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Evaluating the effects of chitosan, plant oils, and different diets on rumen metabolism and protozoan population in sheep

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Abstract: Chitosan (CH) has been shown to be a promising natural antimicrobial agent. This study examined the effects on rumen metabolism when providing diets containing high forage (HFD), low forage (LFD), and maize silage (MSD) to a 24-h batch culture in which the diets were not supplemented (control) or were supplemented with CH (100 mg/L), sunflower oil (SF, 35.0 g/kg dry matter), rapeseed oil (RP, 35.0 g/kg dry matter), or SF and RF with chitosan combinations (SFCH and RPCH). Dry matter digestibility in the batch culture was influenced by CH and was decreased in HFD compared with the control ($P < 0.01$). Decreasing total number of protozoa and the number of *Entodinium* spp. was evident, especially in LFD and MSD, with all additives. RPCH had a predominantly adverse effect on *Dasytricha ruminantium* in MSD. Interactions of the diets × additives in the majority of rumen ciliate populations were detected. The concentrations of trans-vaccenic acid and conjugated linoleic acid (c9, t11-CLA) were unchanged by chitosan. However, the combination of SF and RF with chitosan (SFCH and RPCH) positively affected fatty acid profiles, which might be related to differences in diets, SF or RP composition, and different microbial activity in the batch cultures.

Key words: Chitosan, sunflower oil, rapeseed oil, rumen ciliate protozoa, fatty acids

1. Introduction

A trend among rumen microbiologists and nutritionists is to manipulate the ruminal microbial ecosystem with feed additives that alter the microbial ecosystem in order to improve the efficiency of feed conversion to produce consumable products for humans. Chitosan, a natural antimicrobial agent, is a deacetylated derivative of chitin (N-acetyl-glucosamine polymer); it has a high molecular weight and is the second most abundant natural biopolymer commonly found in the shells of marine crustaceans and the cell walls of fungi (1). Its antimicrobial properties have been widely accepted (2). Possible effects due to its antimicrobial properties on rumen microorganisms may be questionable. Although the negative effects of chitosan on the rumen ciliate protozoa population could be useful, such effects on rumen bacteria, with the consequent decrease in dry matter digestibility, would be harmful. The use of chitosan in ruminant diets influences rumen fermentation processes both in vitro and in vivo (3–6), and it can also inhibit in vitro rumen biohydrogenation when fat sources are included (7). Rumen ciliate protozoa play an important role in increasing the concentration of conjugated linoleic acid (cis9, trans11$C_{18:2}$; c9, t11-CLA) and trans-vaccenic acid (trans11$C_{18:1}$; TVA) in the rumen fluid (8,9), and their alteration by chitosan or by a combination of both additives (i.e. chitosan and plant oil) in different diets could influence rumen fermentation and metabolism. Therefore, the aim of the present study was to determine the influence of chitosan (CH), sunflower oil (SF), rapeseed oil (RP) and their combination (i.e. SFCH and RPCH) on rumen fermentation, rumen ciliate protozoa, and the composition of fatty acids in rumen fluid of sheep incubated with a high forage diet (HFD), low forage diet (LFD), and maize silage diet (MSD).

2. Materials and methods

2.1. Animals and sampling

Rumen inoculum was obtained from 5 rumen-cannulated rams (Lacaune versus Suffolk; 1 year of age; 25.0 ± 0.5 kg in weight) that had been fed 800 g dry matter (DM) of meadow hay and 300 g DM of crushed barley grain in 2 equal meals per day. The rams were housed separately in pens and had free access to water. Rumen fluid was collected before the morning feeding using a manual vacuum pump into a prewarmed (39 ± 0.5 °C) thermos flask filled with CO_2. The rumen fluid from all sheep was

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combined proportionally and blended under CO_2 in a prewarmed blender for 30 s, squeezed through 4 layers of cheesecloth into a pre-warmed flask under a constant stream of CO_2, and kept in a water bath at 39 ± 0.5 °C.

The rumen fluid was mixed (1:1), under continuous flushing with CO_2, with McDougall's buffer (10). After mixing, 35 mL of rumen content inocula was pumped by an automatic pump into preheated fermentation bottles (100-mL serum bottles) containing the diet substrates. The fermentation bottles were then filled up with CO_2 and closed with butyl rubber stoppers and aluminum screw caps. The incubation was performed in the incubator for 24 h at 39 ± 0.5 °C.

Meadow hay and barley grain were used as the components (substrates) of both a HFD (800:200 w/w) and a LFD (500:500 w/w), respectively. The MSD consisted of maize silage. The substrates were ground and sieved (0.15–0.4 mm) and added in a total amount of 0.25 g of absolutely DM into each individual batch culture supplemented (35.0 g/kg DM) with SF or RP from commercial sources. Chitosan (poly (D-glucosamine)-deacetylated chitin, Sigma-Aldrich Co., St. Louis, MO, USA) was added in a dose of 100 mg/L of culture fluid. Nutrient and fatty acid composition of the diet substrates is shown in Table 1.

The experiment consisted of an in vitro batch fermentation trial with 3 diets (i.e. HFD, LFD, or MSD). Ten replications were used for all the experimental groups—rumen inoculum plus a diet substrate (i.e. HFD, LFD or MSD) plus an additive (i.e. CH, SF, RP, SFCH, or RPCH). Ten replicates were used as controls (rumen inoculum plus diet substrate, but no additives). Blanks containing 35 mL of buffered rumen fluid (inoculum, no diets and no additives) were run simultaneously for examination of fermentation activity of the media (results are not shown). At least 2 independent experiments were performed.

Samples of the fermentation fluid for microscopically counting ciliate protozoa were fixed with an equal volume of 8% formaldehyde (11).

Table 1. Nutrient and fatty acid composition of diet substrates and plant oils.

Dry matter (g/kg)	Meadow hay	Barley grain	Maize silage	Sunflower oil	Rapeseed oil
	924	900	320	–	–
Nutrient composition (g/kg of dry matter)					
Nitrogen	8.90	22.1	11.7	–	–
Crude protein	53.3	120	73.2	–	–
Neutral detergent fiber	576	261	410	–	–
Acid detergent fiber	368	67.4	245	–	–
Fat	22.7	24.1	30.0	–	–
Ash	80.0	37.0	52.1	–	–
Starch	–	550	275	–	–
Fatty acid composition (g/kg of fatty acids)					
$C_{14:0}$ myristic	21	12	8.20	1.0	0.5
$C_{16:0}$ palmitic	330	288	238	57	47
$C_{16:1}$ palmitoleic	21	11	7	1.4	2.2
$C_{18:0}$ stearic	48	27	29.5	32	36
$C_{18:1}$ oleic	101	204	193	329	539
$C_{18:2}$ linoleic	183	364	366	535	205
$C_{18:3}$ α-linolenic	138	32	122	10	94
Saturated fatty acids	400	330	308	97	89
Monounsaturated fatty acids	145	224	200	333	558
Polyunsaturated fatty acids	352	402	492	545	306

2.2. Chemical analysis

Standard methods were used for the chemical analysis of substrates: DM (No. 967 03), ash (No. 942 05), nitrogen (No. 968 06), fat (No. 983 23), crude protein (No. 990 03), and starch (No. 948 02) (12). Dried samples were analyzed for neutral detergent fiber (NDF) and acid detergent fiber (ADF) (13) using a Fibertec 2010 (Tecator Comp., Höganäs, Sweden).

The volume of released gas was measured by the pressure transducer technique, and in vitro DM digestibility (IVDMD) was estimated from the difference in dietary substrate weight before and after incubation. Gases from each fermentation bottle were analyzed for methane concentration using a PerkinElmer Clarus 500 gas chromatograph (PerkinElmer, Inc., Shelton, CT, USA). The concentration of short-chain fatty acids (SCFA) in the medium was determined by gas chromatography with a PerkinElmer Clarus 500 gas chromatograph (14). The fatty acid (FA) content was determined from freeze-dried samples using a Thermo Savant Micromodulyo freeze-drier (Thermo Savant Micro Modulyo, NY, USA). Lipids were extracted from 500 mg of freeze-dried sample with a mixture of chloroform:methanol (2:1). The FA methyl esters peaks were identified by authentic standards of C_4–C_{24} FA methyl ester mixture (Supelco, Bellefonte, PA, USA) by gas chromatography (PerkinElmer Clarus 500 gas chromatograph, PerkinElmer, Inc. Shelton, CT, USA).

The results were analyzed statistically by analysis of variance (Graphpad Instat, Graphpad Software Inc., San Diego, CA, USA). All values are shown as means with pooled standard errors of the means (SEM). Effects included in the model were diets (D), 2 levels of chitosan (present versus absent), 2 kinds of oils (SF and RP), combinations of oils with chitosan (OCH), and the interactions between parameters (D × CH, D × O, D × OCH). The treatment effect (i.e. control versus CH, control versus SF, control versus RP, control versus SFCH, and control versus RPCH) was considered significant when no significant diet × additive interaction occurred. Differences from the control were analyzed using a Bonferroni post-test and considered to be significant when $P < 0.05$.

3. Results

The diets (D) affected the results in all fermentation parameters except acetate and propionate ($P < 0.001$; Table 2). IVDMD in the batch culture was influenced by chitosan and in the HFD was decreased compared with the control ($P < 0.01$). The diet × oil interaction (D × O) and diet × oil with chitosan (D × OCH) affected the results in IVDMD ($P < 0.001$). Total gas production was influenced by chitosan ($P < 0.001$), oil ($P < 0.001$), and oil with chitosan ($P < 0.001$), and it decreased statistically or numerically during fermentation in all diets compared with the control. An effect of CH ($P < 0.01$) and the interaction D × CH ($P < 0.001$) in methane production was detected. The D × CH, D × O, and D × OCH interactions affected the total SCFA production ($P < 0.01$ and $P < 0.001$). The molar proportion of n-butyrate was influenced by the combination of CH with oil ($P < 0.01$), and it decreased in MSD with RPCH ($P < 0.01$).

The diets affected all rumen ciliate protozoa numbers ($P < 0.05$ and $P < 0.001$), with the exception of *Enoploplastron triloricatum* (Table 3). The D × CH, D × O, and D × OCH interactions affected the total protozoa number as well as the count of the majority of the individual rumen ciliate genera. The rumen ciliate *Dasytricha ruminantium* was influenced by oil ($P < 0.05$) and OCH ($P < 0.01$), and it decreased in MSD with RPCH ($P < 0.05$).

The diet, oil, and oil with chitosan affected the outputs of all fatty acids ($P < 0.001$; Table 4). The D × O and D × OCH affected the outputs of all fatty acids ($P < 0.05$, $P < 0.01$, and $P < 0.001$) except for myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), and margaric acid ($C_{17:0}$) outputs. Compared with the control, the outputs of $C_{14:0}$, $C_{16:0}$, and $C_{17:0}$ of all diets with additives were lower. The output of stearic acid ($C_{18:0}$) was lower in HFD and MSD with RP ($P < 0.05$) and LFD with SF ($P < 0.01$) when compared with the control.

4. Discussion

There is currently a worldwide trend of producing ecologically clean foods by adding natural supplements to animal nutrition that have a positive influence on the human organism. Our results show that chitosan had a tendency to decrease IVDMD; however, this was significantly decreased only in the high forage diet. A reduction of in vitro digestibility for maize silage diet and a different forage-concentrate diet with chitosan (325, 750, and 1500 mg/L of culture fluid) has also been observed previously (3–5). A slight decrease in the digestibility in sheep given chitosan (136 mg/kg of BW) daily via rumen fistula has also been reported (6). In addition, the lipid supplementation (up to 60 g/kg DM) of diets mostly reduced rumen degradation of fiber and organic matter in vitro and in vivo (15,16). In our experiment, the interactions in IVDMD were detected, and then the P values were hard to interpret. Because IVDMD is associated with microbial fermentation activity, chitosan and oil additives also influenced the total gas production, with a slight effect on methane production. In addition, the molar proportion of n-butyrate was decreased in the maize silage diet with rapeseed oil combined with chitosan, probably because of the induced changes in size and activities of the microbial populations (i.e. *Dasytricha ruminantium*) in the batch cultures. However, chitosan has a predominantly adverse effect on the rumen cellulolytic

Table 2. Effect of diets incubated with chitosan, sunflower oil, rapeseed oil, and their combinations on rumen fermentation patterns.

Diet	Additive	IVDMD (g/kg DM)	Total gas (mL/g DM)	Methane (mmoL/g DM)	SCFA (mmol/L)	Molar proportion of SCFA		
						Acetate	Propionate	*n*-Butyrate
HFD	Control	631	230	3.98	60.0	70.4	17.4	7.42
	Chitosan	574_{B}	205	3.89	75.9	70.0	18.1	7.18
	SF	583	200^{a}	3.01	75.0	69.0	18.4	7.48
	RP	589	210	3.91	67.7	70.0	18.5	7.11
	SFCH	597	195a	2.68	69.8	69.5	18.5	7.43
	RPCH	547	200a	4.18	68.8	70.3	18.2	6.99
LFD	Control	761	240	3.07	71.5	68.3	18.2	8.27
	Chitosan	717	220	2.41	66.3	68.7	18.3	8.15
	SF	734	200^{b}	3.10	68.1	67.7	18.3	8.48
	RP	625	220	3.32	68.4	67.8	19.1	8.20
	SFCH	634	190c	2.57	70.5	68.5	18.3	8.29
	RPCH	615	205a	3.98	69.4	68.3	19.0	8.08
MSD	Control	539	240	8.13	87.9	66.4	19.0	10.9
	Chitosan	532	210_{A}	4.26	85.0	67.0	18.8	10.2
	SF	491	210^{a}	6.86	104	67.0	18.7	10.0
	RP	555	220	6.79	62.0	66.7	19.9	9.95
	SFCH	471	205a	6.11	103	66.5	19.1	10.4
	RPCH	536	210a	7.55	62.7	66.3	20.7	9.46b
SEM		12.6	8.1	0.689	0.64	2.23	1.13	0.441
Diets (D)		***	***	***	***	Ns	Ns	***
Chitosan (CH)		**	***	**	**	Ns	Ns	Ns
Oils (O)		***	***	Ns	**	Ns	Ns	Ns
OCH		***	***	*	***	Ns	Ns	**
D × CH		Ns	Ns	***	**	Ns	Ns	Ns
D × O		***	Ns	Ns	***	Ns	Ns	Ns
D × OCH		***	Ns	Ns	***	Ns	Ns	Ns

HFD: high forage diet; LFD: low forage diet; MSD: maize silage diet; SF: sunflower oil; RP: rapeseed oil; SFCH: sunflower oil with chitosan; RPCH: rapeseed oil with chitosan; OCH: oil + chitosan; IVDMD: in vitro dry matter digestibility; SCFA: short-chain fatty acids; ^{a,b}: Significant differences ($P < 0.05$) between control vs. oil; $_{A,B}$: significant differences ($P < 0.05$) between control vs. chitosan; a,b,c: significant differences ($P < 0.05$) between control vs. oil with chitosan. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Ns: not significant.

Table 3. Effect of diets incubated with chitosan, sunflower oil, rapeseed oil, and their combinations on ciliate protozoa number (n/mL).

Diet	Additive	Total protozoa	*Entodinium* spp.	*Dasytricha ruminantium*	*Isotricha* spp.	*Enoploplastron triloricatum*	*Polyplastron multivesiculatum*	*Ophryoscolex c. tricoronatus*
HFD	Control	75,400	72,000	2800	200	115	120	115
	Chitosan	74,300	71,200	2500	220	130	145	75
	SF	71,500	68,400	2300	200	110	150	90
	RP	63,000	59,800	2600	240	130	100	80
	SFCH	57,300	54,400	2400	165	135	140	20
	RPCH	63,000	59,600	2600	210	120	150	35
LFD	Control	81,700	78,000	2900	350	170	150	100
	Chitosan	64,000	59,600	3100	300	120	145	110
	SF	60,300	56,200	3330	330	130	150	110
	RP	54,600	52,400	2053	58	40	30	10
	SFCH	70,500	66,800	3000	320	130	190	60
	RPCH	58,100	56,000	1900	80	50	35	10
MSD	Control	110,000	105,000	2700	170	130	375	200
	Chitosan	92,000	88,200	1800	330	170	390	200
	SF	108,000	104,400	2000	320	200	370	180
	RP	75,000	72,600	1100	170	35	80	30
	SFCH	80,500	77,000	2100	390	180	400	240
	RPCH	65,300	63,200	900a	75	30	45	35
SEM		6500	6300	360	14	13	19	14
Diets (D)		***	***	*	***	Ns	***	***
Chitosan (CH)		***	***	Ns	***	Ns	Ns	Ns
Oils (O)		***	***	*	***	***	***	***
OCH		***	***	**	***	*	***	***
D × CH		***	***	Ns	***	Ns	***	Ns
D × O		***	***	Ns	***	***	***	**
D × OCH		***	***	Ns	***	***	***	***

HFD: high forage diet; LFD: low forage diet; MSD: maize silage diet; SF: sunflower oil; RP: rapeseed oil; SFCH: sunflower oil with chitosan; RPCH: rapeseed oil with chitosan; OCH: oil + chitosan; a: significant difference ($P < 0.05$) between control vs. rapeseed oil with chitosan. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Ns: not significant.

Table 4. Effect of diets incubated with chitosan, sunflower oil, rapeseed oil, and their combinations on fatty acid profiles.

Diet	Additive	Fatty acids (g/kg of FA)												
		$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{18:1n9}$	TVA	$C_{18:2n6}$	CLA	$C_{18:3n3}$	SFA	MUFA	PUFA
HFD	Control	43.4	46.2	366	31.1	375	13.1	13.9	7.48	0.0	6.29	924	41	35
	Chitosan	30.1	35.3	307	29.5	327	22.3	18.6	8.64	0.0	5.79	906	61	34
	SF	21.3[a]	21.7	199[c]	17.2	324	162	136	9.68	10.3	3.91	734	210	56
	RP	18.0c	19.3	199[c]	10.6[b]	304[a]	220	141	12.2	11.8	3.51	672	294	34
	SFCH	18.5c	18.3	182c	13.0a	319	193	155	12.4	13.2	2.95	698	265	37
	RPCH	19.0b	18.5	191c	10.3b	309	212	137	10.7	10.8	3.52	679	284	37
LFD	Control	35.4	37.7	335	24.1	409	21.8	32.6	9.70	0.0	4.58	912	67	20
	Chitosan	31.8	32.2	296	20.5	358	43.1	37.7	9.17	0.0	5.13	927	51	22
	SF	21.8	21.7	214[b]	13.6	328[b]	167	130	12.9	19.3	3.54	883	86	31
	RP	8.21[c]	8.46	133[c]	5.96[a]	360	214	145	15.0	12.9	4.31	558	406	36
	SFCH	26.2	24.3	244a	14.4	343	150	111	8.37	13.7	5.15	800	178	22
	RPCH	9.38c	9.76	127c	6.84a	367	195	155	15.3	14.2	5.95	660	299	41
MSD	Control	26.0	25.2	258	11.6	426	36.0	42.5	34.0	0.0	8.46	858	110	42
	Chitosan	27.2	24.9	240	12.5	401	39.5	51.3	31.2	0.0	9.61	851	113	36
	SF	19.1	18.8	201	10.5	393	104	57.6	120	6.03	8.85	681	187	132
	RP	10.2[a]	25.0	136[b]	6.15	360[a]	248	52.3	79.5	6.53	23.3	562	326	112
	SFCH	16.5	16.9	191	12.0	378	106	58.3	121	12.0	10.5	649	206	145
	RPCH	6.68a	19.6	100c	4.05	218	435	55.1	112	7.21	39.7	361	478	161
SEM		4.403	3.585	23.8	4.29	17.2	11.5	4.69	4.92	1.29	1.120	14.3	10.7	8.2
Diets (D)		***	***	***	***	***	***	***	***	***	***	***	***	***
Chitosan (CH)		Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Oils (O)		***	***	***	***	***	***	***	***	***	***	***	***	***
OCH		***	***	***	***	***	***	***	***	***	***	***	***	***
D × CH		Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
D × O		Ns	**	Ns	Ns	Ns	***	***	***	***	***	***	***	***
D × OCH		Ns	**	Ns	Ns	***	***	***	***	*	***	***	***	***

HFD: high forage diet; LFD: low forage diet; MSD: maize silage diet; SF: sunflower oil; RP: rapeseed oil; SFCH: sunflower oil with chitosan; RPCH: rapeseed oil with chitosan; OCH: oil + chitosan; FA: fatty acids; TVA: trans11$C_{18:1}$; CLA: cis9, trans11$C_{18:2}$; SFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA; [a,b]: Significant differences ($P < 0.05$) between control vs. oil; a,b,c: significant differences ($P < 0.05$) between control vs. oil with chitosan. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Ns: not significant.

ciliate population, and it is possible that chitosan also modifies the microbial ecosystem of the rumen through cellulolytic bacteria in a high forage diet.

In our experiment, the diets high in starch (low forage diet and maize silage diet) affected the relative proportion of ciliate protozoa as well as the extent of fermentation in the batch culture. The growth efficiency of the majority of rumen ciliates depended on the amount of starch (concentrate) in the diets. However, rumen ciliate protozoa did not show a uniform response to the tested diets and additives. On the other hand, some in vivo and in vitro studies have shown that most species of ciliate protozoa are unable to grow with a diet rich in starch (17,18). In addition, rumen fibrolytic ciliates (e.g., *Ophryoscolex*, *Eremoplastron*, *Diploplastron*, *Polyplastron*, *Eudiplodinium*) prefer complex substrates with fiber and protein and a small proportion of starch (19,20). The combination of chitosan with rapeseed oil (RPCH) has a predominantly adverse effect on *Dasytricha ruminantium* in maize silage diet. Decreasing total number of protozoa and number of *Entodinium* spp. was evident especially in the low forage diet and maize silage diet with all additives. However, as interactions of diets and additives occurred in the count of the majority of rumen ciliate genera, we speculate that all additives used (chitosan, SF, RP, SFCH, and RPCH) had an antiprotozoan effect, although potentiated effects were observed only in the maize silage diet. It is known that plant oils rich in polyunsaturated FAs (PUFAs) alter the ruminal microbial population at a dose of less than 5% of dietary DM (21). The FA profile of protozoan cells also depends on the species of ciliates and presumably may be influenced by dietary FAs (22). However, more than 5% of dietary DM being of C_{18} unsaturated FAs has a stronger antiprotozoan effect and can lead to the total elimination of rumen protozoa (23).

In our experiment, we also examined the effect of chitosan with sunflower oil and rapeseed oil on lipid metabolism. Chitosan has been shown to reduce biohydrogenation in vitro by increasing TVA and total CLA proportions and by decreasing $C_{18:0}$ regardless of the dietary FA source (7). In our experiment, the use of only chitosan had no effect on the FA profiles of rumen fluid. On the other hand, it is known that plant oils could positively affect TVA, and c9, t11-CLA flow from the rumen (24). We found an increase in TVA and c9, t11-CLA concentrations in all diets with oil additives. However, when the interactions were significant in lipid metabolism, we can speculate that differences may be caused by differences in microbial populations developed during fermentation in the batch cultures or by differences in the amount of concentrate in the diets. Recent studies have reported a relationship between the proportion of C_{18} FA isomers and the forage-to-concentrate ratio in diets (25,26). Increasing the amount of concentrate has been shown to increase c9, t11-CLA content in vitro and in vivo (27), but other authors found no changes in the proportion of c9, t11-CLA when concentrates were increased in diets (28,29).

In conclusion, the supplementation of 3 different diets (i.e. diets containing high forage, low forage, and maize silage) with chitosan at a dose 100 mg/L of culture fluid for 24 h in vitro incubation has an effect on IVDMD, total gas, methane production, and the growth of some rumen ciliate genera. Chitosan did not affect the FA profiles, and it was not effective in increasing conjugated linoleic acid and trans-vaccenic acid concentrations in batch cultures. However, the combination of both additives (sunflower oil with chitosan, rapeseed oil with chitosan) had the opposite effect, suggesting that the effects of oils dominated the effects of chitosan. However, more studies are needed to determine the impact of chitosan as a component of ruminant diets.

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