseudomonas* spp., *Bacteroides-Prevotell-Porphyromonas* and *C. perfringens* were not detected, their abundances were possibly lower than detectable minimum concentrations of 1.63, 2.88, 1.64, 1.01 and 3.29 log copy number/ml, respectively. Abundance of *C. coccoides-E. rectale* from both treatments at each fermentation time showed no significant difference ($P > 0.05$), while *Enterobacteriaceae* and *Lactobacillus* spp. at 24 h

displayed significant difference ($P < 0.05$). Abundance of Enterobacteriaceae from the control and COPRA-MOS treatment decreased to 7.49 log copy number/ml and non-detectable, respectively, resulting in growth suppression in COPRA-MOS treatment. However, abundance of *Lactobacillus* spp. from the COPRA-MOS treatment increased at 24 h to 11.85 log copy number/ml, while the ones of control were stable.

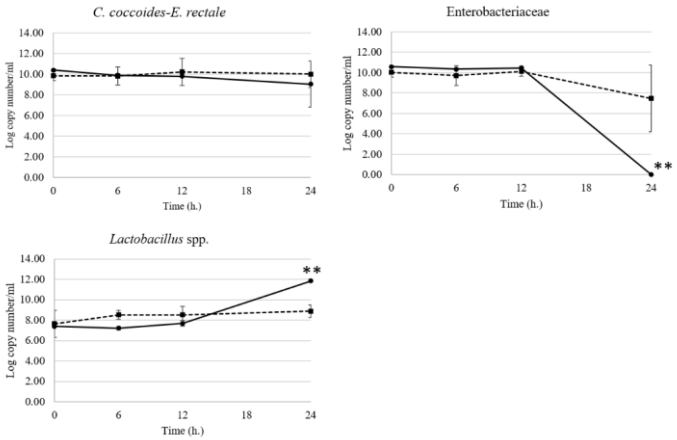

Figure 5 Cecal bacteria found in simulated chicken gastrointestinal tract. Control or basal medium alone ( ); Basal medium supplemented with 1% (w/v) COPRA-MOS ( ); Treatment in the graphs was indicated by double star (**) with significant differences ($P \leq 0.05$)

Four SCFA of lactic, acetic, propionic and butyric acids were determined as shown in the figure 6. Three acids of lactic, acetic and propionic acids from both treatments were detected during 24 h fermentation. Concentration of both acetic acid and lactic acid from the control significantly increased to 22.94 mM and 13.77 mM, respectively at 24 h while COPRA-MOS treatment gave higher concentrations of 45.20 mM and 16.32 mM, respectively. However, concentrations of lactic acid in the COPR-MOS treatment were not significantly different from the control, while acetic acid concentrations of COPRA-MOS treatment showed significant higher during 12-24 h. Propionic acid concentration of the control were 38.71-57.58 mM during fermentation, while the ones of the COPRA-MOS treatment remained stable at 33.85-39.47 mM and undetectable at 24 h which were significantly lower than the control during 6-24 h.

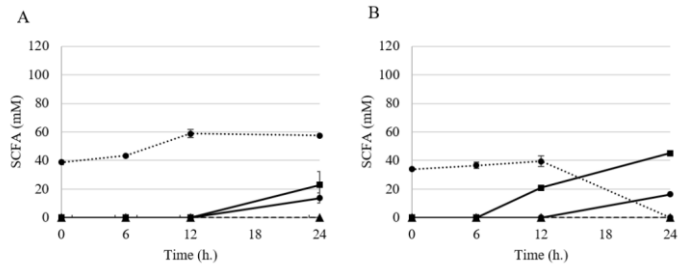

Figure 6 SCFA concentrations of culture fermentation in simulated chicken gastrointestinal tract (A) Control, basal medium alone; (B) basal medium supplemented with 1% (w/v) COPRA-MOS; ( ) Lactic acid; ( ) Acetic acid; ( ) Propionic acid; ( ) Butyric acid

DISCUSSION

Various oligosaccharides are nondigestible and have beneficial effects on hosts which can confer prebiotic properties. One of them, manno-oligosaccharides (MOS) has been previously documented to potentially promote microorganisms such as bifidobacteria and lactobacilli in the colon. In our previous study, the recombinant *E. coli* KMAN-3 produced mannanase KMAN which hydrolyzed copra mannan to produce COPRA-MOS at 24 h (**Pongsapipatana *et al*. 2016)**. However, prolonged reaction time can affect to the activity of enzyme and cause high production cost. In this study, the time course of hydrolytic activity of mannanase KMAN were performed during 1-24 h resulting in the highest productivity at 1 h and then decreasing. However, their TLC chromatograms presenting different intensities of each product showed that the treatment 6-24 h trended to contain higher MW of M2-M4 than the ones 1-3 h and could enhance the growth of lactobacilli, especially two probiotics studied. Therefore, only short reaction time of 6 h was enough to produce effective COPRA-MOS. Comparing to MOS quality from other mannanase sources, the outputs obtained were different from mannanase CHM produced by *Bacillus circulans* NT6.7 reported by **Prayoonthien *et al*. (2019)**. Its main products consisted of M5, M6 and trace amount of M1. However, the hydrolytic reaction of its recombinant mannanase MAN6.7 expressed by *E. coli* strain BL21(DE3) harboring plasmid pET21d-(+)/man6.7 produced different COPRA-MOS from its wild type which mainly consisted of M2-M5 with trace amount of M1 **(Piwpankaew, 2014)**. **Sritrakul *et al.* (2020)** also reported that the hydrolytic pattern between COPRA-MOS from mannanase KMAN and MAN6.7 was different from each other with M5 found from KMAN only. Whilst copra meal hydrolysate produced by β-mannanase from *Streptomyces* sp. BF3.1 mainly consisted of M2 and small amount of glucose, M1 and M3-M6 **(Ariandi *et al*., 2015)**. It was indicated that copra meal hydrolysate produced by different mannanase sources yielded different amount of products and oligosaccharide profiles which would lead to different source of carbohydrate for bacterial growth.

Further characterization of two main functions of prebiotic COPRA-MOS as a decoy receptor and as a substrate for selectively fermentation by gut microbiota were presented. The first function was tested by observation of COPRA-MOS influencing on autoaggregation of two probiotic strains and one pathogen with the expectation that COPRA-MOS would enhance autoaggregation of probiotic strains as it would influence their adherence later on gut mucosa and it would capture pathogen which prevent its competitive colonization with indirectly enhance the one of the probiotics **(Trunk *et al*., 2018)**. In this study, COPRA-MOS accelerated autoaggregation on *L. reuteri* KUB-AC5 at 5 h of incubation at both 37 and 42°C. Their activities at 24 h increased even higher than *L. johnsonii* and *S.* Enteritidis treatment for 3.5-5.4 and 2.3-8.6 times, respectively. It seemed that COPRA-MOS acted as a flocculant causing floc cells which later precipitated. Many studies have reported the possible adherence mechanism of *L. reuteri* to mucus layer on gut epithelial cells of the host due to mucus-binding protein (MUB) or MUB-like protein which is identified as adhesins **(Mu *et al*., 2018)**. The adhesins possessed by lactobacilli are also similar to those of bacterial pathogens which interact to the specific receptors on host cell surface **(Singh *et al*., 2017)**. The binding site of adhesin possessed by *L. reuteri* for binding to mucus was reported to be carbohydrate chains containing galactosyl residues in mucin glycoproteins structure **(Dudík *et al*., 2020; Nishiyama *et al*., 2016)**. In this study, COPRA-MOS contained M2, M3, M4 and M4-H which contain galactosyl residue at the side chain based on the chemical structure of copra meal as well. Such characters possibly stimulated aggregation activity. Lower aggregation of *L. johnsonii* KUNN19-2 and *S.* Enteritidis DMST17368 did occur as well. In pathogens, pili are used for attachment to the host mucosal surface **(Solanki *et al*., 2018; Soto & Hultgren, 1999; Teng & Kim, 2018)**. Similar to pathogenic bacteria, the gene coding for pili was also found in *L. johnsonii* which is essential for adhesion to mucosal surface **(Nishiyama *et al*., 2016)**. By using carbohydrates as potential receptor decoys, *L. johnsonii* and enterobacteria also shared carbohydrate-binding specificities to mannose in yeast cell wall mannoprotein **(Neeser *et al*., 2000)** and β-1,4-mannan from plants **(Marshall *et al*., 2016)**, respectively. This might be the reason why the aggregation of *L. johnsonii* KUNN19-2 and *S.* Enteritidis DMST17368 in the presence of COPRA-MOS happened in similar manner. However, in the presence of COPRA-MOS, their aggregate cells were found more in the remaining suspension causing slow aggregation rate. It would be proposed that COPRA-MOS delayed aggregation activity for both *L. johnsonii* KUNN19-2 and *S.* Enteritidis DMST17368. Therefore, these finding suggested that aggregation activities in the presence of COPRA-MOS was impacted depending on the cell wall characters of each bacterial species.

The latter function was tested by COPRA-MOS influencing on gut microbiota in two selected models, human and chicken, with the expectation that it would enhance the growth of beneficial bacteria and suppress the growth of pathogenic bacteria in order to apply in the food and/or feed industries in the future. The gastrointestinal (GI) tract harbors a complex community of 10 million to 100 trillion microbial cells in various life **(Sender *et al*., 2016; Shang *et al*., 2018)** which influence physiology, metabolism, nutrition and immune function **(Dekaboruah *et al*., 2020)**. However, the host may shape its gut microbiota via specific and nonspecific factors such as antibiotics, age and diet including those nutritional factors, prebiotics, probiotics influencing to the health **(Holscher, 2017).** In this study the effect of one prebiotic, COPRA-MOS, on bacterial populations in the gastrointestinal tract was performed by using a human and chicken GI model containing fecal and cecal microbes, respectively. In order to minimize individual variation which might cause by the difference in diets and lifestyles **(La-ongkham *et al*., 2015; Ruengsomwong *et al*., 2014)**, a single fecal source from healthy adult volunteer and a single cecal one from 21-d Arbor Acres Plus Broiler were tested for the effect of COPRA-MOS on gut microbiota.

In fecal fermentation, COPRA-MOS affected the increasing abundance of *C. coccoides-E. rectale* group but had no effect on Enterobacteriaceae and *Lactobacillus.* In addition, high amount of acetic acid followed by propionic acid were observed. It seemed that COPRA-MOS might enhance the growth of *C. coccoides-E. rectale* group and consequently produced more acetic acid and propionic acid However, **Mukherjee *et al*. (2020)** reported its metabolites are not only acetic acid and propionic acid but also butyric acid. It might be that both acids in this study came from different conversion of lactic acid which are common end products from carbohydrate fermentation of lactic acid bacteria, bifidobacterial and proteobacteria **(Asano *et al*., 2003; Prayoonthien *et al*., 2019; Russell *et al*., 2013; Tungland, 2018; Vacca *et al*., 2020)**. Possibly, *Clostridium* belonging to cluster IX involved in lactic acid conversion to more propionic acid while clostridia cluster XIVa converts lactic acid to butyric acid **(Flint *et al*., 2014; Louis**

& Flint, 2009; Oliphant & Allen-Vercoe, 2019; Ramakrishna, 2013). Therefore, different clostridia cluster may provide different metabolites

In cecal fermentation, abundance of *Enterobacteriaceae* decreased along with the increasing of *Lactobacillus* abundance in BM supplemented with COPRA-MOS. Consequently, the increase of acetic and lactic acid concentration was detected in the COPRA-MOS treatment. The increase of acetic acid and lactic acid were also coincided with the increase in the growth of lactobacilli. The decrease of *C. coccoides-E. rectale* group which is lactate-utilizing bacteria and propionate producer in the COPRA-MOS treatment comparing to the control might cause the decrease of propionic acid concentration observed. It was interesting that growth inhibition of gram-negative bacteria belonging to Enterobacteriaceae was observed. Its inhibition activity could be due to acetic and lactic acid by diffusion into bacterial cells which caused the disruption of osmotic and reduction of intracellular pH **(Lamas *et al*., 2019; Tan *et al*., 2014)** or various antimicrobial substances produced by the *Lactobacillus* group **(Šušković *et al*., 2010; Tungland, 2018)** which were different from previous work reported by **Prayoonthien et al. (2017)**. The CMH containing M1, M5 and M6 **(Prayoonthien *et al*., 2019)** significantly increased *Lactobacillus* and *Enterococcus* spp. after 12, 18 and 24 h of cecal bacteria fermentation *in vitro*, but it had no inhibition effect on Enterobacteriaceae after 24 h fermentation which were different to this study. The results suggested that disparate oligosaccharides produced by diverse mannanase sources cause different microbial communities and subsequently produce typical SCFA composition.

It was obviously shown that COPRA-MOS treatment of both human and chicken gut models affected the difference of the abundances of both bacterial group and their metabolites of SCFAs. In addition, disparate sources of MOS also cause diverse microbial abundance. Therefore, MOS produced from different substrates and enzymes will show different interactions to the bacterial community and SCFA generally produced from dietary fiber fermentation by anaerobic microorganisms supported by **Ricke *et al*. (2020)**. Based on overall results, COPRA-MOS obtained by this proposed reaction could benefit more by increasing the growth of *Lactobacillus* and suppressing the growth of Enterobacteriaceae in cecal environment of chicken. The increasing of lactic acid and acetic acid during fermentation could also benefit the gut environment with its inhibitory effect on pathogens. Moreover, the autoaggregation activity of bacteria in the present of COPRA-MOS suggested its benefit on autoaggregation of *L. reuteri* KUB-AC5, a probiotic strain, which could contribute to its adherence to gut mucosa of host and act as a barrier to prevent the infection of pathogens. COPRA-MOS could also be used as a decoy to capture *S.* Enteritidis with end results of infection prevention and improve gut health. From all the expected benefits, COPRA-MOS could be potentially applied for feed supplement in the future.

CONCLUSION

COPRA-MOS, comprising of M2, M3, M4 and higher MW (M4-H) was obtained from enzymatic hydrolysis of defatted copra meal by mannanase KMAN from the recombinant *E. coli* KMAN-3. The highest productivity occurred at the early stage of 1 h under optimal condition of pH 4.0 and 40°C, while high contents of 2.13-2.38 g/l were achieved at 6-24 h and sufficient to enhance the growth of *L. johnsonii* KUNN19-2. High contents of 2.38 g/l enhanced the growth of *L. reuteri* KUB-AC5. COPRA-MOS had no effect on the growth of *Lactobacillus* spp. on human gut microbiota but affected cecal bacteria by increasing the growth of *Lactobacillus* spp. and simultaneously decreasing the growth of Enterobacteriaceae. The ability of COPRA-MOS to accelerate aggregation was observed in *L. reuteri* KUB-AC5, while aggregation prevention ability was observed in *L. johnsonii* KUNN19-2 and *S.* Enteritidis DMST17368. These findings provided potential oligosaccharides to prevent colonization of pathogenic bacteria in the gastrointestinal tract.

Acknowledgements: This work is partially supported by the Center for Advanced Studies for Agriculture and Food, Institute for Advanced Studies, Kasetsart University under the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Ministry of Education, Thailand. This research is supported in part by the Graduate Program Scholarship from The Graduate School, Kasetsart University including by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE).

REFERENCES

Ariandi, Yopi, & Meryandini, A. (2015). Enzymatic Hydrolysis of Copra Meal by Mannanase from Streptomyces sp. BF3.1 for The Production of Mannooligosaccharides. *HAYATI Journal of Biosciences*, *22*(2), 79-86. http://dx.doi.org/10.4308/hjb.22.2.79

Asano, I., Hamaguchi, K., Fujii, S., & Iino, H. (2003). *In Vitro* Digestibility and Fermentation of Mannooligosaccharides from Coffee Mannan. *Food Science and Technology Research*, *9*(1), 62-66. https://doi.org/10.3136/fstr.9.62

Association of Official Analytical, C., & Helrich, K. (1990). *Official methods of analysis of the Association of Official Analytical Chemists*. The Association, Arlington, VA.

Bartosch, S., Fite, A., Macfarlane, G. T., & McMurdo, M. E. T. (2004). Characterization of Bacterial Communities in Feces from Healthy Elderly Volunteers and Hospitalized Elderly Patients by Using Real-Time PCR and Effects of Antibiotic Treatment on the Fecal Microbiota. *Applied and Environmental Microbiology*, *70*(6), 3575-3581. https://doi.org/10.1128/aem.70.6.3575-3581.2004

Chauhan, P. S., Puri, N., Sharma, P., & Gupta, N. (2012). Mannanases: microbial sources, production, properties and potential biotechnological applications. *Applied Microbiology and Biotechnology*, *93*(5), 1817-1830. https://doi.org/10.1007/s00253-012-3887-5

Dekaboruah, E., Suryavanshi, M. V., Chettri, D., & Verma, A. K. (2020). Human microbiome: an academic update on human body site specific surveillance and its possible role. *Archives of Microbiology*, *202*(8), 2147-2167. https://doi.org/10.1007/s00203-020-01931-x

Dudík, B., Kiňová Sepová, H., Bilka, F., Pašková, Ľ., & Bilková, A. (2020). Mucin pre-cultivated *Lactobacillus reuteri* E shows enhanced adhesion and increases mucin expression in HT-29 cells. *Antonie Van Leeuwenhoek*, *113*(8), 1191-1200. https://doi.org/10.1007/s10482-020-01426-1

Flint, H. J., Duncan, S. H., Scott, K. P., & Louis, P. (2014). Links between diet, gut microbiota composition and gut metabolism. *Proceedings of the Nutrition Society*, *74*(1), 13-22. https://doi.org/10.1017/S0029665114001463

Holscher, H. D. (2017). Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut microbes*, *8*(2), 172-184. https://doi.org/10.1080/19490976.2017.1290756

Isenring, J., Geirnaert, A., Lacroix, C., & Stevens, M. J. A. (2021). Bistable auto-aggregation phenotype in Lactiplantibacillus plantarum emerges after cultivation in in vitro colonic microbiota. *BMC Microbiology*, *21*(1), 268. https://doi.org/10.1186/s12866-021-02331-x

Khan, I. U. H., & Yadav, J. S. (2004). Real-time PCR assays for genus-specific detection and quantification of culturable and non-culturable Mycobacteria and Pseudomonads in metalworking fluids. *Molecular and Cellular Probes*, *18*(1), 67-73. http://dx.doi.org/10.1016/j.mcp.2003.09.004

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., & Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology*, *94*(6), 981-987. https://doi.org/10.1046/j.1365-2672.2003.01915.x

La-ongkham, O., Nakphaichit, M., Leelavatcharamas, V., Keawsompong, S., & Nitisinprasert, S. (2015). Distinct gut microbiota of healthy children from two different geographic regions of Thailand. *Archives of Microbiology*, *197*(4), 561-573. https://doi.org/10.1007/s00203-015-1089-0

Lamas, A., Regal, P., Vázquez, B., Cepeda, A., & Franco, C. M. (2019). Short chain fatty acids commonly produced by gut microbiota influence *Salmonella enterica* motility, biofilm formation, and gene expression. *Antibiotics (Basel, Switzerland)*, *8*(4), 265. https://doi.org/10.3390/antibiotics8040265

Lei, F., Yin, Y., Wang, Y., Deng, B., Yu, H. D., Li, L., Xiang, C., Wang, S., Zhu, B., & Wang, X. (2012). Higher-level production of volatile fatty acids *in vitro* by chicken gut microbiotas than by human gut microbiotas as determined by functional analyses. *Applied and Environmental Microbiology*, 78(16), 5763-5772. https://doi.org/10.1128/AEM.00327-12

Louis, P., & Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiology Letters*, *294*(1), 1-8. https://doi.org/10.1111/j.1574-6968.2009.01514.x

Lukic, J., Strahinic, I., Milenkovic, M., Nikolic, M., Tolinacki, M., Kojic, M., & Begovic, J. (2014). Aggregation Factor as an Inhibitor of Bacterial Binding to Gut Mucosa. *Microbial Ecology*, *68*(3), 633-644. https://doi.org/10.1007/s00248-014-0426-1

Markowiak, P., & Śliżewska, K. (2018). The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut pathogens*, *10*, 21. https://doi.org/10.1186/s13099-018-0250-0

Marshall, J., Rossez, Y., Mainda, G., Gally, D. L., Daniell, T. J., & Holden, N. J. (2016). Alternate thermoregulation and functional binding of *Escherichia coli* type 1 fimbriae in environmental and animal isolates. *FEMS Microbiology Letters*, *363*(22). https://doi.org/10.1093/femsle/fnw251

Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., Oyaizu, H., & Tanaka, R. (2002). Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Applied and Environmental Microbiology*, *68*(11), 5445-5451. https://doi.org/10.1128/aem.68.11.5445-5451.2002

Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T., & Tanaka, R. (2004). Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Applied and Environmental Microbiology*, *70*(12), 7220-7228. https://doi.org/10.1128/aem.70.12.7220-7228.2004

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing Sugar. *Analytical Chemistry*, *31*(3), 426-428. https://doi.org/10.1021/ac60147a030

Monteagudo-Mera, A., Rastall, R. A., Gibson, G. R., Charalampopoulos, D., & Chatzifragkou, A. (2019). Adhesion mechanisms mediated by probiotics and

prebiotics and their potential impact on human health. *Applied Microbiology and Biotechnology*, *103*(16), 6463-6472. https://doi.org/10.1007/s00253-019-09978-7
Mu, Q., Tavella, V. J., & Luo, X. M. (2018). Role of *Lactobacillus reuteri* in Human Health and Diseases [Review]. *Frontiers in Microbiology*, *9*(757). https://doi.org/10.3389/fmicb.2018.00757
Mukherjee, A., Lordan, C., Ross, R. P., & Cotter, P. D. (2020). Gut microbes from the phylogenetically diverse genus Eubacterium and their various contributions to gut health. *Gut microbes*, *12*(1), 1802866. https://doi.org/10.1080/19490976.2020.1802866
Nakphaichit, M., Phraephaisarn, C., Keawsompong, S., Sukpiriyagul, O., & Nitisinprasert, S. (2014). Effect of increasing dietary protein from soybean meal on intestinal microbiota and their fatty acids production in broiler chicken. *Advances in Animal and Veterinary Sciences*, *2*(6), 337-343. https://doi.org/10.14737/journal.aavs/2014/2.6.337.343
Neeser, J.R., Granato, D., Rouvet, M., Servin, A., Teneberg, S., & Karlsson, K.-A. (2000). *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. *Glycobiology*, *10*(11), 1193-1199. https://doi.org/10.1093/glycob/10.11.1193
Nisbet, D. J. (2000). Modeling the survivability of *Salmonella typhimurium* in the chicken cecae using an anaerobic continuous-culture of chicken cecal bacteria. *Microbial Ecology in Health and Disease*, *12*(1), 42-47. https://doi.org/10.1080/089106000435581
Nishiyama, K., Sugiyama, M., & Mukai, T. (2016). Adhesion properties of lactic acid bacteria on intestinal mucin. *Microorganisms*, *4*(3), 34. https://doi.org/10.3390/microorganisms4030034
Nitisinprasert, S. (2006). *In vitro* adhesion assay of lactic acid bacteria, *Escherichia coli* and *Salmonella* sp. by microbiological and PCR methods. *Songklanakarin Journal of Science and Technology (Thailand)*, *28*(Suppl. 1), 99-106.
Nitisinprasert, S., Nilphai, V., Bunyun, P., Sukyai, P., Doi, K., & Sonomoto, K. (2000). Screening and identification of effective thermotolerant lactic acid bacteria producing antimicrobial activity against *Escherichia coli* and *Salmonella* sp. resistant to antibiotics. *Kasetsart Journal (Natural Sciences) (Thailand)*, *34*(3), 387-400.
Nwoko, E.-s. Q. A., & Okeke, I. N. (2021). Bacteria autoaggregation: how and why bacteria stick together. *Biochemical Society Transactions*, *49*(3), 1147-1157. https://doi.org/10.1042/bst20200718
Oliphant, K., & Allen-Vercoe, E. (2019). Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome*, *7*(1), 91. https://doi.org/10.1186/s40168-019-0704-8
Onumpai, C., Kolida, S., Bonnin, E., & Rastall, R. A. (2011). Microbial utilization and selectivity of pectin fractions with various structures. *Applied and Environmental Microbiology*, *77*(16), 5747-5754. https://doi.org/10.1128/AEM.00179-11
Pangsri, P., Piwpankaew, Y., Ingkakul, A., Nitisinprasert, S., & Keawsompong, S. (2015). Characterization of mannanase from *Bacillus circulans* NT 6.7 and its application in mannooligosaccharides preparation as prebiotic. *SpringerPlus*, *4*, 771. https://doi.org/10.1186/s40064-015-1565-7
Piwpankaew, Y. (2014). Cloning and expression of mannanase from *Bacillus circulans* NT 6.7. *Thesis, Kasetsart University*.
Pongsapipatana, N., Damrongteerapap, P., Chantorn, S., Sintuprapa, W., Keawsompong, S., & Nitisinprasert, S. (2016). Molecular cloning of *kman* coding for mannanase from *Klebsiella oxytoca* KUB-CW2-3 and its hybrid mannanase characters. *Enzyme and Microbial Technology*, *89*, 39-51. http://dx.doi.org/10.1016/j.enzmictec.2016.03.005
Prayoonthien, P., Nitisinprasert, S., & Keawsompong, S. (2017). *In vitro* fermentation of copra meal hydrolysate by chicken microbiota. *3 Biotech*, *8*(1), 41. https://doi.org/10.1007/s13205-017-1058-1
Prayoonthien, P., Rastall, R. A., Kolida, S., Nitisinprasert, S., & Keawsompong, S. (2019). In vitro fermentation of copra meal hydrolysate by human fecal microbiota. *3 Biotech*, *9*(3), 93. https://doi.org/10.1007/s13205-019-1633-8
Prommadee, P., Garnjanagoonchorn, W., Lange, K. d., & Nitisinprasert, S. (2012). Characterization of *Lactobacillus johnsonii* KUNN19-2 and *Pediococcus pentosaceus* KUNNE6-1 isolated from Thai-style fermented pork (Nham) for their probiotic properties in the gastrointestinal tract and immunomodulation. *Kasetsart Journal (Natural Sciences) (Thailand)*, *46*(3), 440-450.
Ramakrishna, B. S. (2013). Role of the gut microbiota in human nutrition and metabolism. *J Gastroenterol Hepatol*, *28 Suppl 4*, 9-17. https://doi.org/10.1111/jgh.12294
Ricke, S. C., Lee, S. I., Kim, S. A., Park, S. H., & Shi, Z. (2020). Prebiotics and the poultry gastrointestinal tract microbiome. *Poultry Science*, *99*(2), 670-677. https://doi.org/10.1016/j.psj.2019.12.018
Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., & Palva, A. (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *Journal of Applied Microbiology*, *97*(6), 1166-1177. https://doi.org/10.1111/j.1365-2672.2004.02409.x
Ruengsomwong, S., Korenori, Y., Sakamoto, N., Wannissorn, B., Nakayama, J., & Nitisinprasert, S. (2014). Senior Thai fecal microbiota comparison between vegetarians and non-vegetarians using PCR-DGGE and real-time PCR. *J Microbiol Biotechnol*, *24*(8), 1026-1033. https://doi.org/10.4014/jmb.1310.10043
Russell, W. R., Hoyles, L., Flint, H. J., & Dumas, M.-E. (2013). Colonic bacterial metabolites and human health. *Curr Opin Microbiol*, *16*(3), 246-254. http://dx.doi.org/10.1016/j.mib.2013.07.002
Sakamoto, N., Tanaka, S., Sonomoto, K., & Nakayama, J. (2011). 16S rRNA pyrosequencing-based investigation of the bacterial community in nukadoko, a pickling bed of fermented rice bran. *International Journal of Food Microbiology*, *144*(3), 352-359. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.10.017
Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., & Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nature Reviews Gastroenterology & Hepatology*, *16*(10), 605-616. https://doi.org/10.1038/s41575-019-0173-3
Sender, R., Fuchs, S., & Milo, R. (2016). Revised estimates for the number of human and bacteria cells in the body. *PLOS Biology*, *14*(8), e1002533. https://doi.org/10.1371/journal.pbio.1002533
Shang, Y., Kumar, S., Oakley, B., & Kim, W. K. (2018). Chicken gut microbiota: importance and detection technology [Review]. *Frontiers in veterinary science*, *5*(254). https://doi.org/10.3389/fvets.2018.00254
Singh, T. P., Kaur, G., Kapila, S., & Malik, R. K. (2017). Antagonistic activity of *Lactobacillus reuteri* strains on the adhesion characteristics of selected pathogens. *Frontiers in Microbiology*, *8*(486). https://doi.org/10.3389/fmicb.2017.00486
Solanki, V., Tiwari, M., & Tiwari, V. (2018). Host-bacteria interaction and adhesin study for development of therapeutics. *International Journal of Biological Macromolecules*, *112*, 54-64. https://doi.org/10.1016/j.ijbiomac.2018.01.151
Soto, G. E., & Hultgren, S. J. (1999). Bacterial adhesins: common themes and variations in architecture and assembly. *J Bacteriol*, *181*(4), 1059-1071. https://doi.org/10.1128/JB.181.4.1059-1071.1999
Sritrakul, N., Nitisinprasert, S., & Keawsompong, S. (2020). Copra meal hydrolysis by the recombinant β-mannanase KMAN-3 and MAN 6.7 expressed in *Escherichia coli*. *3 Biotech*, *10*(2), 44. https://doi.org/10.1007/s13205-019-2005-0
Srivastava, P. K., & Kapoor, M. (2017). Production, properties, and applications of endo-β-mannanases. *Biotechnology Advances*, *35*(1), 1-19. http://dx.doi.org/10.1016/j.biotechadv.2016.11.001
Sundu, B., & Dingle, J. G. (2003). Use of enzymes to improve the nutritional value of palm kernel meal and copra meal. *Queensland Poultry Science Symposium 2003, Queensland Poultry Science Symposium 2003, Gatton, Queensland, Australia, (1-15). 24 July 2003.*, *11*, 1-15.
Šušković, J., Kos, B., Novak, J., Pavunc, A., Habjanič, K., & Matoć, S. (2010). Antimicrobial activity - The most important property of probiotic and starter lactic acid bacteria. *Food Technology and Biotechnology 48*(3), 296–307.
Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., & Macia, L. (2014). Chapter Three - The role of short-chain fatty acids in health and disease. *In* F. W. Alt (Ed.), *Advances in Immunology* (Vol. 121, pp. 91-119). Academic Press. https://doi.org/10.1016/B978-0-12-800100-4.00003-9
Teng, P. Y., & Kim, W. K. (2018). Review: Roles of prebiotics in intestinal ecosystem of broilers. *Frontiers in veterinary science*, *5*, 245. https://doi.org/10.3389/fvets.2018.00245
Titapoka, S., Keawsompong, S., Haltrich, D., & Nitisinprasert, S. (2007). Selection and characterization of mannanase-producing bacteria useful for the formation of prebiotic manno-oligosaccharides from copra meal. *World Journal of Microbiology and Biotechnology*, *24*(8), 1425-1433. https://doi.org/10.1007/s11274-007-9627-9
Trunk, T., Khalil, H. S., & Leo, J. C. (2018). Bacterial autoaggregation. *AIMS microbiology*, *4*(1), 140-164. https://doi.org/10.3934/microbiol.2018.1.140
Tungland, B. (2018). Chapter 2 Short-chain fatty acid production and functional aspects on host metabolism. In *Human Microbiota in Health and Disease* (pp. 37-106). https://doi.org/10.1016/b978-0-12-814649-1.00002-8
Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., & De Angelis, M. (2020). The controversial role of human gut Lachnospiraceae. *Microorganisms*, *8*(4), 573. https://doi.org/10.3390/microorganisms8040573
Vanbroekhoven, K., Ryngaert, A., Wattiau, P., De Mot, R., & Springael, D. (2004). *Acinetobacter* diversity in environmental samples assessed by 16S rRNA gene PCR–DGGE fingerprinting. *FEMS Microbiology Ecology*, *50*(1), 37-50. https://doi.org/10.1016/j.femsec.2004.05.007
Walker, G. K., Jalukar, S., & Brake, J. (2017). Effect of refined functional carbohydrates from enzymatically hydrolyzed yeast on the presence of *Salmonella* spp. in the ceca of broiler breeder females. *Poultry Science*, *96*(8), 2684-2690. https://doi.org/10.3382/ps/pex054
Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., & Hammes, W. P. (2001). Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* Species in Human Feces by Using Group-Specific PCR Primers and Denaturing Gradient Gel Electrophoresis. *Applied and Environmental Microbiology*, *67*(6), 2578-2585. https://doi.org/10.1128/aem.67.6.2578-2585.2001