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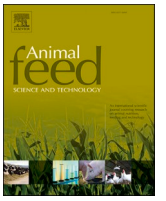

In vitro fermentation of substrates from *Saccharina latissima* by broiler chicken's caecal microbiota

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ABSTRACT

This study investigated seasonal variations in the composition of the kelp species *Saccharina latissima* cultivated on the Swedish west coast and, using an in vitro fermentation system, evaluated the effect of fermented whole *S. latissima* biomass or biomass components on the gut microbiota of broiler chickens. Caecal contents of six Ross 308 broilers fed a standard wheat and soy-based diet were retrieved on two different occasions, with three birds sampled per occasion. Two in vitro fermentation batches (A, B) were established using caecal contents as inoculum, and whole *S. latissima*, ethanol-washed *S. latissima*, laminarin extract or inulin (control) as substrate. Total short-chain fatty acid (SCFA) content, gas production, pH and changes in microbiota composition were studied after 6, 12 and 24 h of fermentation. Analysis of seasonal variations revealed that *S. latissima* harvested in June had the highest laminarin content and lowest ash and crude protein content. Broiler microbiota, SCFA profile, gas production and pH mainly varied depending on fermentation inoculum, but there were also some variations depending on substrate. For instance, uncultured bacterium from *Clostridiales*_vadinBB60_group showed higher relative abundance (RA) in batch A, while *Faecalibacterium* showed higher RA in batch B. Whole and ethanol-washed *S. latissima* resulted in highest RA of unclassified *Ruminococcaceae* and *Tyzzerella* for both batches, while ethanol-washed *S. latissima* and laminarin gave highest RA of *Erysipelatoclostridium*. Inulin resulted in highest RA for the genus *Subdoligranulum*. Acetic, n-butyric and propionic acid were the main fermentation products and total SCFA level was higher in batch B. Within batch B, inulin and laminarin generated higher levels of acetic and butyric acid. When using inulin and laminarin, gas production was lower in batch B compared with batch A. In summary, this study showed that *S. latissima* is a good source of laminarin, especially when harvested in summer. Within the in vitro system, the microbiota was affected by different substrates, but inoculum source was identified as an important contributor to microbial community development during fermentation.

Abbreviations: DM, dry matter; GI, gastrointestinal; M/G ratio, mannuronic acid/guluronic acid ratio; RA, relative abundance; SCFA, short-chain fatty acid.

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1. Introduction

The gut microbiota in broiler chickens is important for host health, as it has a positive effect on gastrointestinal (GI) homeostasis (Brisbin et al., 2008). For instance, the microbiota is involved in reducing or preventing colonisation by enteric pathogens, through the process of competitive exclusion and production of bacteriostatic and bactericidal substances (Clavijo and Flórez, 2018). In addition, the short-chain fatty acids (SCFAs) produced by commensal bacteria in the GI tract, such as acetate, propionate and butyrate, are important for GI homeostasis (De Vadder et al., 2014). For example, it has been shown that SCFAs stimulate gut epithelial cell proliferation and differentiation and increase villus height, thereby increasing the absorptive surface area (De Vadder et al., 2014). SCFAs have also been shown to reduce the amount of undesirable bacterial species in the caecum (Snel et al., 2002). In addition, environmental factors, such as biosecurity level, housing, litter, feed access and climate, affect the chicken gut microbiota (Kers et al., 2018). In particular, feed composition is known to be one of the key factors affecting chicken gut microbiota (Bindari and Gerber, 2022).

Brown macroalgae are an interesting candidate feed additive within the concept of sustainable farming, since they do not compete for terrestrial land or fresh water for growth and since they contain various compounds that can be beneficial in animal production (Coudert et al., 2020; Sørensen et al., 2021). Brown macroalgae of the order Laminariales (kelps) typically contain the polysaccharides alginates, cellulose, fucoidans and laminarin and these bioactive compounds, especially laminarin, may improve intestinal health and immune function (Sweeney and O'Doherty, 2016). Sweeney et al. (2017) found improved growth performance, improved villus architecture and upregulation of some immunological markers when feeding broiler chickens diets supplemented with 250 mg/kg laminarin. Furthermore, Venardou et al. (2021) found improved growth performance and promotion of beneficial bacteria when feeding broiler chicken diets supplemented with 300 mg/kg laminarin. Therefore, supplementation of the diet with laminarin can be a promising strategy to enhance growth performance and promote a beneficial GI microbiota profile in broiler chickens. However, these and other components in kelp species undergo seasonal fluctuations, so harvest time should be adjusted to maximise yield of the target component (Schiener et al., 2015; Vilg et al., 2015; Thomas et al., 2022). In addition, the structure and bioactivity of macroalgal polysaccharides differ between seaweed species as well as extraction method (Garcia-Vaquero et al., 2017), and there is a lack of data on the impact of using whole kelp as feed substrate on the GI microbiota in broiler chickens.

In vitro models allow gut fermentation to be conducted in a highly controlled environment, in order to study non-host-associated factors that shape the gut microbiota. In addition, in vitro modelling reduces the need for animal testing, as it enables quickly testing of a large number of substrates and thereby lowers the ethical burden and enables time- and cost-efficient studies (Lacroix et al., 2015; Li et al., 2019). However, the in vitro models does not study the effect on host functionality and it has been recommended that a combination of in vitro and in vivo models are used to fully reveal the effect of fibre fermentation on host health (Kang et al., 2022).

The aim of this study was to investigate seasonal variations in composition of biomass from the kelp *Saccharina latissima* and explore effects of fermenting different substrates derived from *S. latissima* on the gut microbiota, SCFA, pH and gas production, using an in vitro fermentation system with caecal content from broilers as fermentation inoculum.

2. Material and methods

2.1. Analysis of S. latissima compositions

S. latissima cultivated on longlines in the sea around Koster archipelago outside Tjärnö Marine Laboratory (Strömstad, Sweden), off the Swedish west coast, was harvested in April, May, June and July 2017. The *S. latissima* biomass was freeze-dried and milled (1 mm mesh) and samples were analysed for dry matter and ash (Jennische and Larsson, 1990), crude protein (Kjeldahl method: NMKL, 2003; conversion factor 5,6 as advised by Bogolitsyn et al. (2014), non-starch polysaccharides (Uppsala method: AOAC 994.13), carbohydrate monomers (HPAEC-PAD; Manns et al. (2014), minerals with ICP-SFMS according to (US Environmental Protection Agency, 1994; International Organization for Standardization, 2016) and for glucan content using the yeast and mushroom enzymatic kit and assay procedure (K-YBGL 12/16, Megazyme, Scotland, UK) according to manufactures instructions.

Table 1
Composition of *Saccharina latissima* biomass harvested over the spring-summer season and of ethanol-washed biomass from the June harvest (June_e).

g/ kg of dry matter	April*	May*	June*	July*	June_e*
Ash	362.9	361.4	252.2	284.8	134.5
Crude protein	78.8	69.8	57.7	74.3	73.2
Dietary fibre	229.0	263.8	396.6	363.0	NA[1]
Soluble fibre	38.0	46.8	205.3	176.4	NA
Insoluble fibre	78.1	88.8	70.3	65.0	NA
Laminarin	5.3	6.5	19.7	15.3	25
Glucose	76.6	92.3	232.7	196.5	NA
Fucose	17.1	18.0	14.6	18.3	NA
Mannitol	113.0	95.2	99.2	93.0	66.0
Alginate	430	476.0	340	280	490
M/G ratio[2]	3.5	2.9	3.2	2.0	2.9

[1]Not analysed. [2]Mannuronic/Guluronic acid residues ratio.
* Analyses were performed on one pooled sample from each harvest time.

2.2. Substrates used for fermentation

Three substrates derived from the milled *S. latissima* biomass harvested in June were used for the in vitro fermentation, due to high laminarin content in that month (Table 1). These substrates were: whole algal biomass (Algae_w), algal biomass washed with ethanol (Algae_e) and a laminarin extract (laminarin). Long-chain length inulin (inulin) was included as a control (Raftiline HP, Beneo-Orafti SA, Oreye, France). The ethanol wash was intended to remove easily digestible carbohydrates, as those parts would typically be digested in the small intestine of the chicken. Washing was performed by mixing 4 g of algae biomass with 80% ethanol for 30 min. The samples were centrifuged at 3000 *g* for 10 min, the ethanol was removed and the procedure was repeated twice more. The pellet obtained was dried at 40 °C overnight in a vacuum cabinet.

Laminarin was extracted from 40 g portions of air-dried algal biomass, which were mixed with 800 mL 0.3 M HCl and ultrasonicated for 30 min, with an end temperature of 73 °C, to precipitate alginate. The samples were directly cooled on ice and centrifuged at 4000 rpm for 10 min. The supernatant containing the dissolved laminarin was saved and 99.8% ethanol was added to give a concentration of 70% ethanol. The samples were stored at 4 °C overnight to allow more efficient precipitation of laminarin, and on day two of the procedure all samples were decanted and centrifuged at 4000 rpm for 10 min. The pellet obtained was further washed in 90% ethanol to rinse out salts and stored at 4 °C overnight, and this process was repeated on day three. On day four, the pellets were freeze-dried after centrifugation. The β-glucan content in the extract (K-YBGL 12/16, Megazyme) was used as a proxy for laminarin content, in accordance with Lynch et al. (2010), and was determined to 75%.

2.3. In vitro culture fermentation

On two occasions (each constituting one incubation batch), caecal contents were retrieved from three Ross 308 broilers (5–10 mL/bird) fed a standard wheat and soy-based diet and kept in the same pen throughout the rearing period. The birds were 35 and 38 days old on the respective sampling and the samples (n = 3 per occasion) were pooled to reduce the variation caused by individual birds. To retrieve the caecal contents, the birds were euthanized as approved by the Ethics Board for the Uppsala region (5.8.18–07947/2017). In brief, the birds were injected with an overdose of sodium pentobarbital in the wing vein and the caecum was removed. Within an hour of collection, the caecal contents were diluted 1:25 in a standard bicarbonate phosphate buffer for ruminant in vitro incubation (Lindgren's VOS buffer), as described by Eriksson and Murphy (2004), under a constant flow of CO_2 to provide anaerobic conditions, and filtered through one layer of cheesecloth. The filtrate was used as the culture inoculum, with 60 mL poured into 100 mL Duran bottles pre-weighed with 1 g organic matter of each substrate. There were three replicates for each substrate per fermentation batch, i.e. a total of six replicates per substrate. Blank controls (bottles with filtrate but no substrate) were included to show the system effect. All bottles were connected to an automatic gas volume recorder (Gas Endeavour, Bioprocess Control AB, Lund, Sweden) and placed in a shaking water bath at 39 °C to resemble caecum conditions. Gas volume was measured continuously and stored in a computer program of the Gas Endeavour system (Bioprocess Control). Fermentation was allowed to proceed for 24 h, and the fermentation liquid was sampled at 6, 12 and 24 h, through syringes attached to the bottle lids. On each sampling occasion, 0.5 mL aliquots of liquid were sampled for SCFA and microbiota analysis and stored at − 80 °C until use.

2.4. Short-chain fatty acids and pH

Short-chain fatty acid concentration in 0.5 mL samples of fermentation liquid from the in vitro system was analysed as previously described (Udén and Sjaunja, 2009), using an HPLC system consisting of an Alliance 2795 separation module and 2414 RI Detector (Waters Corp. Milford, MA, USA). Column packet ReproGel H 9 μ 300 * 8 mm was used as the separation column and a ReproGel H, 9 μ 30 * 8 mm (Dr. A. Maisch, Ammerbuch, Germany) was used as pre-column. Sample pH was measured using a glass electrode (Metrohm 654, Herisau, Switzerland).

2.5. DNA extraction, sequencing and bioinformatic analysis

DNA was extracted from 0.5 mL samples of fermentation liquid using the QIAamp Fast DNA Stool Mini Kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's protocol, but with the addition of using bead beating to break down bacterial cell walls. The bead-beating step was carried out by adding 0.3 g sterilised 0.1 mm zirconia/silica beads to each sample and running it in a Precellys24 sample homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France). Sequencing library construction and sequencing were conducted by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). In brief, 16 S rRNA gene V3-V4 regions were amplified using Illumina primer set (341 F: CCTAYGGGRBGCASCAG, 806 R: GGACTACNNGGGTATCTAAT) with a barcode. PCR reactions were performed with Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, MA, USA). Sequencing libraries were generated using NEBNext Ultra DNA Library Prep Kit (New England Biolabs, MA, USA). The raw sequencing data have been deposited in the Sequence Read Archive at the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/sra), under accession number PRJNA896740. The barcode and primer sequence of raw demultiplexed reads were trimmed off. Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) and quality filtering using Quantitative Insights into Microbial Ecology 2 (Core 2020.11) (Bolyen et al., 2019). Vsearch was used to perform dereplication, de novo chimera filtering and clustering of sequencing reads (Rognes et al., 2016). Sequences with ≥ 97% similarity were assigned to the same operational taxonomic unit (OTU). A phylogenetic tree was built using FastTree and MAFFT alignment (Katoh et al., 2002; Price et al., 2010). The SILVA SSU Ref NR 97 132 dataset was first trimmed to the corresponding primer region and trained as classify-sklearn taxonomy

classifier (Pedregosa et al., 2011; Quast et al., 2013; Bokulich et al., 2018). OTUs were then assigned taxonomy using the resulting classifier. The OTU table was rarefied at 11233 reads per sample. A generalised UniFrac distance matrix (alpha = 0.5) was generated using the QIIME2 diversity plugin (Bolyen et al., 2019), and subsequently used for Principal coordinate analysis (Gower, 2015).

2.6. Statistical analysis

All statistical analysis was performed with R (R Core Team, 2021). For SCFA data, one-way ANOVA was used for significance analyses and Tukey honest significant difference (Tukey HSD) was used for multiple pairwise-comparison between the means of groups. For sequencing data, Quasi-Poisson regression was used for significance analyses, and Tukey HSD was used for multiple pairwise-comparisons. The SCFA profile and sequencing results were evaluated statistically only after 24 h of fermentation. The effect of fermentation batch (i.e. the effect of inoculum) and of substrate within each batch were evaluated. A value of $P < 0.05$ was considered statistically significant. Permutational multivariate analysis of variance (PERMANOVA) test of generalized UniFrac distance matrix with Benjamini and Hochberg correction was conducted to evaluate the difference among groups (Anderson, 2001).

3. Results

3.1. Seasonal variation in S. latissima composition

Alginate and ash constituted the largest proportions of dry matter (DM) in *S. latissima* biomass composition (Table 1). There was a seasonal effect in *S. latissima* biomass composition, e.g. on a DM-basis ash and crude protein concentrations were lowest, and laminarin concentration was highest, in June harvest. The content of soluble dietary fibre was also highest in June samples. The insoluble fibre content ranged between 6.5% and 8.6% in the four sampling months, with the highest value observed in May. Glucose and fucose were the two largest sugar components. The mannuronic acid/guluronic acid (M/G) ratio was 2.9–3.5 in *S. latissima* biomass harvested in April, May and June, but dropped to 2by July harvest. Mineral content also showed seasonal variation (Table 2). For instance, potassium, magnesium, sodium, phosphorus, sulphur and chloride levels were lowest in June, while iodine level was highest. The content of calcium increased gradually from April to July.

3.2. Gas production and pH

The inoculum batch and substrate used had an impact on the final gas volume (Fig. 1). For instance, with the inulin and laminarin substrates, batch B showed lower gas yields than batch A, whereas for the Algae_w and Algae_e substrates the two batches provided similar gas yield. Average gas yield for both batches was higher when using Algae_w compared with Algae_e. Although there was a batch effect, the average gas yield was higher for the inulin control than for laminarin. Regarding the time taken for gas production to plateau, Algae_w and Algae_e reached a plateau at 7.75–9.00 h after the start of the experiment. Inulin and laminarin took more time than Algae_w and Algae_e to reach a gas production plateau, and there was an effect of batch. For inulin, batch A and B reached a plateau after 23 and 19.75 h, respectively, while for laminarin the time needed was 24 and 19.75 h, respectively. Both batch and substrate had an impact on pH, with batch B generally having lower pH for all substrates used (Table 3). For Algae_w and Algae_e, the pH drop stopped after 12 h, while for laminarin and inulin the lowest pH value was observed after 24 h, which agreed with the gas production data.

3.3. Microbial community analysis by 16S rRNA gene sequencing

Principal coordinate analysis based on generalised Unifrac distance matrix revealed that incubation batch (i.e. inoculum) was the

Table 2
Concentrations of different minerals in *Saccharina latissima* biomass harvested over the spring-summer season and in ethanol-washed biomass from the June harvest (June_e).

	April*	May*	June*	July*	June_e*
g/kg dry matter					
Ca	8.8	9.7	13.7	20.5	21.6
K	82.1	62.2	26.1	28.2	20.4
Mg	6.9	8.1	5.7	6.5	4.1
Na	38.8	43.8	29.8	39.5	13.4
P	1.6	1.7	1.2	1.7	1.4
S	8.7	9.1	6.9	8.8	10.7
Cl	64.8	58.2	32.7	51.8	3.7
I	3.8	3.6	5.1	4.5	0.6
mg/kg dry matter					
Cu	1.3	1.0	0.8	1.1	1.7
Fe	310.6	80.5	113.0	42.8	147.2
Zn	25.2	20.6	19.5	13.5	21.8

* Analyses were performed on one pooled sample from each harvest time.

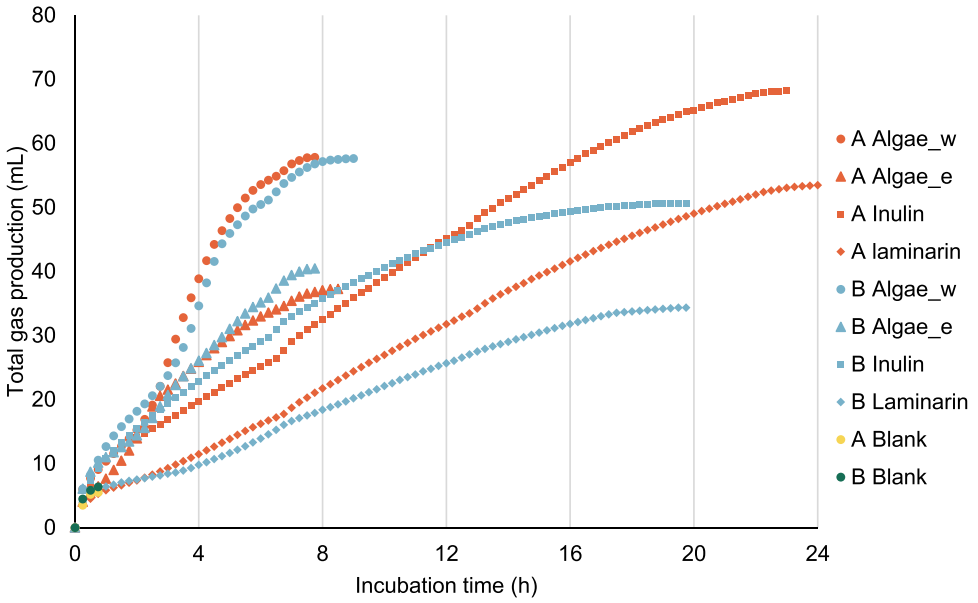

Fig. 1. Gas profile of the fermented substrates: whole *S. latissima* (Algae_w), ethanol-washed *S. latissima* (Algae_e), laminarin, and inulin. Substrate fermentation samples from batch A is orange colored whereas those from batch B is colored blue.

Table 3
Measured pH in batch A and batch B at different sampling points during in vitro fermentation of different substrates and a blank control without substrate.

		Batch A		Batch B	
		Mean	SD[a]	Mean	SD
Inoculum	0 h	6.86	NA[2]	6.83	NA
Blank	6 h	7.12	NA	7.15	0.00
	12 h	7.24	NA	7.21	0.00
	24 h	7.25	NA	7.27	0.01
Algae_w	6 h	6.52	0.04	6.57	0.01
	12 h	6.41	0.00	6.38	0.02
	24 h	6.42	0.02	6.40	0.04
Algae_e	6 h	6.71	0.01	6.65	0.00
	12 h	6.57	0.01	6.50	0.01
	24 h	6.56	0.02	6.52	0.02
Laminarin	6 h	7.00	0.04	6.84	0.01
	12 h	6.72	0.05	6.57	0.00
	24 h	6.02	0.01	5.82	0.09
Inulin	6 h	6.84	0.03	6.72	0.01
	12 h	6.52	0.00	6.41	0.02
	24 h	5.80	0.03	5.73	0.02

[a] Standard deviation. [2]Not available.

most influential factor for microbial community composition (Fig. 2a). Within each batch, the most influential factor for microbial community composition was the substrate used for fermentation (Figs. 2b,c). Within each substrate, the microbial community also differed between different sampling points during fermentation.

There were differences in the initial microbial community in the inocula used in batches A and B (Fig. 3). However, because inoculum was sequenced as a single replicate for the pooled sample of each batch, differences in relative abundance (RA) of the main microbial groups were not evaluated statistically. Within the dominant genera, uncultured bacterium from *Clostridiales*_vadinBB60_group, unclassified *Lachnospiraceae*, *Ruminococcaceae*-UCG-014, *Ruminococcus-torques*-group and *Anaeroplasma* showed higher RA in batch A, whereas *Faecalibacterium*, *Escherichia-Shigella*, *Subdoligranulum* and *Tyzzerella* showed higher RA in batch B. Compared with the initial inoculum, by the end of the fermentation some dominant genera showed a similar trend of change in RA for all substrates in both batches, while others showed opposing changes in RA in the different batches. For instance, the genera *Faecalibacterium*, *Clostridiales*_vadinBB60_group, unclassified *Lachnospiraceae*, *Ruminococcaceae*-UCG-014 and *Eubacterium-coprostanoligenes*-group in general showed deceased RA, and the genera *Ruminococcus-torques*-group, *Anaeroplasma*, *Erysipelatoclostridium* and *Tyzzerella* in general showed increased RA, for all substrates in both batches.

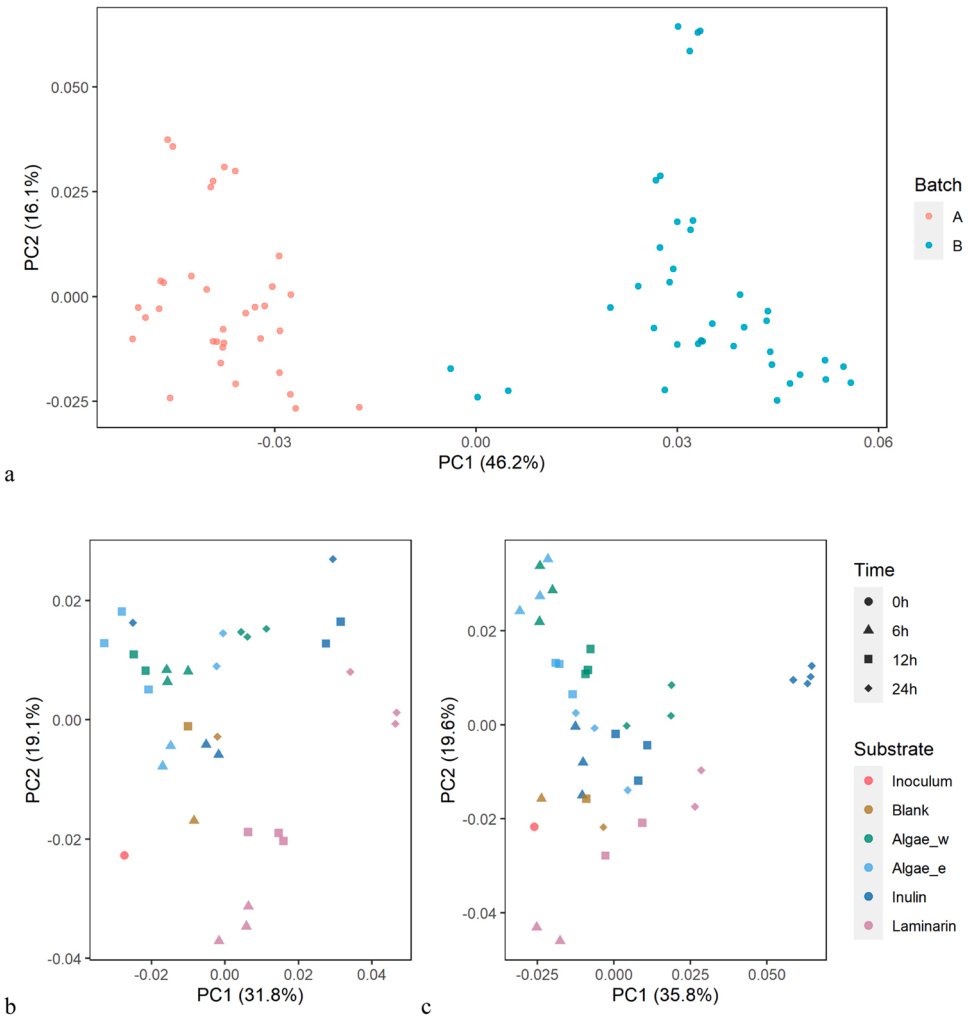

Fig. 2. Principal coordinate plots by generalised Unifrac distance of the bacterial community in inoculum and in vitro fermentation liquid of (a) batch A and B, (b) within batch A and (c) within batch B. For each batch, (b, c), sampling time is represented by different symbols and substrate including whole *S. latissima* (Algae_w), ethanol-washed *S. latissima* (Algae_e), laminarin, and inulin is represented by different colours, see key in diagrams.

The dynamics of microbial community composition during fermentation also differed (Fig. 3). For instance, *Faecalibacterium* was present in the highest RA after 6 h, but its RA decreased as fermentation continued. Higher RA of unclassified *Lachnospiraceae* was more commonly observed after 12 h of fermentation, with a slight decrease at 24 h. *Anaeroplasma* in general showed a continuous RA increase, especially for batch B.

At the end of fermentation, comparing all four substrates used, certain substrates enriched the same genus regardless of batch (Table 4). For instance, Algae_W and Algae_e had higher RA of unclassified *Ruminococcaceae* and *Tyzzerella* than inulin and laminarin for both batches. Furthermore, Algae_e and laminarin had higher RA of *Erysipelatoclostridium* than Algae_W and inulin. While inulin resulted in the highest RA for the genus *Subdoligranulum*. On the other hand, the same substrate also had a differential impact on a genus among two batches. For example, inulin resulted in the numerically highest RA of *Faecalibacterium* in batch A, but the numerically lowest RA in batch B. In addition, for batch B, inulin resulted in highest RA for *Escherichia-Shigella* and *Anaeroplasma* while no differences was observed in Batch A. Likewise laminarin resulted highest RA of Ruminococcaceae-UCG-014 in batch B, while no differences among substrates was observed in batch A.

3.4. Short-chain fatty acid concentrations

The inoculum used in batch B had higher initial total SCFA content, 6.0 mmol/L compared with 3.4 mmol/L in batch A. The blank control fermentation (without substrate) gave similarly low levels of SCFA over time (Fig. 4). For all substrates, SCFA generally accumulated during the 24 h of fermentation, with acetic, n-butyric, and propionic acid as the main products (Fig. 4). There was a difference in production of SCFA between the two batches. Batch A resulted in higher lactic acid production when using inulin and

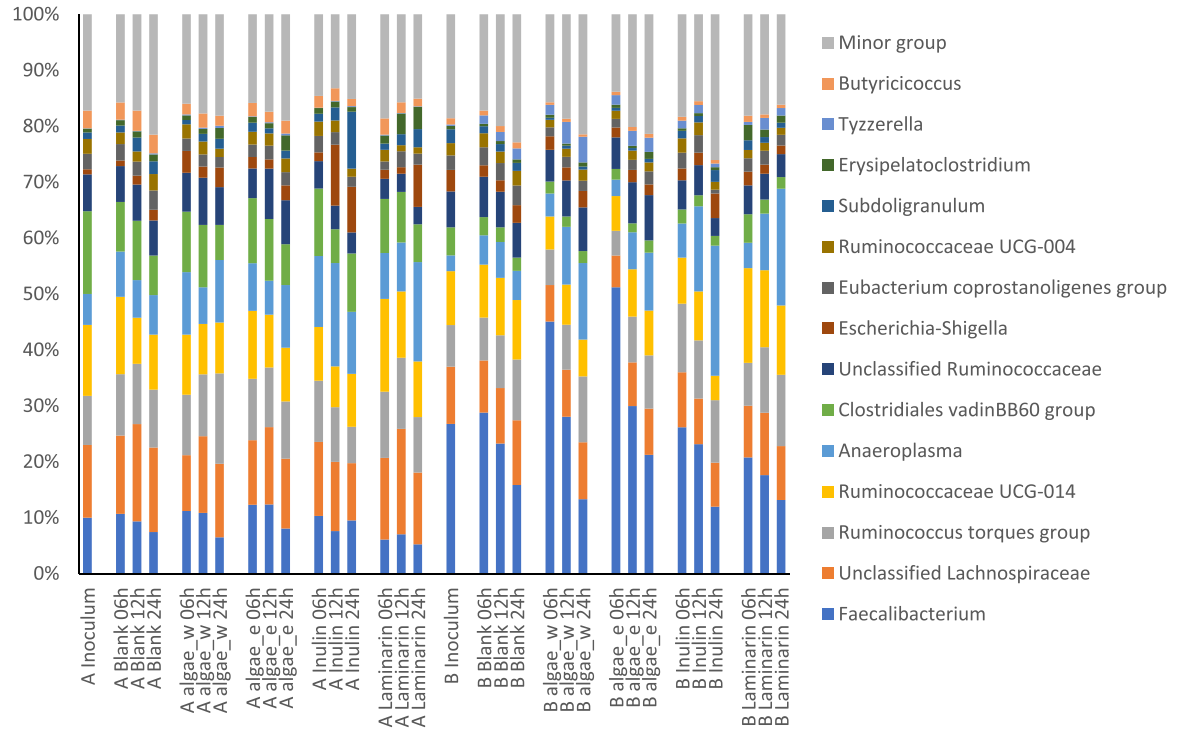

Fig. 3. Bacteria genera with relative abundance higher than 1% in the microbial community in batch A and B fermentations. The bars represents inoculum, blanks and the different substrates used for fermentation: whole *S. latissima* (Algae_w), ethanol-washed *S. latissima* (Algae_e), laminarin and inulin at different sampling points (6, 12 and 24 h of fermentation).

Table 4
The mean and SD of relative abundance (%) of top 12 genera of microbial community in batch A and B fermentations using substrate: whole *S. latissima* (Algae_w), ethanol-washed *S. latissima* (Algae_e), laminarin and inulin at the end of fermentation (24 h).

	Batch A			
Genus	Algae_w	Algae_e	Inulin	Laminarin
Tyzzerella	0.3 ± 0^{ca}	0.4 ± 0^{c}	0.2 ± 0.1^{b}	0.1 ± 0^{a}
Erysipelatoclostridium	1.9 ± 0.1^{b}	2.6 ± 0.5^{c}	0.8 ± 0^{a}	3.9 ± 0.4^{d}
Subdoligranulum	1.7 ± 0.2^{a}	1.5 ± 0.2^{a}	10.1 ± 5.3^{b}	3.1 ± 0.1^{a}
Eubacterium coprostanoligenes group	1.9 ± 0.3^{a}	2.4 ± 0.2^{b}	1.7 ± 0.2^{a}	1.8 ± 0.1^{a}
Escherichia-Shigella	3.8 ± 0.5	2.7 ± 0.3	8.3 ± 8.4	7.9 ± 0.9
Anaeroplasma	11 ± 1.7	11.2 ± 0	11.2 ± 9.8	17.7 ± 2
Unclassified Ruminococcaceae	6.9 ± 1.5^{b}	8 ± 0.1^{b}	3.6 ± 0.7^{a}	3 ± 0.6^{a}
Ruminococcus torques group	16.1 ± 0.4^{c}	10.4 ± 0.2^{b}	6.5 ± 0.5^{a}	10 ± 0.2^{b}
Ruminococcaceae UCG-004	8.8 ± 0.5	9.2 ± 0.8	9.6 ± 1	9.8 ± 0.5
Unclassified Lachnospiraceae	13 ± 0.2^{b}	12.5 ± 0.9^{b}	10.3 ± 0.1^{a}	12.8 ± 0.4^{b}
Clostridiales vadinBB60 group	6.2 ± 0.8	7.6 ± 0.3	10.6 ± 5.4	6.9 ± 0.6
Faecalibacterium	6.6 ± 0.3^{ab}	8 ± 0.4^{ab}	9.4 ± 3.3^{b}	5.1 ± 1.3^{a}
	Batch B			
Genus	Algae_w	Algae_e	Inulin	Laminarin
Tyzzerella	4.7 ± 0.6^{d}	2.7 ± 0.3^{c}	0.8 ± 0.1^{a}	1.4 ± 0.1^{b}
Erysipelatoclostridium	0.8 ± 0.2^{a}	1.3 ± 0.1^{b}	0.5 ± 0.2^{a}	1.3 ± 0.3^{b}
Subdoligranulum	0.5 ± 0.1^{a}	0.6 ± 0^{a}	1.9 ± 0.1^{c}	0.9 ± 0.3^{b}
Eubacterium coprostanoligenes group	1.9 ± 0.2^{b}	2.2 ± 0.2^{b}	0.8 ± 0^{a}	2.1 ± 0.3^{b}
Escherichia-Shigella	2.8 ± 0.2^{b}	2 ± 0.4^{a}	4.1 ± 0.4^{c}	1.4 ± 0.4^{a}
Anaeroplasma	13.7 ± 2.1^{a}	10.2 ± 1.8^{a}	23.4 ± 1.2^{b}	20.8 ± 2.5^{b}
Unclassified Ruminococcaceae	8 ± 0.6^{c}	7.9 ± 1^{c}	3 ± 0.3^{a}	4 ± 0.1^{b}
Ruminococcus torques group	11.5 ± 0.9^{b}	9.3 ± 0.8^{a}	11.8 ± 0.7^{b}	13.3 ± 0.1^{b}
Ruminococcaceae UCG-004	6.8 ± 0.2^{b}	8.4 ± 1.2^{c}	4.2 ± 0.2^{a}	12.2 ± 0.3^{d}
Unclassified Lachnospiraceae	9.9 ± 0.6^{c}	8.5 ± 0.6^{ab}	8.1 ± 0.5^{a}	9.8 ± 0.3^{bc}
Clostridiales vadinBB60 group	2.1 ± 0.1^{ab}	2.3 ± 0.2^{b}	1.6 ± 0.4^{a}	2.1 ± 0.1^{ab}
Faecalibacterium	13.2 ± 2^{ab}	21 ± 6.3^{b}	11.7 ± 0.9^{a}	13.1 ± 1^{a}

[a] Vales within rows with different superscripts are significantly different ($P < 0.05$).

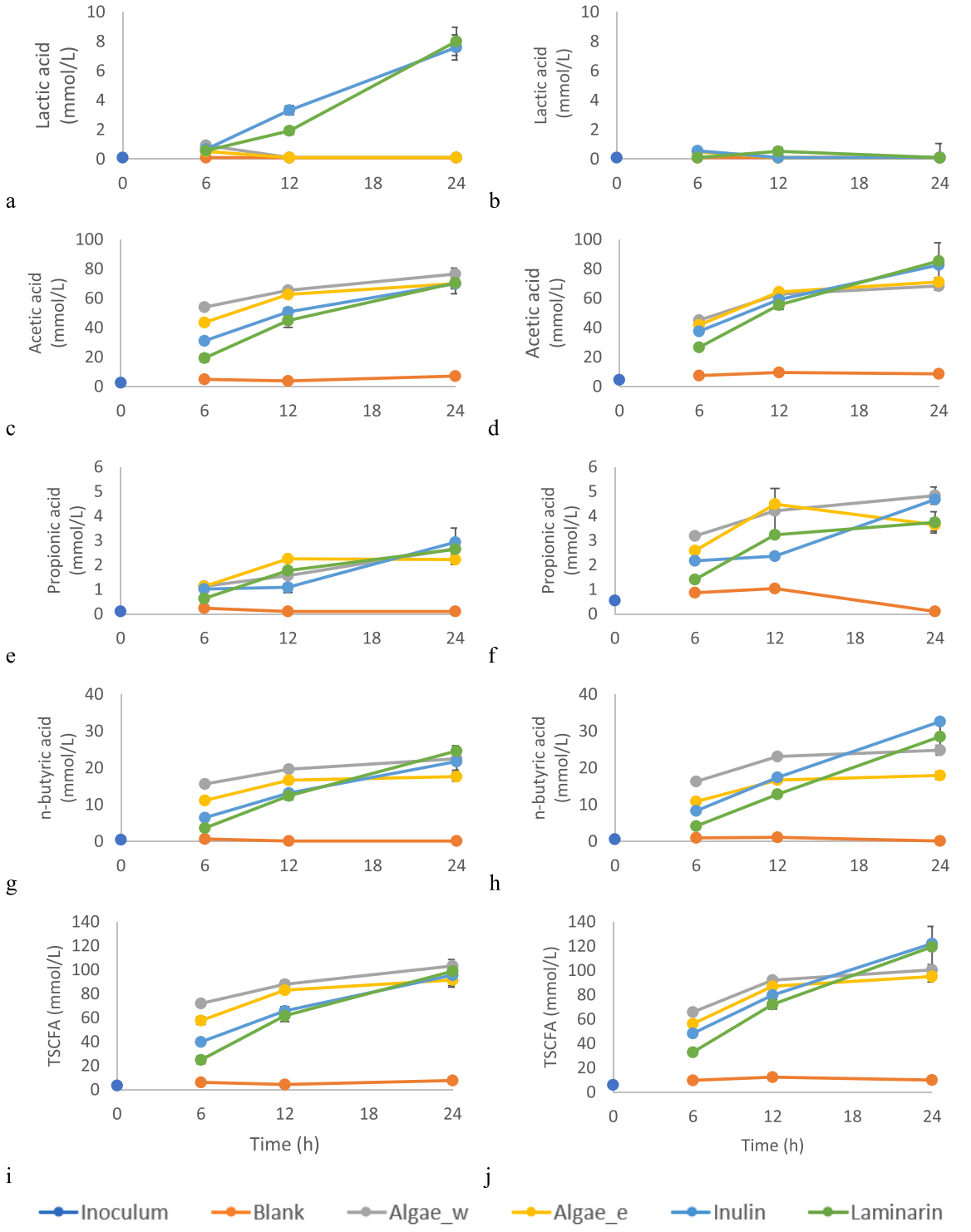

Fig. 4. Concentrations of short-chain fatty acids (SCFAs) generated during fermentation of whole *S. latissima* (Algae_w), ethanol-washed *S. latissima* (Algae_e), laminarin and inulin at different sampling points (0, 6, 12 and 24 h of fermentation). The left panels shows data from batch A whereas the right panels shows data from batch B. Panel a, b) lactic acid, c, d) acetic acid, e, f) propionic acid, g, h) n-butyric acid, i, j) total SCFA). Error bars in panels indicate standard deviations of the mean for in vitro fermentation replicates (n = 3).

laminarin as substrate compared with batch B. Batch A also resulted in higher acetic acid production than in batch B when using Algae_w as substrate. In contrast, batch B resulted in higher acetic, propionic and n-butyric acid production, and higher total SCFA production than batch A, when using inulin as substrate. In addition, batch B resulted in higher propionic acid levels than batch A when using laminarin, Algae_w and Algae_e as substrate.

In addition to the observed differences between the batches, the substrate used induced differences in SCFA levels within each batch. Within batch A, all substrates yielded similar levels of total SCFA, acetic acid and propionic acid after 24 h of fermentation. Laminarin yielded more n-butyric acid than Algae_e, while inulin and laminarin resulted in higher lactic acid production than Algae_w and Algae_e. Within batch B, inulin and laminarin yielded the highest levels of total SCFA and acetic acid. Inulin fermentation resulted in the highest level of n-butyric acid, whereas Algae_w and inulin gave higher levels of propionic acid than Algae_e and laminarin.

4. Discussion

4.1. Composition of S. latissima biomass

Laminarin content was found to be higher, and ash content lower, in *S. latissima* harvested in summer (June and July) compared with spring (April and May). This is in agreement with previous findings of seasonal variations in biomass composition in this species, with the highest levels of ash and metals reported for winter months and early spring and the highest laminarin content in summer months (Adams et al., 2009; Schiener et al., 2015; Vilg et al., 2015; Sharma et al., 2018; Øverland et al., 2019). Laminarin has been found to accumulate in *S. latissima* during summer and autumn (Adams et al., 2011).

4.2. Gas production and pH

Algae_w and Algae_e gave higher gas production rate and needed a shorter time for gas yield to plateau compared with inulin and laminarin. This can likely be explained by utilisation of easily available and degradable biomass components. The ethanol wash treatment of the algal biomass succeeded in removing part of the easily digestible carbohydrates, as indicated by lower gas yield compared with whole algal biomass during the fermentations. However, the fast gas production rate shows that Algae_e had different fermentation characteristics compared to laminarin and inulin and feeding intact algae therefore might result in a different animal response compared to feeding pure polysaccharides. A slow fermentation of laminarin was also reported by Seong et al. (2019) using human in vitro human faecal fermentation. The authors suggested that the slower fermentation would imply fermentation in the distal parts of colon and it can be hypothesised that this also could apply to broiler chickens, however this needs to be further studied in vivo. For inulin and laminarin, there was also a difference in fermentation between the batches, where batch B showed lower gas yield and pH compared with batch A. Batch B inoculum had higher initial levels of SCFA and higher total SCFA content after 24 h of fermentation, which likely explains its lower pH. The low pH in turn resulted in lower gas yield for batch B when using inulin and laminarin, which in agreement with previous findings that gas production decreases with decreasing medium pH in in vitro systems (Amanzougarene and Fondevila, 2017).

4.3. Microbial community

In the in vitro experiment in the present study, the two fermentation batches were intended to act as replicates, i.e. the caecal contents of the three chickens sampled at 35 and 38 days of age, respectively, were pooled to minimise the variation caused by individual birds. It was surprising to find that inoculum had a larger impact than the substrates tested. An impact of age on caecal microbiota has been observed in many studies, but reported differences apply for periods much longer than three days (Oakley et al., 2014; Kers et al., 2018; Ivarsson et al., 2022). Moreover, the sampling was taken at the age from 35 days, at this time point, the gut microbiota was assumed to be more stable than at early age. Therefore, age is not considered as the driving factor for the observed differences in microbial composition between inoculum, instead the individual variation between chickens is likely the cause. It has been reported that bacterial communities in the chicken GI tract differ significantly between gut segments and individuals (Torok et al., 2008; Sekelja et al., 2012; Choi et al., 2014). One possible cause for the observed differences between inoculum could be that the number of donors used for microbiota inoculum was not sufficient to counteract the microbiota variation between individuals. Based on our findings, future in vitro studies that aim to study only the effects of substrates should increase the number of donors to reduce the variation in inoculum.

In both batches, the microbiota present in inoculum was dominated by the phylum Firmicutes, which was in line with recent findings by Ivarsson et al. (2022) for 40-day-old broilers of the Ross 308 hybrid. Previous in vitro fermentations of brown seaweed-based substrate using human gut microbiota have resulted in higher RA of *Faecalibacterium* (Charoensiddhi et al., 2017; Fu et al., 2018). In the present study, RA of *Faecalibacterium* decreased for all substrates by the study endpoint compared with the level in inoculum. *Ruminococcus torques* group, which has been associated with better performance measured as feed efficiency (Torok et al., 2011), had highest RA with Algae_w followed by laminarin,and Algae_e in and inulin having the lowest RA batch A. However, in Batch B the *Ruminococcus torques* group had the lowest RA in Algae_e and did not differ in RA between laminarin, Algae_w and inulin. Differences in alternations of microbiota composition when different inoculums ferment the same substrates was also found by Pirkola et al. (2023) which demonstrated that differences in microbiota composition may partly account for variations between individuals in health outcomes related to diet due to different metabolite production. Furthermore, in poultry production it is well-known that results of using a feed additives such as a prebiotic on animal performance are often contradictory (Bucław, 2016) and differences in initial

microbiota composition is likely a contributing factor. Kim and Lillehoj (2019) stated that a better understanding of the microbiotas role of gut health is needed if we want to use microbes to enhance growth performance in poultry. Interestingly, Venardou et al. (2021) found that diets with supplement of 300 mg/kg laminarin improved growth performance and increased abundance of *Bifidobacterium* in caecum of broiler chickens. An effect of *Bifidobacterium* was not found in the current study while using laminarin as the sole substrate. Several factors apart from inclusion level, such as the use of in vivo versus in vitro system and differences in technical factors might have contributed to differences in between the studies.

Anaeroplasma showed increased RA for all substrate groups in batch B, but inulin and laminarin gave higher RA of this genus compared with Algae_w and Algae_e. *Anaeroplasma* is reported to have some anti-inflammatory properties and has been suggested for use in prevention and treatment of chronic inflammation in humans (Beller et al., 2019). *Tyzzerella piliforme* (formerly *Clostridium piliforme*), a known pathogenic bacteria that causes Tyzzer's disease (Yutin and Galperin, 2013), showed higher RA when Algae_w and Algae_e was used at substrate. The RA of this genus was higher in batch B, although detected at low levels (Fig. 3), its presence requires further study, to confirm pathogenicity.

4.4. Short-chain fatty acids

All substrates were fermentable and resulted in production of SCFA, but the SCFA profile mainly differed between the two batches after 24 h of fermentation. Although some differences in SCFA production were observed for the substrates tested, these changes differed between batches, highlighting the importance of starting inoculum and corresponding effects on metabolic functions.

Since the SCFA level differed between the inoculum used for batch A and that used for batch B, the observed batch differences in SCFA fermentation profile likely originated from initial differences in gut microbiota in the donor chickens. Acetic and n-butyric acid were the most abundant SCFAs. A previous study by Fukuda et al. (2011) found that acetate produced by bifidobacteria can improve intestinal defence mediated by epithelial cells and thereby protect a mouse host against enteropathogens, in batch B both inulin and laminarin stimulated the acetic acid production. Butyrate is better-known for its function on gut health (Onrust et al., 2015; Rubio, 2019), for instance improved GI tract mucosa integrity (Peng et al., 2009) and reduced *Salmonella enteritidis* infection (Fernández-Rubio et al., 2009). Interestingly laminarin supported higher concentration of butyrate than Algae_e in batch A, and although not statistically significant, numerically higher concentrations were also shown in batch B. Propionic acid was generally present in higher levels in batch B fermentation, while within batch B Algae_w and inulin resulted in higher propionic acid levels. Propionate has been found to improve gut barrier function (Tong et al., 2016) and inhibit growth of *Salmonella typhimurium* (Jacobson et al., 2018). Lactic acid production was only observed for inulin and laminarin in batch A and there was no significant difference in RA of lactic acid bacteria that could explain this difference. However, considering the lower level of propionate in batch A, inhibition of the acrylate pathway was a possible reason. Apart from lactic acid production in batch A the inulin and laminarin showed several similarities in fermentation characteristics such as fermentation kinetics, increased RA of *Anaeroplasma* and higher acetic acid concentration in batch B as well as decreased RA of Unclassified Ruminococcaceae in Batch A. The inulin was used as a positive control, as it is known to have prebiotic properties in poultry although results from different studies often are contradictory and depend both on different host and environmental factors (Bucław, 2016). We also saw this with different responses to inulin in batch A and B for both gut microbiota and SCFA. Therefore are further studies needed both in vivo and in vitro to further elucidate those different host and environmental factors and in the long term better predict the response to a certain substrate.

5. Conclusions

S. latissima is good resource for extraction of laminarin, although there are seasonal variations in its laminarin content. In this study, the different *S. latissima* derived substrates all showed potential to modulate broiler gut microbiota, however the fermentation rate was slower in laminarin compared to intact algae. Acetic, n-butyric and propionic acid were the main products formed after fermentation of *S. latissima* derived substrates and inulin. Inoculum source was found to be an important factor affecting fermentation highlighting the importance of microbial species composition for concentration of fermentation end products.

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CRediT authorship contribution statement

Ivarsson Emma: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Writing – review & editing. **Andersson Roger:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Writing – review & editing. **Cervin Gunnar:** Methodology, Resources, Validation, Writing – review & editing. **Pavia Henrik:** Funding acquisition, Resources, Writing – review & editing. **Dicksved Johan:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Sun Li:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Eriksson Torsten:** Investigation, Methodology, Validation, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest for this manuscript.

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