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Article

Ruminal Ecology, Microbial Protein Synthesis and Milk Production in Lactating Dairy Cows Fed Glycerin-Based Diet: A Comparison Study on Chitosan Sources Supplementation

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Abstract: The study compared the influence of chitosan sources on rumen fermentation, methane emission and milk production in lactating dairy cows fed a glycerin-based diet. Six, lactating Holstein-Frisian crossbreeds (410 ± 5.0 kg BW, 120 ± 21 day-in-milk), were arranged in a 3 x 3 replicated Latin square design. In addition to control, a 2% chitosan extract supplement and a 2% commercial chitosan supplement of dry matter intake were the treatments. The results denoted that no significant differences on daily dry matter, nutrients or estimated energy intake were noted when cows received different sources of chitosan. Nutrient digestibility was not influenced differently by extraction based or commercial chitosan supplements. The pH, temperature, ammonia nitrogen, blood urea and microbial count were similar among treatments. The different sources of chitosan supplements did not change the totals of volatile fatty acids, acetate and butyrate; in contrast, different chitosan sources influenced ($P<0.05$) propionate content. The ruminal acetate to propionate ratio was markedly ($P<0.05$) reduced with chitosan supplement, but no change appeared between sources of chitosan. At 4 hours after feeding, the methane estimation significantly decreased with the addition of chitosan supplementation ($P<0.05$) compared to the control group. The purine derivatives and microbial protein synthesis were not altered by the treatments. No significant differences existed on milk yield, milk composition or milk urea nitrogen when cows received different sources of chitosan ($P>0.05$). In sum, supplementing extracted chitosan showed more potential than did commercial chitosan for enhancing economic efficiency and recycling shrimp residues, therefore, reducing environmental waste.

Keywords: chitosan; microbial synthesis; milk composition; volatile fatty acids; purine derivatives

1. Introduction

The natural biopolymer chitosan is produced from N-acetyl-d-glycosamide. The organic compound chitin is abundant in nature [1]. Chitosan has a antimicrobial effect on bacteria, molds and yeasts, so chitosan has been widely applied in medicine and in food [2-3]. Additionally, Goiri et al. [4-5] suggested that chitosan could enhance fermentation in the rumen, mainly by increasing the propionate concentration and decreasing the methane and acetate to propionate ratio. This yields an efficient energy supply and decrease ruminal protein disappearance [4].

In ruminant nutrition, Paiva et al. [6] demonstrated that chitosan could reduce *in vitro* CH_4 production and increase C3 concentration, thus improving energy usage [7]. Previous studies evaluated chitosan's influence on ruminal fermentation during *in vivo* trials, especially with lactating cows [6,8]. Besides its effects on volatile fatty acid (VFA) production, evidence suggests that chitosan decreases biohydrogenation in the rumen [4,8]. Recently, Del Valle et al. [8] suggested that chitosan

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improved feed efficiency and increased the milk unsaturated fatty acid (UFA) concentration of cows fed a soybean-oil-free diet.

Most chitosan product supplements for ruminants come from commercial products, which have high prices and increase feed cost when introduced to an animals. Recently, abundant residue from shrimp has been generated by local restaurants and plant factories in Thailand. In addition, shrimp has the highest production in an aquaculture system, comprising around 40% (398,500 tons per year) of Thailand's total aquaculture output yield. Over 40% of the residue was wasted in processing, including shrimp shells, head shells and tail shells [9]. Therefore, chitosan extracted from shrimp residues could be comparable to the commercial chitosan product for handling rumen fermentation, enhancing the production of dairy cows while reducing feed costs and environmental pollution. The study compared the influence of chitosan sources on rumen fermentation, CH_4 emission and milk production in lactating dairy cows fed a glycerin-based diet.

2. Materials and methods

The approval No. 38/2561 was provided by the Ethical committees of Animal Welfare of Khon Kaen University.

2.1. Study location, cows, ration, and design

This study was performed at a dairy farm of Khon Kaen University. Six, lactating Holstein-Frisian crossbreeds (410 ± 5 kg BW, 120 ± 21 day-in-milk), were arranged in a 3 x 3 replicated Latin square design. In addition to control, a 2% chitosan extract supplement and a 2% commercial chitosan supplement of dry matter intake were the treatments. The commercial chitosan had 90% deacetylation, 99% solubility in acetic, 8.9% moisture, a pH of 7.8 and an 80 mesh particle size (Tonggoa chitosan of Gritnarong Part., Ltd. Thailand). Chitosan extract supplement was prepared as stated by the method of Toan [10]. In brief, fresh shrimp shells were obtained from a local market in Khon Kaen province, Thailand, and washed with clean water. The autolysis of the shrimp shells was carried out by adding 0.68 M HCl solution (1:5 w/v) at 26–30 °C for 2 days. The dregs were washed and soaked in tap water for 6–8 h. It was then removed from the water, and protein was eliminated using NaOH solution (0.62 M; 1:5 w/v) at 26–30 °C for 20 h. The chitin yield was eliminated of acetyl groups using a solution of NaOH at 65 °C for 20 h, after which the chitosan was achieved. The chitosan was washed and sundried 1-2 days before being applied for the test. Chitosan was twice equally supplemented and mixed into the TMR diet before feeding it to the animals. The total mixed ration (TMR) contained energy from crude glycerin at 21% and was fed to animals *ad libitum*.

The ingredients of the TMR and chitosan extraction are presented in Table 1. Each cow had an individual house equipped with fresh water and mineral blocks. Three periods 14 days to adjust to the diet and 7 days to collect data were included. The experiment was performed with double square in 3 periods, each of which lasted 21 days. The first 14 days allowed for treatment adaptation and feed intake measurements, and the last 7 days were for diet, fecal, urine, and milk sample collection. Cows were weighed prior to starting the experiment and on the last day of each period. Milk yields were recorded daily.

Table 1. Ingredients and chemical composition used in the total mixed ration (TMR).

Items	TMR	Chitosan extraction
Ingredients (kg dry matter; DM)		
Rice straw	30.00	
Crude glycerin	21.00	
Cassava chips	20.00	
Rice bran	6.31	
Palm kernel meal	9.00	
Soybean meal	9.00	
Molasses, liquid	1.00	

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Urea	2.19	
Pure sulfur	0.50	
Mineral premix	0.50	
Salt	0.50	
Chemical composition		
Dry matter, %	92.84	98.90
Organic matter, %DM	94.20	99.73
Ash, %DM	5.80	0.27
Crude protein, %DM	14.05	0.53
Ether extract, %DM	8.19	-
Neutral detergent fiber, %DM	40.98	-
Acid detergent fiber, %DM	18.23	-
Solubility, %	-	98.70
Deacetylation degree, %	-	88.00

2.2. Sample collection and analysis

During the collection period TMR was collected daily and compounded by the time before the chemical analysis. TMR, fecal, and urine samples were randomly collected in the last 7-days and deposited during a separate period. Rectal sampling was performed for fecal collection, spot sampling was conducted to collect urine and 10% hydrochloric acid was added to prevent nitrogen loss. The deposited TMR and fecal samples were subsampled for the later analysis. The subsamples of deposited TMR and fecal samples were analyzed for dry matter and then oven-dried at 60 °C and milled to pass through a 1-mm screen to analyze the crude protein (CP) and ash [11]. Van Soest et al. [12]'s procedure was followed for neutral detergent fiber-NDF and acid detergent fiber-ADF analyses. An acid insoluble ash (AIA) marker was performed for digestibility estimation [13].

Rumen fluid and blood were taken at 0 hours before offering TMR and 4 hours after offering TMR in the morning, on the last day of each period. An adapted vacuum pump with stomach tube was used to withdraw approximately 200 ml of ruminal fluid. A portable pH meter connected to a pH-temperature probe (HI 8424, Singapore) was used to immediately measure the pH and temperature value. Subsequently, 4 pieces of cheesecloth were applied to obtain the clear ruminal fluid. A plastic bottle containing 5 ml of 1 molar concentration of sulfuric acid was filled with 45 ml of ruminal fluid and immediately closed. Then a centrifugation of 3,000×g for 10 min was performed. All samples were prepared with the same procedure. Samuel et al. [14]'s procedure was performed to analyze acetate, propionate and butyrate under a high-performance liquid chromatography, and the method of AOAC [11] was used for an ammonia nitrogen (NH_3-N) analyses. The above analysis was performed with a sample size of 25-ml of the clear rumen fluid after being centrifuged. The CH_4 estimation followed Moss et al. [15] calculation:

$$CH_4 \text{ production} = 0.45 \text{ (acetate; C2)} - 0.275 \text{ (propionate; C3)} + 0.4 \text{ (butyrate; C4)} \quad (1)$$

The plastic bottle containing 9 ml of 10% formalin was filled with 1 ml of ruminal fluid and placed in a refrigerator at 4 °C for a subsequent count of protozoa and fungi, following Galyen et al. [16]'s method of using a microscopic and haemacytometer (Boeco, Hamburg, Germany).

The blood collection tubes containing EDTA were prepared and filled with 5 ml of blood samples withdrawn from the jugular vein and then centrifuged for 20 min. The plasma portion was placed in a freezer at minus 20°C. The Crocker [17] procedure to measure blood urea nitrogen (BUN) was performed.

A high performance liquid chromatography (HPLC) was used to analyze urine samples for allantoin and creatinine, as described in Chen and Gomes [18]'s procedure. Chen and Gomes [18]'s equation was used to calculate absorption and excretion of purine.

The samples of milk were taken in the morning and afternoon and then combined at a 60:40 ratio. The milk samples were preserved in the plastic bottles containing potassium dichromate ($K_2Cr_2O_7$) and then stored at 4 °C to analyze milk components and milk urea nitrogen (MUN) using Milko-Scan (Foss Electric, Denmark).

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2.3. Statistics determination

The observed data were analyzed according to a replicated 3 × 3 in Latin square design using the PROC MIXED procedure with following model:

$$Y_{ijkl} = \mu + T_i + P_j + A_k + \varepsilon_{ijkl} \quad (2)$$

where Y_{ijkl} are the variances, μ is the overall mean, T_i is the fixed effect of treatment (i = 1, . . . , 3), P_j is the random effect of period (j=1,….,3), A_k is the random effect of cow (k = 1, . . . , 6) and ε_{ijkl} is the residual. Duncan multiples ranging test [19] was performed to check the statistical differences of treatments' mean at $P < 0.05$.

3. Results

3.1. TMR ingredients and nutrient composition

Table 1 shows the nutrient composition of chitosan and TMR containing crude glycerin. Crude glycerin was included at 21% as an energy source in 20% of cassava chips. Urea was added at 2.19% of the TMR ration to upgrade the N content to meet dairy cows' recommended requirements by NRC [20]. The TMR diet contained 14.05% of CP, which is suitable for a cow producing about 10-12 kg of milk daily. The chitosan's consistency was calculated to characterize the preparation process including the acetylation degree and solubility. The shrimp-based shell extract chitosan was of uniform consistency, with 98.90% DM, 99.73% OM, 0.53% CP, and less than 1% ash. The 88% solubility and 98.7% acetylation of the chitosan-based extract indicates it as a potential additive for ruminant rations. The commercial chitosan had a 90% acetylation degree and 99% solubility, 43 mpa.s viscosity, 8.9% moisture, pH of 7.8, and 80 mesh particle size.

3.2. Intake and digestibility study

Table 2 denotes chitosan sources' impact on voluntary dry matter, nutrients and the energy estimation from intake and digestibility. No significant differences on daily dry matter, nutrients or estimated energy intake were noted when cows received different sources of chitosan. The daily intake ranged from 12.46-13.80 kg/day, and ME intake ranged from 23.29-25.69 Mcal/day. Nutrient digestibility was not influenced differently by extraction based or commercial chitosan supplements. The dry matter digestibility ranged from 63.34- 66.81%.

Table 2. Effect of chitosan supplementation on voluntary feed intake and nutrient digestibility in lactating dairy cows.

Items	No-chitosan	CH extraction	CH commercial product	SEM	P value
		Total feed intake			
kg of DM/cow/day	12.46	13.36	13.80	1.01	0.75
% BW of daily	2.93	3.36	3.50	0.29	0.55
Nutrient intake, kg/day					
Organic matter	8.00	8.86	9.03	0.46	0.48
Crude protein	0.83	1.03	1.16	0.16	0.53
Neutral detergent fiber	4.06	4.26	4.26	0.24	0.85
Acid detergent fiber	2.03	2.40	2.80	0.89	0.59
		Estimated energy intake			
ME, Mcal/day	23.29	25.35	25.69	1.38	0.60
ME, Mcal/kg DM	2.25	2.38	2.80	0.27	0.75
		Nutrient digestibility, %			
Dry matter	66.81	63.35	63.34	2.02	0.34
Organic matter	68.92	65.51	65.13	0.81	0.15
Crude protein	66.16	65.19	64.93	2.27	0.94
Neutral detergent fiber	55.32	54.06	56.59	1.54	0.66
Acid detergent fiber	48.47	46.21	46.39	1.82	0.73

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CH= chitosan; SEM=standard error of the mean.

3.3. Ruminal ecology, microbes, and metabolites in blood

The influence of supplemented chitosan sources on ruminal ecology and microorganisms in the rumen of dairy cows is shown in Table 3. The mean values of ruminal pH were not influenced (P>0.05) in all treatments their average ranged from 6.56- 6.74, and the temperature ranged from 39.16-39.35 °C. The NH_3-N at 2 and 4 hours after feeding did not change with the addition of chitosan from different sources, and ranged from 14.56-15.18 mg/dl. The BUN between incorporated and supplemented levels at 4 hours after feeding was not significant, averaging 14.10 and 15.04 mg/dl, respectively. The rumen microorganism populations, such as that of protozoa and fungal zoospores were not altered by the chitosan supplement sources (P>0.05). The mean concentration of protozoa and fungi were 1.25-1.83 x 10^6 cells/ml and 1.20-1.92 10^4 cells/ml, respectively.

Table 3. Effect of chitosan supplementation on rumen fermentation, rumen microorganism population, and blood metabolite

Items	No-chitosan	CH extraction	CH commercial product	SEM	P-value
Ruminal pH					
0 h post feeding	6.76	6.73	6.71	0.03	0.67
4 h post feeding	6.70	6.62	6.69	0.02	0.28
Mean	6.70	6.56	6.74	0.07	0.39
Ruminal temperature, °C					
0 h post feeding	38.77	38.87	39.30	0.36	0.61
4 h post feeding	39.53	39.75	39.39	0.42	0.82
Mean	39.16	39.31	39.35	0.20	0.84
NH_3-N concentration, mg/dL					
0 h post feeding	14.38	14.75	14.60	0.30	0.77
4 h post feeding	14.73	15.60	16.14	0.69	0.56
Mean	14.56	15.18	15.04	0.34	0.60
Blood urea-nitrogen concentration, mg/dl					
0 h post feeding	13.67	14.33	15.83	1.14	0.58
4 h post feeding	14.54	15.67	16.62	3.64	0.94
Mean	14.11	15.00	16.23	1.51	0.73
Protozoa, x 10^6 cells/ml					
0 h post feeding	1.00	1.33	1.50	0.08	0.13
4 h post feeding	1.50	2.00	2.17	0.58	0.79
Mean	1.25	1.67	1.83	0.31	0.58
Fungal zoospore, ×10^4					
0 h post feeding	1.07	0.83	1.00	0.14	0.13
4 h post feeding	1.33	1.83	2.83	1.14	0.75
Mean	1.20	1.33	1.92	0.63	0.86

CH= chitosan; SEM=standard error of the mean.

3.4. Ruminal volatile fatty acids and methane estimation

Table 4 shows the total ruminal concentration of VFA, VFA profiles and CH_4 production in lactating dairy cows supplemented with various sources of chitosan. The different sources of chitosan supplements did not change the totals of VFA, C2 and C4; in contrast, different chitosan sources influenced (P<0.05) C3 content. At 4 hours after feeding, C3 increased by 12.73% when compared to the group without a chitosan supplement. In addition, a comparison between chitosan sources indicated no change in C3 concentration. The ruminal C2 to C3 ratio was markedly (P < 0.05) reduced with chitosan supplement, but no change appeared between sources of chitosan. The chitosan supplementation's effect on CH_4 estimation is shown in Table 4. The average value and the 0 hours after feeding CH_4 estimations from the rumen of cows were similar. At 4 hours after feeding, the CH_4 estimation significantly decreased with the addition of chitosan supplementation (P < 0.05) compared

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to the control group. Chitosan supplementation reduced the CH_4 estimation at 4 hours after feeding by 7.37% compared to the control group.

Table 4. Effect of chitosan supplementation on ruminal volatile fatty acids and methane (CH_4) estimation.

Items	No-chitosan	CH extraction	CH commercial product	SEM	P-value
Total VFA, mmol/L					
0 h post feeding	114.91	116.11	115.16	2.61	0.96
4 h post feeding	111.31	118.21	112.63	1.58	0.20
Mean	113.11	117.16	113.89	0.51	0.07
Acetic acid, mmol/L					
0 h post feeding	65.54	64.95	64.82	1.62	0.96
4 h post feeding	64.84	65.13	63.67	2.38	0.93
Mean	65.19	65.04	64.25	1.88	0.95
Propionic acid, mmol/L					
0 h post feeding	20.73	23.64	22.94	1.48	0.56
4 h post feeding	21.72^{b}	24.26^{a}	24.89^{a}	0.26	0.03
Mean	21.23	23.95	23.92	1.04	0.37
Butyric acid, mmol/L					
0 h post feeding	13.25	11.60	12.06	2.24	0.90
4 h post feeding	13.40	11.88	12.22	3.07	0.95
Mean	13.33	11.74	12.14	2.52	0.93
Acetic acid-to-propionic acid ratio, mmol/L					
0 h post feeding	3.18	2.79	2.84	0.17	0.46
4 h post feeding	2.98^{a}	2.69^{b}	2.60^{b}	0.04	0.03
Mean	3.08	2.73	2.70	0.10	0.22
CH_4 estimation, mM/L					
0 h post feeding	29.09	27.37	27.69	0.88	0.55
4 h post feeding	28.69^{a}	26.44^{b}	26.72^{b}	0.19	0.03
Mean	28.91	26.90	27.21	0.61	0.29

CH= chitosan; SEM=standard error of the mean; $^{a, b}$Differing letters across rows indicate significant differences ($P < 0.05$).

3.5. Purine derivatives and microbial nitrogen synthesis

The effect of chitosan sources on purine derivatives (PD) and on microbial nitrogen synthesis are denoted in Table 5. No differences among treatments were not found for allantoin excretion, absorption or creatinine concentration ($P>0.05$). Microbial nitrogen synthesis (MNS) and the efficiency of microbial N synthesis (EMNS) showed no difference regarding chitosan supplement sources, ranging from 81.89-85.68 g/d, 28.35-33.17 g/kg organic digested in the rumen (OMDR), respectively.

Table 5. Effect of chitosan supplementation on microbial protein synthesis.

Items	No-chitosan	CH extraction	CH commercial product	SEM	P-value
Urinary purine derivatives (mmol/day)					
Allantoin excretion	166.60	168.97	166.46	2.68	0.83
Allantoin absorption	116.02	131.20	124.90	3.90	0.26
MNS (g/day)[1]	81.89	85.68	84.87	0.95	0.23
EMNS (g/kg OMDR)[2]	28.35	33.17	29.86	2.11	0.49

CH= chitosan; SEM=standard error of the mean.[1]Microbial nitrogen supply (MNS), calculated from (PD absorption × 0.727) (Chen and Gomes, 1995). [2]Efficiency of microbial N synthesis (EMNS, g/kg of OM digested in the rumen (OMDR) = [(MCP (g/d) × 1000)/MOMR (g)], assuming that rumen digestion = 65% of digestion in total tract.

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3.6. Milk production

Table 6 presents the effect of chitosan supplementation sources on milk yield and on its composition and economic efficiency return. No significant differences existed on milk yield, milk composition or milk urea nitrogen when cows received different sources of chitosan (P>0.05). The milk yield and the 3.5% fat-corrected milk yield ranged from 10.97-11.60 kg/d and from 11.82-12.64 kg/d, respectively. Economic efficiency is shown in Table 6, demonstrating similar milk incomes among treatments, ranging from 177.30-189.60 baht /cow/day (P>0.05).

Table 6. Effect of chitosan supplementation on milk yield, and milk composition in lactating dairy cows.

Items	No-chitosan	CH extraction	CH commercial product	SEM	P-value
Milk yield, kg/cow/day	10.97	11.43	11.60	0.95	0.92
3.5%FCM, kg/cow/day[1]	11.82	13.26	12.64	1.25	0.80
Milk composition, %					
Protein	3.40	3.37	3.43	0.16	0.97
Fat	3.97	4.47	4.03	0.13	0.24
Lactose	4.67	4.90	4.63	0.25	0.79
Solids-not-fat	8.72	8.92	8.67	0.31	0.91
Total solid	12.68	13.38	12.75	0.41	0.60
Milk urea nitrogen, mg/dl	10.97	11.23	11.37	0.33	0.78
Economic efficiency (Baht)[2]					
Feed cost	64.79[c]	133.60[b]	232.56[a]	9.23	0.04
Milk income	177.30	198.90	189.60	20.56	0.93
Income over feed	112.51[a]	65.30[b]	-33.96[c]	5.63	0.02

CH= chitosan; SEM=standard error of the mean; [a-c] different letters across rows indicate significant differences (P < 0.05).[1]FCM (fat collected milk) = 0.432 (kg of milk/day) +16.23 (kg of fat); [2]TMR= 5.2 baht/kg; Chitosan extraction= 240 baht/kg; Chitosan product= 550 baht/kg; Milk price = 18 baht/kg.

4. Discussion

Chitosan reportedly has a negative effect on microorganisms [21]. However, the chitosan's antimicrobial activities depend on its acetylation degree, on the mass weight and on microbial properties [22]. The extract-based chitosan in the current study had a 98.07% acetylation degree and 88.0% solubility, and commercial chitosan had a 90% acetylation degree and 99% solubility. This resembled the acetylation degree in the study of Araújo et al. [23], who used commercial chitosan with over 92% acetylation degree. Pereira et al. [24], however, used chitosan as an additive with an over 85% deacetylation degree. The variation of acetylation degree in each study might cause the studies' inconsistent results.

Compared to the control group, feed intake was slightly higher in the chitosan supplemented groups. However, chitosan sources did not affect feed intake, which ranged from 2.93- 3.50% BW/d. Feed intake seem higher than recommend by NRD (2001), who suggested that lactating dairy cows with a 410 kg BW and milk production of 12±2 kg/d required a feed intake of about 2.6-2.7% of their BW. This might be due to TMR diet's low rice straw proportion (30%) when 21% glycerin was added, resulting in high palatability and high intake compare to normal TMR. Previous studies demonstrated that chitosan supplementation had no effect on dry matter intake [25]. Goiri et al. [5] also found that chitosan had no effect on sheep's intake of dry matter when chitosan was incorporated at 136 mg /kg BW. Similarly, Mingoti et al. [26] found that chitosan supplementation at 50-150 mg /kg BW had no effect on feed intake.

Nutrient digestibility was not altered by the source of chitosan supplementation (P>0.05). This result aligns with our previous study conducted *in vitro.* Seankamsorn et al. [9] reported that the effects chitosan supplementation at 2% with TMR containing crude glycerin showed no differences in nutrient digestibility for native Thai beef cattle. Furthermore, Holstein cows supplemented with chitosan at 75-225 mg/kg BW had no influence on DM, OM, EE or NDF digestibility [6]. Chitosan

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reduced *in vitro* DM and NDF digestibility in rations containing high fiber [4-5]. Also, sheep fed rations containing chitosan showed no effect on NDF and OM digestibility [5]. However, the present study demonstrates that adding 2% chitosan (either via extraction or commercial product) showed no adverse effect on digestibility and thus might be the optimum levels to manipulate rumen fermentation.

Van Soest et al. [12] reported the optimum environment for rumen ecology maintenance and microbial activity as a pH of 6.5-7.0 and a temperature of 39.5 °C. The ruminal NH_3-N concentration depends on the diet's protein content and N utilization. In this study, cows were fed with similar CP content in the TMR diet which showed no difference in CP digestibility. Thus, no change in ruminal NH_3-N concentration was observed. The ruminal NH_3-N concentration was within the normal range for rumen fermentation, as previously reported (14-30 mg/dl; [27]). Chitosan supplementation did not change the BUN concentration. This agrees with Araújo et al. [23], who found that the BUN was not affected in Nellore steers. However, Mingoti et al. [26] revealed that BUN increased in Holstein dairy cows. Additionally, Garcia-Rodriguez et al. [28] studied sheep that had a high BUN with supplemented chitosan. A higher BUN might relate to a high ruminal protein digestion. Protozoal and fungi numbers were not influenced by the chitosan source. No significant change occurred in the microbial population, so no effect on nutrient digestion was found in present experiment. Anyway, Belanche et al. [29] revealed that a chitosan addition with more than 85% deacetylation decreased protozoal activity. In addition, evidence indicates that chitosan can inhibit ciliate protozoal populations to a lower degree when compared with vegetable oils. However, the current experiment's results might be due to a short period of chitosan supplementation because protozoa and fungal rumen can adapt well with the rumen condition of dairy cows.

Enhancement of C3 and of the ratio of C2 to C3 by chitosan addition may occur because chitosan's mechanical effect was the same as monensin involving an increase of C3 and a decrease of C2, although low dietary intake was included [30]. Zanferari et al. [31] reported that chitosan supplementation in dairy cows at 4 g/kg DM could improve C3 concentration. This aligns with our *in vitro* study [9], which indicated that 26.41% of C3 was enhanced *in vitro* with 21% glycerin plus 2% chitosan. Belanche et al. [2] revealed that adding 2 g/L of chitosan resulted in the greatest C3 concentration. In a Rusitec system study, 36.8% of C3 was increased by chitosan [2].

The stoichiometrical model used for estimating CH_4 from VFA composition was as following Moss et al. [15]. Even though the determination of CH_4 production is usually counted by using a respiratory chamber or by using the gas chromatography technique, unfortunately very costly, and such facilities may not be available especially in the developing countries [32]. Thus, the calculation of CH_4 production from VFA profiles is expected to be a solution to the problem. Jayanegara et al. [32] observed that the estimated CH_4 model line of Moss et al. [15] was constantly closer to the ideal line than the estimated model of Hegarty and Nolan [33] as well as the model, showed a quite accurate result to explain the variation of CH_4 emission. A few theories explaining the mode of action for chitosan's decrease of CH_4 production in ruminants have been suggested [7]. Firstly, chitosan breaks down the cell components of microbes via a reaction between positive and negative charges [22, 34]. Furthermore, reductions in CH_4 production by chitosan could result from the increase of C3 and the decrease C2 to C3 ratio, leading to improve energy usage. Similarly, Goiri et al. [5] showed that chitosan decreased *in vitro* CH_4 concentration, although a high-concentrate diet was provided.

No effect of chitosan sources on purine derivative, on microbial synthesis or on the efficiency of microbial synthesis was found. This result indicates that chitosan supplementation had no adverse effects on microbial protein synthesis in cows receiving TMR containing a glycerin-based diet. In agreement with Paiva et al. [6], this demonstrated that the nitrogen balance and microbial protein synthesis were unaffected by chitosan addition. Additionally, chitosan and soybean oil had no influence on microbial protein synthesis, the allantoin to creatinine ratio, the N urinary excretion or the N balance [8].

Supplemented chitosan showed no adverse effects on milk yield or its composition. The daily milk yield were close to those previously observed in average in the country (12-13 kg/d). Supplementation of high chitosan sources at 2.0% DMI showed no negative effect on milk yield of in

lactating dairy cows receiving TMR containing a glycerin-based diet [35-37]. The milk fat content and total solids were slightly higher than in standard Thai milk, indicating that an average composition should contain 3.5% fat and 12.25% total solids. This indicated that cows received adequate nutrient supply [26]. Similarly, Mingoti et al. [26] demonstrated that a chitosan addition did not influence milk yield or its composition. Furthermore, chitosan supplementation had not effect on milk composition in cows fed diets without oil [8]. Non-chitosan supplementation results in the lowest feed cost and highest income compared to feed with added chitosan. In addition, feed costs increased via chitosan sources. Commercial chitosan was priced higher, increasing feed costs by 89.69 baht/day compared to extracted chitosan. Extracted chitosan supplementation significantly increased income over feed by 99.26 baht/day compared to the commercial chitosan. This could be because the commercial chitosan had a higher price than the chitosan extraction, thus increasing the cost. A comparison between chitosan sources indicates that chitosan extraction might be more economically efficient than commercial chitosan in maintaining milk production and decreasing CH_4 production.

5. Conclusion

This study show that chitosan supplementation resulted in no negative effect on feed utilization, rumen fermentation, milk yield or milk composition. Chitosan supplementation at 2% of dry matter intake could increase the propionic concentration and the ratio of acetate to propionate, while there is a potential of lowering methane production. Furthermore, supplementing extracted chitosan showed more potential than did commercial chitosan for enhancing economic efficiency and recycling shrimp residues, therefore, reducing environmental waste.

Author contributions: Planned and designed the study, A.S., A.C.; Accomplished the animal sampling and laboratory work, A.S.; Analyzed data and writing the manuscript, A.S., A.C.; Provided guidelines for the study and assisted in revision of the manuscript A.S., A.C.; Revised and corrected the manuscript A.S., A.C., S.S., M.W.; All the authors contributed to, read, and approved the final manuscript.

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