



Feeding milk supplemented with *Ulva* sp., *Ascophyllum nodosum*, or *Saccharina latissima* to preweaning dairy calves: Effects on growth, gut microbiota, gut histomorphology, and short-chain fatty acids in digesta

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ABSTRACT

Emerging knowledge shows the importance of preweaning nutrition on programming the gastrointestinal microbiome and development of the gut barrier function. The aim of this study was to assess the effects of supplementing cow milk with either intact dried *Ulva* sp., *Ascophyllum nodosum*, or *Saccharina latissima* on growth performance and several gut health parameters of preweaning dairy calves. Forty male Holstein calves were selected based on birth weight (41 ± 4 kg) and plasma Brix percentage ($\geq 8.7\%$) at d 2 of life. From d 2 to d 42 of life, the control calves ($n = 10$) were fed with cow milk (8 L/d) without seaweed supplementation, and the experimental calves were fed with cow milk (8 L/d) supplemented with either *Ulva* sp. ($n = 10$), *A. nodosum* ($n = 10$), or *S. latissima* ($n = 10$) at a concentration of 50 g/8 L of cow milk per day (i.e., 5% on a dry matter basis). Calves were weighed every week, and body weight gain and calf starter intake were monitored weekly. At $d 42 \pm 3$ of life, calves were slaughtered. The organ weights and digesta pH from the reticulorumen, mid- and end small intestine, and mid-colon were recorded. A tissue sample (5 cm) collected from the mid-small intestine was analyzed for histomorphology. Digesta from the mid-small intestine and mid-colon were analyzed for lactobacilli, *Escherichia coli*, and *Enterobacteriaceae*, and short-chain fatty acid profile. Weight gain of the calves was not affected by seaweed supplementation. Proportional organ weights were not affected by seaweed supplementation except for reticulorumen weight, which was higher in calves fed *Ulva* sp.

Both the mid-small intestinal and mid-colonic digesta populations of lactobacilli, *Enterobacteriaceae*, and *E. coli*, as well as the mid-small intestinal histomorphology in seaweed-supplemented calves were not different from control calves. However, acetic acid proportion in mid-colonic digesta was increased in calves fed *Ulva* sp. and *A. nodosum*, whereas butyric acid proportion was decreased compared with the control calves. Digesta pH in mid- and end small intestine and mid-colon were not affected, whereas ruminal pH was increased in calves fed *Ulva* sp. compared with the control calves. In conclusion, intact dried seaweed supplementation did not improve the growth or selected gut health parameters (i.e., histomorphology, digesta pH, bacteria, and short-chain fatty acids) in preweaning Holstein calves. **Key words:** gut health, prebiotic, intact seaweed, ruminant

INTRODUCTION

Preweaning dairy calves have high susceptibility for several infectious diseases, including neonatal calf diarrhea (Svensson et al., 2003). Neonatal calf diarrhea has a substantial negative impact on the economy of dairy farmers, causing severe health and welfare issues in calves (Lorenz et al., 2011; Walker et al., 2012; USDA, 2018). These include increased morbidity, mortality, and reduced growth rate, subsequently leading to increased age and difficulty at first calving (Windeyer et al., 2014). Furthermore, unhealthy calves have reduced milk yield at first lactation as well as reduced lifetime milk production (Heinrichs and Heinrichs, 2011; Dunn et al., 2018).

In recent years, several studies have focused on improving health of preweaning calves through manipulation of the gastrointestinal microbiome (Malmuthuge et al., 2015, 2019). Mammalian fetuses have a sterile digestive tract, and microbial colonization starts at birth. This complex colonization process plays a cru-

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cial role in the development of the immune system of neonatal calves (Malmuthuge et al., 2015; Hulbert and Moisé, 2016). Therefore, during this early stage of life, the gut microbiome of host calves can be manipulated in a beneficial way (Malmuthuge et al., 2019), which can reduce diarrhea incidences and improve growth and health in these animals (Malmuthuge et al., 2015).

Prebiotics are nonviable substrates favoring beneficial microorganisms such as lactobacilli and bifidobacteria in the gut microbiome, resulting in a competitive advantage over pathogenic microorganisms (i.e., pathogenic *Escherichia coli*), thus conferring health benefits for the host animal (Ashaolu, 2020). Most commonly used prebiotics in calf nutrition are fermentable oligosaccharides such as mannanoligosaccharides and fructooligosaccharides, among others (Cangiano et al., 2020). Seaweed-derived functional polysaccharides such as laminarin and fucoidan have gained increasing interests as prebiotics for neonatal pigs (Sweeney et al., 2011). However, such beneficial effects were not always visible, resulting in inconsistent results, especially when the seaweeds were fed in intact form (Dierick et al., 2009; Michiels et al., 2012). To our knowledge, the present study is the first to investigate the functional effects of feeding intact seaweeds to preweaning dairy calves. In addition, this study will also increase our knowledge on the potential benefits of these seaweeds in calf nutrition. Whole seaweeds were preferred over extracts, as they are cheaper and already considered as feed ingredients by the European Union (EU); thus, no EU registration is needed when they are used as feed additives.

We hypothesized that feeding whole seaweeds, either *Ulva* sp., *Ascophyllum nodosum*, or *Saccharina latissima*, would improve the gut health and consequently growth of the preweaning dairy calves. The objective of this study was to evaluate the effects of feeding milk supplemented with intact dried and ground *Ulva* sp., *Ascophyllum nodosum*, or *Saccharina latissima* on growth and selected gut health parameters (i.e., digesta microbial composition, pH, short-chain fatty acid profile, and intestinal histomorphology) of preweaning Holstein calves. A companion paper investigated the effects of feeding milk supplemented with these seaweeds on systemic immune responses in preweaning Holstein calves (Samarasinghe et al., 2021a).

MATERIALS AND METHODS

All experimental procedures and care and handling of the experimental animals were conducted in compliance with the Danish Ministry of Justice Law No. 474 (May 15, 2014).

Seaweeds

Three different seaweeds grown in Denmark and Iceland were used (Samarasinghe et al., 2021a). Harvesting locations, processing procedure, and in-depth chemical characterization of the 3 seaweeds have been reported in companion papers (Samarasinghe et al., 2021a,b). In brief, *Ulva* sp. and *Saccharina latissima* were harvested in Denmark, and were dried and ground to a screen size of 0.8 mm. *Ascophyllum nodosum* (Thorverk HF) was harvested in Iceland, dried, and ground to a screen size of 0.2 mm. The term “*Ulva* sp.” is used throughout this paper due to the uncertainty of exact species name, as the species diversity of *Ulva* has recently been proven considerably larger than previously expected (Steinhagen et al., 2019). Therefore, exact species determination of *Ulva*, based on algae morphology, is not currently possible (Steinhagen et al., 2019).

Animals and Feeding

Experimental animals, housing, and other management practices are reported in a companion paper (Samarasinghe et al., 2021a). In brief, 40 male Holstein calves were selected based on birth BW (41 ± 4 kg) and plasma Brix percentage ($\geq 8.70\%$) at d 2 of life. The calves were fed 4 L of colostrum (Brix $\geq 22.0\%$) within 2 h after birth and had ad libitum access to fresh water, hay, and a standard commercial calf starter throughout the experimental period.

Selected calves were divided into 10 blocks of 4 calves according to birth order, and treatments were randomly assigned within each block. Calves received 4 L of cow milk ($40.0 \pm 2^\circ\text{C}$; 5.00% lactose, 3.50% protein, 13.5% total solids) twice a day without seaweed supplementation ($n = 10$) or with either dried *Ulva* sp. ($n = 10$), *A. nodosum* ($n = 10$), or *S. latissima* ($n = 10$). The treatment calves were fed dried and ground seaweeds from d 2 until d 42 of age, mixed into milk at a concentration of 25 g/4 L of milk (i.e., 5% on DM basis) twice daily using a nipple bucket, as described by Samarasinghe et al. (2021a). The BW of the calves and the individual calf starter intake were recorded weekly. Thereafter, ADG and daily calf starter intake were calculated.

Collection of Samples from the Gastrointestinal Tract

The calves were slaughtered ($n = 10/\text{group}$) at d 42 ± 3 of life, using a captive bolt pistol followed by exsanguination, and the entire digestive tract was removed by blunt dissection. Thereafter, the digestive tract was partitioned using 1.5-mm nylon threads to clamp the

digestive content to prevent mixing between gastrointestinal tract segments.

First, the small intestine was divided into 3 equal-length segments. At the middle point of the second segment, a 5- to 7-cm sample of intestinal tissue was collected. This tissue sample was rinsed with PBS and fixed in 10% neutral buffered formaldehyde for 24 h for histomorphological analysis. Digesta samples from the second and third segments of the small intestine were collected, and pH was measured. The middle point of the colon was marked using a 1.5-mm nylon thread, digesta samples were collected at this site, and pH was measured. Additionally, reticulorumen digesta pH was recorded. The digesta samples for quantitative (q)PCR analysis were collected from the mid-small intestine and mid-colon and immediately transferred into cryotubes and snap-frozen in liquid nitrogen. For short-chain fatty acid (SCFA) analysis, representative digesta samples from the mid-small intestine (20 g) and mid-colon (20 g) were collected into cooled and sterile tubes (50 mL), put into an ice bath, and stored at -20°C within 30 min after collection. Organ weights, including the reticulorumen, omasum, abomasum, and small intestine were recorded after emptying the digesta. Length of the small intestine was recorded.

Quantitative Real-Time PCR

Extraction of DNA from the digesta samples was performed following the manufacturer's guidelines using an EZNA Stool DNA Kit (Omega Bio-Tek) with slight modifications. Briefly, the samples containing the preservation buffer provided by the kit (i.e., SLX-Mlus buffer) were disrupted using a bead beater (Star-Beater, VWR International) operating at a frequency of 30 (1/s) for 5 min. Thereafter, DNA concentration was analyzed using a Qubit fluorometer 3.0 (Life Technologies). DNA-extracted samples were analyzed using qPCR and a set of primers as described in Table 1 (Sigma-Aldrich). Temperature and primer concentration were modified for each pair of primers (Table 1). For each reaction, solution containing 5 μL of Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), primers in concentrations as stated in Table 1, 2 μL of template DNA, and nuclease-free water up to the final volume of 10 μL were used. The qPCR analysis was performed using a MicroAmp Optical 384-well reaction plate (Applied Biosystems) and an ABI ViiA7 real-time PCR system (Thermo Fisher Scientific). Furthermore, specific conditions during each reaction were as follows: sample mixture was pretreated for 2 min at 50°C , followed by initial denaturation (10 min at 95°C)

and subsequently 40 cycles of denaturation for 15 s at 95°C , 30 s for primer annealing at different temperatures (Table 1), and 30 s at 72°C for base extension. Melting curves were derived by increasing the temperature from 60 to 95°C at a rate of $0.05^{\circ}\text{C}/\text{s}$, recording continuously. These curves were used to evaluate the quality of the PCR products. All analyses were performed in triplicate. A standard curve was created using DNA from pure bacterial cultures, and a no-template control was used during each analysis. *Escherichia coli* AUF4 (serotype 0149:F4, virotype F4ac, STb, LT, EAST), *E. coli* K12, and *Lactobacillus reuteri* were used as the reference strains. The genome size and DNA concentration were used to calculate the number of copies of target DNA in the standard, using the DNA to copy calculator (Biostuff, 2021). The concentrations of target DNA in the samples were estimated using PCR cycle threshold values, using QuantStudio real-time PCR software version 3.1 (Thermo Fisher Scientific).

Histomorphology Analysis

The preserved mid-small intestinal tissue samples were cleaned using saline (154 mM NaCl), dehydrated, and processed using standard paraffin-embedding technique. From each sample, a slide containing 6 to 8 sections that were at least 50 μm apart and having a thickness of 4 μm was prepared. The slides were stained using hematoxylin and eosin, as described by (Hedemann et al., 2005). A computer-integrated microscope and an image analysis system (Zeiss Zen Imaging Software version 2.6) were used for analysis of the histomorphology of the stained tissue samples, and the analysis was performed as previously described by Hedemann et al. (2005). Briefly, 15 well-oriented villi and crypts were selected on each slide, and villus height and crypt depth were measured. In addition, density of the villi and crypts and thickness of the *Muscularis externa* were measured. The density was determined as the number of villi or crypts visible over a defined area of the stained slide. A mean of the 15 determinations per sample was used in the statistical analysis.

Short-Chain Fatty Acid Analysis

The digesta samples were thawed in a water bath at room temperature for 4 h, and 20 g of deionized water was added and vortexed. Thereafter, the samples were centrifuged at $3,000 \times g$ for 20 min at 4°C (Rotixa 50 RS, Andreas Hettich GmbH and Co. KG), and the supernatant was harvested for SCFA analysis. A total of 8 mL of supernatant was mixed with 2 mL of 25% meta-

Table 1. Primers and quantitative PCR conditions used for real-time PCR

Primer name ¹	Target sequence	Sequence (5'-3')	Conc. ² (μM)	T _a ³ (°C)	Size (bp)	Reference
Bank-lacto-F	Genus <i>Lactobacillus</i> (23S)	GCGGTGAAATTCCTCAACG	0.30	60	216	Hermann-Bank et al., 2013
Bank-lacto-R		GGGACCTTAACTGGTGAT				
<i>Escherichia coli</i>	All <i>E. coli</i> (<i>ybbW</i> gene)	TGATTGGCAAAATCTGGCCG	0.50	65	211	Walker et al., 2017
401 F						
<i>E. coli</i> 611 R		GAAATCGCCCAAAATCGCCAT				
All ent F	All <i>Enterobacteriaceae</i> (16S rRNA)	TGTCGACTTGGAGGTGTGTC	0.30	65	63	Frydendahl et al., 2001
All ent R		GCGGTCGACTTAACGCGTT				

¹F = forward; R = reverse.²Primer concentration.³Annealing temperature.

phosphoric acid and mixed gently; thereafter the SCFA profile was determined using gas chromatography as described by Kristensen et al. (1996). Concentration of L-lactate was determined using membrane-immobilized substrate-specific oxidases (i.e., L-lactate oxidase) by a YSI Biochemistry Analyzer (YSI 7100, YSI Inc.). Concentration of D-lactate in digesta was analyzed by an enzymatic-fluorometric method, as described by Larsen (2017).

Statistical Analyses

The sample size per group was determined using the Power procedure of SAS software (version 9.4, SAS Institute Inc.) based on the results published by Heins and Chester-Jones (2015) regarding the BW recorded on d 90 of life in calves supplemented with or without *S. latissima*. Based on this analysis, each experimental group should contain 10 animals to provide a power of 0.8 and a significance level of 0.05.

All other statistical analyses were performed using R version 3.6.1 (<https://www.r-project.org/>). The effect of seaweed supplementation on ADG and calf starter intake was analyzed using a linear mixed model, considering seaweed supplementation (SW), age (T), and their interaction (SW × T) as fixed effects, and calf nested within block as random effect, considering compound symmetry as the correlation structure. In addition, the individual calf was considered as the experimental unit. Initially, birth BW was used as a covariate for analysis of starter intake data. However, this addition did not improve the model; hence, it was removed from the model. The model was fitted with REML, and the lmer function from the lme4 package was used (Bates et al., 2015). Final BW, proportional organ weights, and gut parameters (i.e., qPCR data, SCFA concentrations, digesta pH, histomorphology) were analyzed using a linear model considering treatment (SW) as a fixed effect and block as a random effect. For final BW analysis, birth BW was included as a covariate. The model was fitted with REML, and the lmer function from the lme4 package was used (Bates et al., 2015). Residual error was assumed to be independent with constant variance and normal distribution. The qPCR and D-lactate data were log₁₀ transformed to obtain variance homogeneity and normality of residuals.

Least squares means (LSM) and standard error of means (SEM) were obtained using the emmeans package of R (Lenth et al., 2020). Differences between LSM were evaluated using Tukey's method for comparing a family of 8 estimates. The contrast function was used to test the general effect of each seaweed supplementation against control. Statistical significance was set as $P \leq 0.05$, and tendencies were set as $0.05 < P \leq 0.10$.

Table 2. Overall and weekly ADG (kg/d) of calves fed with only milk (control, n = 10) and calves fed with milk supplemented with *Ulva* sp. (SW1; n = 10), *Ascophyllum nodosum* (SW2; n = 10), or *Saccharina latissima* (SW3; n = 10)

Item	Experimental group				SEM	Fixed effect ¹		
	Control	SW1	SW2	SW3		SW	T	SW × T
Overall	0.90	0.91	0.87	0.88	0.04	0.905	0.250	0.218
Wk 1	1.02	0.79	0.98	0.82	0.08			
Wk 2	0.93	0.87	0.93	1.06	0.08			
Wk 3	0.93	1.03	0.88	0.79	0.08			
Wk 4	0.87	1.03	0.89	0.94	0.08			
Wk 5	0.90	0.85	0.81	0.92	0.08			
Wk 6 ²	0.74	0.91	0.75	0.73	0.11			

¹SW = seaweed supplementation; T = time; SW × T = seaweed supplementation and time interaction.²n = 5 per treatment, as half of the calves were slaughtered in the middle of wk 5.

RESULTS

Growth Performance and Calf Starter Intake

Average daily gain was high in all experimental groups (0.88–0.91 kg/d), and ADG of the SW calves was not different from the control calves ($P = 0.905$; Table 2). As shown in Table 3, the average daily calf starter intake was affected by seaweed supplementation ($P = 0.008$), and *Ulva* sp. fed group (86.7 g of DM/d) had higher starter intake than the control group (47.3 g of DM/d). This difference in daily calf starter intake between calves fed *Ulva* sp. and control calves was visible at wk 4 and continued until wk 6 (Table 3).

Gut Health Parameters

Final BW was not affected by the seaweed supplementation ($P = 0.787$; Table 4). Proportional weight of the reticulorumen was affected by seaweed supplementation ($P = 0.024$). Calves from the group fed *Ulva* sp. had the highest proportional reticulorumen weight

(7.52 g/kg of BW), significantly higher than control calves ($P = 0.025$) and with a tendency to be higher than calves fed *S. latissima* ($P = 0.070$; Table 4). Neither proportional weight of the omasum, abomasum, or small intestine, nor proportional length of the small intestine were affected by seaweed supplementation.

Lactobacilli population in mid-small intestinal digesta tended to be affected by seaweed supplementation ($P = 0.095$; Table 5). Nevertheless, no differences were observed in lactobacilli population in mid-small intestinal digesta of calves fed milk supplemented with seaweeds compared with control calves (Table 5). Similarly, a tendency was observed in lactobacilli population in mid-colonic digesta ($P = 0.092$; Table 5), where calves fed *Ulva* sp. tended to have a greater population than that of calves fed *A. nodosum*. The *E. coli* and *Enterobacteriaceae* populations in mid-colonic digesta were not affected by seaweed supplementation (Table 5).

The SCFA concentrations in mid-small intestinal digesta of the calves were below the detection limit of the method used for the SCFA analysis; hence, these

Table 3. Overall and weekly average daily calf starter intake (g of DM/d) of calves fed with only milk (control, n = 10) and calves fed with milk supplemented with *Ulva* sp. (SW1; n = 10), *Ascophyllum nodosum* (SW2; n = 10), or *Saccharina latissima* (SW3; n = 10)

Variable	Experimental group				SEM	Fixed effect ¹		
	Control	SW1	SW2	SW3		SW	T	SW × T
Overall	47.3 ^a	86.7 ^{b,B}	56.5 ^a	59.4 ^{a,A}	8.83	0.008	<0.001	0.340
Wk 2	32.8	37.2	26.1	43.4	13.1			
Wk 3	48.1	76.1	47.5	46.8	12.6			
Wk 4	37.8 ^a	90.4 ^b	45.9 ^a	44.5 ^a	13.1			
Wk 5	58.4 ^a	104.6 ^b	80.5 ^{ab}	75.1 ^{ab}	12.6			
Wk 6 ²	59.4 ^a	125.2 ^{b,B}	82.8 ^{a,A}	87.1 ^{ab}	13.8			

^{a,b}Lowercase letters within a row indicate significant differences ($P \leq 0.05$).^{A,B}Uppercase letters within a row indicate tendencies ($0.05 < P \leq 0.10$).¹SW = seaweed supplementation; T = time; SW × T = seaweed supplementation and time interaction.²n = 5 per treatment, as half of the calves were slaughtered in the middle of wk 5.

Table 4. Final BW and proportional organ weights of calves fed with only milk (control, n = 10) and calves fed with milk supplemented with *Ulva* sp. (SW1; n = 10), *Ascophyllum nodosum* (SW2; n = 10), or *Saccharina latissima* (SW3; n = 10)

Variable	Experimental group				SEM	P-value ¹
	Control	SW1	SW2	SW3		
Final BW (kg)	79.1	79.3	77.8	78.4	1.80	0.787
Proportional organ weights (g/kg of BW)						
Reticulorumen	6.68 ^a	7.52 ^{b,B}	7.12 ^{ab}	6.81 ^{a,A}	0.20	0.024
Omasum	2.03	1.98	2.04	2.01	0.08	0.953
Abomasum	6.15	6.03	6.02	5.85	0.26	0.878
Small intestine (SI)	28.0	28.2	29.5	29.9	1.29	0.604
Proportional SI length (m/kg of BW)	0.15	0.15	0.16	0.17	0.01	0.301

^{a,b}Lowercase letters within a row indicate significant differences ($P \leq 0.05$).

^{A,B}Uppercase letters within a row indicate tendencies ($0.05 < P \leq 0.10$).

¹Seaweed supplementation.

results are not presented. Total SCFA, L- and D-lactate concentrations in mid-colonic digesta were not affected by seaweed supplementation (Table 6), although the proportions of several SCFA were affected. Acetic acid concentration as a percentage of total SCFA concentration was affected by seaweed supplementation ($P < 0.001$). The acetic acid percentage in mid-colonic digesta was highest in calves fed *A. nodosum* ($P < 0.001$), followed by those fed *Ulva* sp. ($P = 0.012$), and was 8 to 13% greater than that of control calves. Propionic acid percentage in mid-colonic digesta of calves fed *A. nodosum* was lower compared with those fed control ($P < 0.001$), *Ulva* sp. ($P = 0.006$), and *S. latissima* ($P = 0.005$; Table 6). In addition, butyric acid percentage in mid-colonic digesta was 26% and 18% lower in calves fed *Ulva* sp. and *A. nodosum*, respectively, compared with the control calves ($P < 0.05$). Isovaleric acid percentage in mid-colonic digesta was lower in calves fed *A. nodosum* compared with the control calves ($P = 0.032$; Table 6).

Neither the mid- and end small intestinal nor the mid-colonic digesta pH were affected by seaweed supplementation (Table 7). Nevertheless, contrast analysis revealed that the reticulorumen pH in the calves fed *Ulva* sp. was 0.34 points greater than that in the control calves ($P = 0.040$).

Considering histomorphology of mid-small intestinal epithelium, none of the studied parameters, including crypt depth and density and muscle layer thickness, were affected by seaweed feeding (Table 8).

DISCUSSION

The use of seaweed extracts (i.e., laminarin, fucoi-dan, fatty acids, and others) for improving health of young livestock, mainly for weanling piglets, has gained great interest in the last 2 decades (Reilly et al., 2008; McDonnell et al., 2010; Øverland et al., 2019). However, there is a paucity of knowledge on the potential of intact (dried and ground) seaweeds in this regard

Table 5. Lactobacilli, *Escherichia coli*, and *Enterobacteriaceae* populations (\log_{10} DNA copies/g of digesta) in mid-small intestinal and mid-colonic digesta of calves fed with only milk (control, n = 10) and calves fed with milk supplemented with *Ulva* sp. (SW1; n = 10), *Ascophyllum nodosum* (SW2; n = 10), or *Saccharina latissima* (SW3; n = 10)

Variable	Experimental group				SEM	P-value
	Control	SW1	SW2	SW3		
Mid-small intestinal digesta ¹						
Lactobacilli	6.95	7.44	7.37	6.97	0.18	0.095
<i>E. coli</i>	ND ²	ND	ND	ND		
<i>Enterobacteriaceae</i>	ND	ND	ND	ND		
Mid-colonic digesta						
Lactobacilli	8.38	8.66	8.19	8.22	0.15	0.092
<i>E. coli</i>	5.80	6.00	5.06	5.27	0.48	0.411
<i>Enterobacteriaceae</i>	6.33	6.56	5.52	5.75	0.44	0.249

¹*E. coli* and *Enterobacteriaceae* populations in mid-small intestinal digesta were below the detection limit of the analysis method.

²ND = not detected.

Table 6. Total short-chain fatty acids (SCFA) concentration and SCFA profile in digesta from mid-colon of control calves (control, n = 10) and calves supplemented with *Ulva* sp. (SW1; n = 10), *Ascophyllum nodosum* (SW2; n = 10), or *Saccharina latissima* (SW3; n = 10)

Variable	Experimental group				SEM	P-value
	Control	SW1	SW2	SW3		
Total SCFA (mmol/L)	65.80	62.80	71.10	59.60	5.73	0.503
SCFA profile (mmol/100 mmol of total SCFA)						
Acetic acid	64.50 ^a	69.60 ^{bc}	72.80 ^b	67.80 ^{ac}	1.27	<0.001
Propionic acid	19.30 ^a	18.20 ^a	14.40 ^b	18.40 ^a	0.85	<0.001
Butyric acid	8.79 ^a	6.54 ^{bc}	7.18 ^{bc}	7.77 ^{ac}	0.54	0.003
Isobutyric acid	2.11	1.68	1.57	1.79	0.18	0.168
Isovaleric acid	1.96 ^a	1.39 ^{ac}	1.19 ^{bc}	1.50 ^{ac}	0.20	0.043
Valeric acid	2.84	2.26	2.48	2.18	0.26	0.268
Caproic acid	0.47	0.46	0.40	0.46	0.04	0.651
L-Lactate (mmol/g)	0.13	0.08	0.16	0.14	0.04	0.540
D-Lactate, log ₁₀ (mmol/g)	0.16	0.18	0.46	0.11	0.19	0.114
D-Lactate ¹ (mmol/g)	0.26	0.26	1.48	0.30	—	

^{a-c}Lowercase letters within a row indicate significant differences ($P \leq 0.05$).

¹LSM from the statistical model using untransformed values.

and inconsistencies among different studies (Dierick et al., 2009; Michiels et al., 2012). In addition, there is currently no literature regarding the use of intact seaweeds in preweaning calf nutrition. Using seaweeds in their intact form can be advantageous, as it avoids expensive extraction procedures and it represents a sustainable feed supplement (Øverland et al., 2019). To the best of our knowledge, the present study is the first to investigate the effects of feeding milk supplemented with dried intact seaweeds (i.e., *Ulva* sp., *A. nodosum*, or *S. latissima*) on growth performance, organ size, and selected gut health parameters of preweaning dairy calves.

In the current study, intact seaweed supplementation at a dose equivalent to 50 g/d (i.e., 5% DM basis) was selected, based on our interest in using it as a supplement to be fed together with milk. In the case of using higher doses, these seaweeds could not be considered feed supplements but feed ingredients. The current supplementation rate of the intact dried seaweeds was 5 to 20 times higher than the rate applied by Michiels et al. (2012), in which intact dried *A. nodosum* was tested for improving the performance of newly weaned piglets.

However, similar to the observations by Michiels et al. (2012), we observed that the intact dried seaweed supplementation did not improve ADG in preweaning calves. The generally good health condition among the experimental calves of the present study (Samarasinghe et al., 2021a) could also explain the lack of differences in ADG between treatment groups. Michiels et al. (2012) observed no differences in feed intake between piglets fed with *A. nodosum* and control piglets. In the present study, calf starter intake was affected by *Ulva* sp. supplementation, which was not expected. This is because seaweeds were supplemented in milk, which bypassed the rumen via the esophageal groove, and all calves had the same milk intake (Samarasinghe et al., 2021a). The increased proportional weight of the reticulorumen of calves fed *Ulva* sp. could probably be explained by the higher calf starter intake by these calves. Nevertheless, increased starter intake does not correspond well with the slightly higher reticulorumen pH in calves fed *Ulva* sp. It should be noted that the calves consumed 8 L of whole cow milk per day and had a similar ADG during the entire experimental period. Thus, the amount of fed cow milk provided approximately 95% of the net

Table 7. Digesta pH in reticulorumen, mid-small intestine, end small intestine, and mid-colon of calves fed only milk (control, n = 10) and calves fed with milk supplemented with *Ulva* sp. (SW1; n = 10), *Ascophyllum nodosum* (SW2; n = 10), or *Saccharina latissima* (SW3; n = 10)

Variable	Experimental group				SEM	P-value
	Control	SW1	SW2	SW3		
Digesta pH						
Reticulorumen	5.20	5.54	5.31	5.36	0.09	0.07
Mid-small intestine	6.30	6.38	6.41	6.32	0.06	0.33
End small intestine ¹	6.99	7.23	7.26	7.10	0.09	0.13
Mid-colon	7.09	7.12	6.96	7.13	0.10	0.55

¹n = 5 per treatment.

Table 8. Mid-small intestinal histomorphology of calves fed with only milk (control, n = 10) and calves fed with milk supplemented with *Ulva* sp. (SW1; n = 10), *Ascophyllum nodosum* (SW2; n = 10), or *Saccharina latissima* (SW3; n = 10)

Variable	Experimental group				SEM	P-value
	Control	SW1	SW2	SW3		
Crypt depth (μm)	431	423	452	441	21.0	0.635
Crypt density (n/mm)	12.7	12.4	12.7	12.2	0.29	0.587
Muscle thickness (μm)	467	473	418	422	28.9	0.407

energy to these calves, and the contribution from the starter intake was negligible regardless of whether the calves ate 87 (*Ulva* sp.) or 47 (control) g of DM/d. Despite several studies showing that supplementation of brown seaweed extracts in piglets improved ADG (Gahan et al., 2009; McDonnell et al., 2010), there is presently no solid literature claiming such effects when intact seaweeds are used. The extracts from brown seaweeds mainly comprised laminarin and fucoidan, which are also considered functional polysaccharides with the potential of improving health.

The limited effects of whole seaweed supplementation on the intestinal microbiome were similar to other available findings. Thus, Michiels et al. (2012) observed neither an increased lactobacilli population nor a decreased *E. coli* population in the small intestinal digesta of weaned piglets due to *A. nodosum* supplementation. The literature claiming improved balance in the gut microbiome is mainly based on dietary seaweed extracts (McDonnell et al., 2010). However, for calves, no studies have reported significant beneficial effects of supplementing intact seaweeds. The improved microbial balance in intestinal digesta by the seaweed extracts is claimed to be due to prebiotic effects exerted by bioactive polysaccharides (McDonnell et al., 2010, 2016). The inability of intact seaweeds to exert similar effects was thought to be an effect of components other than polysaccharides, having counteractive and confounding effects on the beneficial properties of laminarin and fucoidan (Michiels et al., 2012).

If the functional polysaccharides (i.e., laminarin and fucoidan), which were expected to be present in these whole seaweeds (Samarasinghe et al., 2021b), were active, the digesta SCFA profile should have been different among treatment groups. Butyric acid and D-lactate concentrations were expected to increase with potential prebiotic effects, but *Ulva* sp. and *A. nodosum* supplementation in the present study caused increased acetic acid and decreased butyric acid production in mid-colonic digesta. Cows fed a high-fiber diet often produce high amounts of acetic acid and low amounts of propionic acid in the rumen as a result of increased fibrolytic fermentation. Increased acetic acid and de-

creased propionic acid proportions in the mid-colon of the calves supplemented with *A. nodosum* suggest that those fibrolytic fermentation processes might be increased due to *A. nodosum* supplementation. However, it is not predictable from the present results, and similar studies are not available in the literature for pre-weaning calves, regarding which bacterial genera could have been dominating. Nonetheless, unaffected lactate concentrations in mid-colonic digesta indirectly reflect the unaffected lactobacilli population in the seaweed-supplemented groups compared with the control group.

Although a decrease in pH was expected along with seaweed supplementation, digesta pH was not affected in any of the studied intestinal sites. The unaffected lactate concentrations in mid-colonic digesta correspond with the unaffected mid-colonic digesta pH.

According to Tappenden et al. (2003), SCFA production due to bacterial fermentation in the distal small intestine and colon can increase tissue mass and thickness both in the fermentation sites and in other compartments of the gastrointestinal tract. The main SCFA responsible for such effects is reported to be butyric acid (Tappenden et al., 2003). Although the butyric acid proportion in mid-colonic digesta was reduced due to *Ulva* sp. and *A. nodosum* supplementation, proportional weights of the small intestine and other studied organs were not reduced by seaweed supplementation.

The rationale behind investigating the histomorphology of the mid-small intestinal epithelium instead of the distal end of the small intestine, where more bacterial fermentation takes place, was because the majority of digested nutrients are absorbed in the jejunum (Tappenden et al., 2003). Due to technical issues, the villi tips were disintegrated during the tissue preprocessing for histomorphology analysis. Therefore, only crypt depth and density, and not villi height, could be evaluated. The values of crypt depth, density, and muscle thickness in mid-small intestinal epithelium are similar to the values observed by Kosiorowska et al. (2011) in 6-wk-old Holstein bull calves. The crypt depth, density, and muscle thickness in the mid-small intestinal epithelium were not affected by seaweed supplementation, which is in accordance with the lack of effects on animal

growth performance, gut bacteria, and SCFA profile. Similar to the present study, Michiels et al. (2012) did not observe any effect on gut histomorphological variables of piglets fed with intact *A. nodosum*.

The lack of improvements or effects observed in the present study could be due to several reasons. These could include the presence of several bioactive compounds (Samarasinghe et al., 2021b), possible interactions of these compounds in the gut (Michiels et al., 2012), confounding effects by high mineral concentrations (Samarasinghe et al., 2021b), or insufficiency of the supplemented dosage to exert a significant prebiotic effect. Finally, the good health status and high performance of the experimental calves could also be a reason for not seeing significant improvements in ADG and selected gut health parameters of the preweaning calves in the present study. The effect of bulk feeding these intact seaweeds as a feed ingredient to the calves during longer periods is unknown and hence requires further research. In addition, the results presented in this manuscript are based on supplementing whole cow milk with the seaweeds; however, it is noteworthy that the effect of supplementing milk replacer with these intact seaweeds on calves is unknown and will require further research.

CONCLUSIONS

Supplementing milk with either dried intact *Ulva* sp., *A. nodosum*, or *S. latissima* at a dose of 50 g/8 L of milk did not improve the growth performance and selected gut health parameters in preweaning dairy calves. Milk supplemented with either *Ulva* sp. or *A. nodosum* affected the SCFA profile in mid-colonic digesta by increasing acetic acid proportion and decreasing butyric acid proportion. In addition, no effects were observed on selected gut microbial populations (i.e., lactobacilli, *E. coli*, *Enterobacteriaceae*), intestinal histomorphology parameters, intestinal digesta pH, and small intestinal tissue mass due to intact dried seaweed supplementation. Thus, the current study did not document significant beneficial effects of feeding intact dried and ground *Ulva* sp., *A. nodosum*, or *S. latissima* to calves with good health status and high performance.

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