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Quality Changes of Cultivated *Saccharina latissima* as Influenced by Preservation Method During Short Term Storage

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ABSTRACT

Post-harvest changes in the quality of cut and whole *S. latissima* upon 23 days storage with or without acidification at different temperatures were studied by bacterial counts, amino acids composition, and ^{1}H-NMR profiling of extracted metabolites. Lowering the temperature inhibited bacterial growth, and seaweed stored 10 days at 4°C had the same bacterial counts as seaweed stored 2 days at room temperature. Cutting reduced the bacterial degradation of mannitol, free amino acids, and other low molecular components but made proteins and carbohydrates more available for enzymatic hydrolysis. Acidification hindered microbial growth and reduced degradation of amino acids and other components.

KEYWORDS

Proteins; feed; preservation; silage; NMR metabolomics

Introduction

Seaweed is increasingly recognized as a sustainable source of valuable components for food or feed and other applications. Globally, seaweed farming is a large industry, constituting in volume 27% of all marine aquaculture (FAO 2020). Most of the farming takes place in Asia. However, seaweed farming has gained an increasing focus in Europe (Chauton et al. 2021; Hafting et al. 2015). *Saccharina latissima*, or sugar kelp, is a promising seaweed for large-scale cultivation due to its chemical composition and ability to achieve high biomass yields in a short time. *S. latissima* contains carbohydrates (alginate, cellulose, laminaran, fucoidan, and mannitol), proteins, lipids, polyphenols, pigments, vitamins, and minerals (Choudhary et al. 2021; Forbord et al. 2020; Holdt and Kraan 2011; Roleda et al. 2019; Schiener et al. 2015; Stevant et al. 2018) that make it relevant for multiple applications, such as food, bioactive components, feed, fertilizer, or biofuels (Øverland et al. 2019; Sandbakken et al. 2018; Zhang and Thomsen 2021). However, the harvesting season is short, typically 2–3 months, and large volumes are to be harvested in a short period of time. Seaweeds are characterized by a high moisture content and rapid microbial decomposition after harvesting. There is a need to develop handling and effective preservation methods onboard that maintains the quality of such large biomass volumes and ensures stability during storage. Year-round availability of stable and high-quality raw material for further processing is important to facilitate growth in the macroalgae product sector.

The suitable preservation methods to be used on algae-biomass post-harvest varies greatly according to the use of the biomass. Drying (Stevant et al. 2018) and freezing (Albers et al. 2021) are commonly used in Europe today, but they are both expensive due to the high energy demand. Blanching, which is a short holding time in hot water, is used to stop microbial/enzymatic activity and for mineral reduction and is commonly used for algae intended for human consumption (Nielsen et al. 2020). Storage in seawater is also used for short term storage (Stevant et al. 2017) but requires

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large storage volumes. Conservation by lowering pH is a commonly used technology for preservation of food and feed raw materials and allows for preservation of large volumes of biomass in an energy efficient way. The pH is reduced either by adding acid (ensiling) or by microbial acid production (fermentation).

The use of macroalgae as a sustainable feed ingredient for finfish (Wan et al. 2019) and as a source of protein and bioactive compounds source in feed for animals (Øverland et al. 2019) has recently been reviewed. Proteins were in earlier years considered a by-product of polysaccharide extraction, and therefore significantly less attention was addressed to the nutritional value of the seaweed proteins. However, in recent years, the interest in seaweed as a protein source for food or feed has been increasingly recognized (Albers et al. 2021; Bleakley and Hayes 2017; Larsen et al. 2021; Mæhre et al. 2016), and it has been reported that seaweed proteins contain significant amounts of essential amino acids (AA) (Biancarosa et al. 2017; Fleurence 1999; Kazir et al. 2019; Stevant et al. 2018; Wong and Cheung 2000).

There is, however, limited knowledge on the changes in seaweed proteins, both after harvesting and after pre-treatments, such as lowering pH. There are several studies describing ensiling and fermentation of seaweeds for production of biofuels (Herrmann et al. 2015; Sandbakken et al. 2018)), food (Bruhn et al. 2019), or feeds (Cabrita et al. 2017; Campbell et al. 2020; Novoa-Garrido et al. 2020; Yen et al. 2022), but effects on the valuable proteins were not evaluated in these studies. A recent study by Albers et al. (2021) evaluated different preservation methods on *S. latissima*, such as drying, freezing, and silage, and found that ensiling lead to a decrease in the molecular weight of alginate and also that protein solubility was reduced. Larsen et al. (2021) studied ensiling of *S. latissima* both in lab and pilot scale and found that ensiling may be used in a biorefinery concept. However, several chemical changes were observed, such as the reduction of both total amino acids and protein-bound amino acids (Larsen et al. 2021).

Effective preservation should start immediately after harvesting, possibly onboard the harvesting vessels. In this regard, reduction of volume by pressing, cutting, or mincing is highly relevant. Little is known about how such pressing and mincing could influence both quality and preservation. For example, the amount of drip loss may vary between seaweed stored whole and minced. Increased drip loss may be unfavorable due to the loss of soluble nutrients (e.g. amino acids, carbohydrates) into the water phase, but a reduction of water, salt content, and iodine by draining or pressing may also be favorable for the use of *S. latissima* as a food or feed resource (Biancarosa et al. 2017; Gallagher et al. 2021; Nielsen et al. 2020). Moreover, it is a possibility that compounds with antibacterial or antioxidative effects in the seaweed (Balboa et al. 2013; Cox et al. 2010; Gupta et al. 2012; Pérez et al. 2016) are released into the drip loss, leading to better preservation when stored in bulk compared to drained. Studies on ensiling *Palmaria palmata* reported a high volume of silage juice, containing 16–34% of the silage dry matter (Gallagher et al. 2021), but a detailed characterization of this effluent was not done. Leakage/solubilization of sugars could be desirable when seaweeds are to be preserved by bacterial cultures, as they are substrates for fermentation and result in lactic acid production/pH reduction if the conditions are suitable (Davies et al. 1998; Rooke and Hatfield 2003).

The current study investigates the changes in quality from harvest to 23 days of storage of whole and cut *S. latissima* stored at different temperatures (4°C and room temperature) and the effect of acid preservation. The quality was evaluated based on microbiological quality and analysis of total amino acid content and composition. In addition, ^{1}H NMR metabolomics was applied for a non-targeted analysis of changes in low molecular metabolites easily extracted during storage. The data provides knowledge of changes in quality during relevant storage conditions for use of *S. latissima* as an ingredient for feed or food.

Materials and methods

Storage and sampling times

Sugar kelp (*Saccharina latissima)* was harvested from the cultivation site at Frøya, Central-Norway (June 2018) and divided into batches for different treatments. Samples for six different storage conditions were prepared as shown in Table 1: whole and cut seaweed were stored at room temperature (W-RT and C-RT), at 4°C (W-4C and C-4C), and at room temperature after acid addition (W-Acid and C-Acid), respectively. Cutting and acid addition were performed directly after harvest as follows: part of the sugar kelp specimen was cut with scissors to make approx. 1–2 × 1–2 cm pieces, and diluted acetic acid was added (10 ml for 100 g of raw material) to pH < 4.0. The samples were put into boxes (polypropylene boxes with silicone tide lock, 15 cm×15cmx7cm), with three biological replicates for each of the treatment/storage conditions, resulting in 36 boxes in total. The boxes were transported to the laboratory within 3 h after harvesting. Seaweeds stored at room temperature were sampled at day 2, 5, and 8. Seaweeds stored at 4°C were sampled on 2, 5, and 12 days of storage. Seaweeds with acid addition were sampled at day 2, 5, 12, and 23.

Sampling and protocol for water extraction and collection of seaweed residue

Seaweed samples were first submitted to microbial and pH measurements. Due to the limited sample amount, there was no clear drip loss at any of the samplings. However, by submitting the seaweed to rapid water extraction and analyzing the resulting extract, evaluation of changes in metabolites due to solubilization or bacterial- or enzymatic degradation were assessed. The extract was performed on 5 g of seaweed from each box, by adding 10 ml distilled water, shaking for some seconds, and centrifugation for 10 min at 6500 g (refrigerated bench centrifuge SIGMA SK-2, Germany). Liquid part was separated, weighed, and frozen (−80°C) prior to analyses of dry matter, ash, and ^{1}H NMR analysis, while the residual seaweed samples were freeze-dried and analyzed for total amino acids.

pH

The pH was measured in seaweed (after cutting) using a shielded glass electrode (WTW SenTix 41) connected to a portable pH meter (model WTW pH3110, WTW, Weilheim, Germany).

Table 1. Experimental design of the storage trial where the effect of acid addition, cutting, and storage temperature were investigated. At each sampling, microbial quality and pH were measured before extraction was performed to collect water soluble components and remaining seaweed residue for further analysis. RT – room temperature, W – Whole, C – Cut.

	Harvest					
	Natural pH		Natural pH		Acid addition	
Treatment →	Whole	Cut	Whole	Cut	Whole	Cut
Label →	W-4°C	C-4°C	W-RT	C-RT	W-Acid	C-Acid
	4°C storage		RT storage		RT storage	
Days of storage ↓	Sampling days ↓					
2	X	X	X	X	X	X
5	X	X	X	X	X	X
8			X	X		
12	X	X			X	X
23					X	X

Microbiological analysis

Samples (1–5 g) were mixed with 45 mL of buffered peptone water plus (Biorad), and further dilutions were prepared by using 9 ml ready prepared tubes with Tryptone Salt Diluent (Biorad). Dilutions (1:1000, 1:100, 1:10) of the homogenate were prepared in maximum recovery diluent (MRD, Thermo scientific). For total viable aerobic bacterial counts, 1 ml of dilution was spread on the MC-Media Pad R-AC. The MC-Media Pad Rapid Aerobic Count (RAC) is a ready-to-use culture device combining a test pad coated with medium and water absorption polymers that are designed for the rapid quantification of total aerobic bacteria in food products. The plates were incubated at 30 ± 1°C, and colonies were enumerated after 48 h (standard enumeration) of incubation.

Dry matter and ash content

Dry matter was determined in the raw material at day 2, and in the water extract at each sampling point. The dry matter content in the samples was determined gravimetrically after drying at 105°C until constant weight of samples was achieved (typically 24 h). Ash content was analyzed in the raw material at day 2 and in the water extract at selected samplings points (day 5 and day 12) to evaluate to which extent the compounds extracted were salt/minerals or organic matter. Ash content was determined after heating dry samples at 590°C for 12 h.

Amino acid analysis

The amino acid profile in freeze-dried ground seaweed residue was analyzed as described previously (Stevant et al. 2018). Tryptophan decomposes during the preparation step and was not quantified.

Protein efficiency ratio (PER)

Protein efficiency ratio (PER) of proteins in seaweed was calculated by using the following equation (Lee et al. 1978).

$$\text{PER} : 0.08084\left[\sum \text{AA7}\right] - 0.1094, \text{where} \sum \text{AA7} = \text{Thr} + \text{Val} + \text{Met} + \text{Ile} + \text{Leu} + \text{Phe} + \text{Lys}$$

Nuclear Magnetic Resonance (NMR) spectroscopy

Sugar kelp extracts were thawed, and pH of the extractswas measured with an Orion Star A111 pH meter from Thermo Scientific. Samples with pH values higher than 8 were adjusted to pH 7 by use of TCA (7.5%). Initially, 540 µL of supernatant from all storage conditions was transferred to new 1.5 mL Eppendorf tubes, 60 µL of 1 mM TSP in 20 mM Sodium Phosphate buffer pH 7 in D_2O was added, and the mixture was transferred to standard 5 mm-NMR tubes. However, for samples with acid addition (C-Acid and Whole-Acid), it was necessary to dilute the samples more prior to NMR, due to the high concentration of acetic acid. For these samples, 50 µL of extracts was added to 500 µL of PBS buffer prior to NMR analysis, but still the peak from acetic acid was highly dominant in the ^{1}H NMR spectra, leading to low S/N ratio for all peaks except for peaks from mannitol.

Chemicals used were deuterium oxide (D_2O, 99.9%) from Cambridge Isotope Laboratories Inc. (Andover, MA, USA); 3-(Trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP, 98 atom % D) from Armar Chemicals (Dottingen, Switzerland); and trichloroacetic acid (TCA) from Sigma-Aldrich (St. Louis, MO, USA).

1D ^{1}H NMR spectra of algal water extracts were acquired at 300 K with a Bruker Avance 600 MHz spectrometer equipped with 5-mm z-gradient TXI (H/C/N) cryoprobe. The NMR spectra were acquired with the Bruker pulse sequences *noesygppr1d* and the following acquisition parameters: ns

= 48/128 (the latter for samples with acid addition); sw = 20, ppm; aq = 2.6 s, rg = 50.8; o1 = 2820 Hz; d1 = 4 seconds.

NMR processing and quantification

NMR spectra were processed with *TopSpin* 3.6.1 (Bruker, Germany) by using lb = 0.3 and one zero filling. NMR spectra were further processed with the software Chenomx NMR suite 7.0 (Chenomx Inc., Edmonton, Alberta, Canada) (Weljie et al. 2006). Quantification of metabolites from ^{1}H NMR data was only performed for water extracted from seaweed samples stored at natural pH. The ^{1}H NMR spectra of extracts from acid-treated seaweed had low S/N ratio of peaks from other components, except for acetic acid and mannitol, leading to inaccurate quantification of other metabolites.

Statistical analysis

Statistical analysis and data processing were performed using Microsoft Excel 2013 and Minitab v20 (Minitab Inc., PA, USA). Results of the chemical analysis are reported as average values with standard deviation of $n = 3$ biological replicates ($n = 3$ boxes for each condition at each sampling day). Technical replicates were $n = 2$ for most analysis, except for the NMR analysis ($n = 1$). One way analysis of variance (ANOVA) was used to determine significant differences of selected components where the normal distribution and equal variance tests were fulfilled (extracted dry matter, ash content, bacterial counts, free amino acids). Data were tested for normal distribution using the Anderson – Darling test and for equal variance by Levene´s test, prior to one-way ANOVA, using the Tukey method for comparison of means (all by using Minitab v20). The statistical significance level was set to $p < 0.05$. However, due to the great variation among replicate samples, individual values of replicates are given. The unsupervised multivariate principal component analysis (PCA) (Wold et al. 1987) was performed to visualize differences and changes in the profile of low molecular weight metabolites in the water extract based on storage conditions and times. The PCA was performed by the use of Minitab v.20 statistical software and employing the correlation matrix as calculated by the software.

Results and discussion

pH changes

Figure 1 shows pH development in the samples during different storage conditions. The pH was stable during the whole storage period in samples where pH was adjusted initially by adding acid (pH < 4) (W-Acid and C-Acid). For samples without pH adjustment, storage at room temperature led to a clear pH increase in whole seaweed (W-RT), implying spoilage, while the cut seaweed (C-RT) showed a different trend of a slight pH decrease. The cut seaweed stored at 4°C (Cut- 4C) had a similar pH development pattern as the latter: pH gradually decreased with storage time; however, the pH was above pH 5 for all samplings. The pH reduction (from 6.2 to 5.5) was in the same range as observed after 13 days storage of cut *S.latissima* stored in plastic trays at 2.8°C (Wirenfeldt et al. 2022). For whole sugar kelp at 4°C (W-4C), the pH varied between 5.8 and 6.2 during storage.

Microbiological quality

In samples with acid addition, no colony forming units (cfu) were detected for any of the samplings (results not shown, detection limit of < 2.6 log cfu/g). Figure 2 shows the results of the microbiological analysis of seaweed during storage at natural pH.

The bacterial counts for seaweed stored at room temperature (W-RT and C-RT) were relatively high at day 2, with values of ~ 6 log cfu/g; while for samples stored at 4°C (W-4C and C-4C), no cfu were detected (detection limit of < 2 log cfu/g of the lowest dilution).

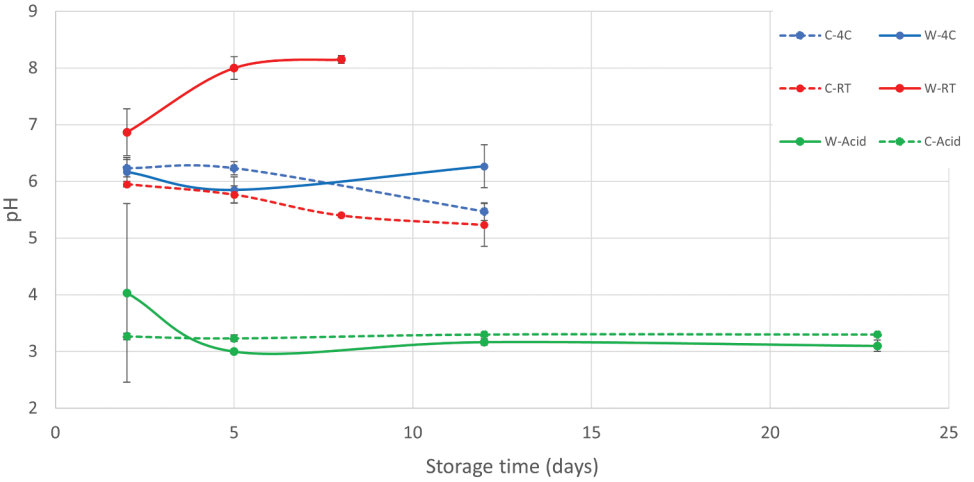

Figure 1. pH development in *S. latissima* during different storage conditions.

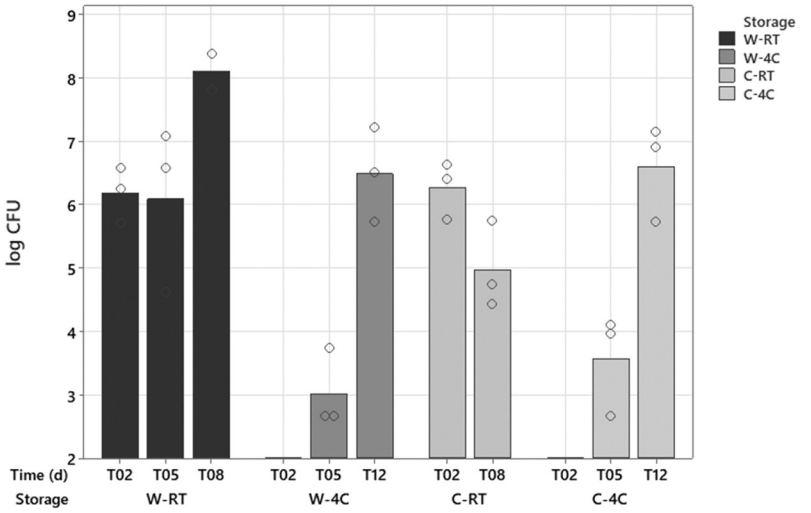

Figure 2. Bacterial counts (log CFU/g) during storage of *S. latissima* at natural pH at different conditions. W-RT: whole in room temperature, C-RT: cut in room temperature, W-4C: whole at 4°C and C-4C: cut at 4°C.

Compared to storage at room temperature, seaweed at 4°C could be stored additional 10 days before reaching similar bacterial counts. This highlights the importance of low storage temperature shortly after harvesting. Values of 1–3 log cfu/g have previously been reported for fresh seaweed (Blikra et al. 2019), while the end of the sensory shelf life was found to correlate with bacterial counts of > 7 log cfu/g in a recent study of *S.latissima storage* (Wirenfeldt et al. 2022).

Interestingly, during storage at room temperature, cut seaweed had lower bacterial counts than whole seaweed at day 8; this is in accordance with the lower pH values for the cut seaweed. While for samples at 4°C, no difference in cfu was seen between the cut and whole seaweed. It is thereby difficult to conclude that the reason for the lower bacterial counts in cut seaweed, compared to whole, when stored at room temperature could be due to the release of antibacterial components such as phenols, pigments, or other constituents of seaweed (Cox et al. 2010; Pérez et al. 2016).

Amount of dry material extracted to the water extract

The results show that a significant part of the dry material was easily extracted by the rapid water extraction (Figure 3). At day 2, the water extract contained 10–25% of the total dry matter that was present in the initial seaweed. A significant increase in extracted dry material was seen from day 2 until day 5 for all samples, except for the cut seaweed stored at 4°C where a slight reduction was seen. A higher amount of dry material was extracted from the cut seaweed compared to whole seaweed for each storage time.

The ash content is a measure of the content of inorganic components, such as salt, and other minerals, such as iodine. The ash content of the water extract was relatively high, ranging from 50% – 78% of the dry weight extracted (Table 2), but these numbers also suggest that some organic components were extracted (50–22% of the extract). The organic components extracted may be in the form of both carbohydrates, amino acids, or other easily soluble components, and the composition of the extract for samples stored at natural pH was described by the use of ^{1}H NMR as discussed later.

However, in addition to possible differences in extractability of organic components between the treatments, the composition of the extract may also be influenced by bacterial activity. The proportion of ash of total dry matter was highest in the water extract of W-RT at day 5, with values of ash content of 77.7 ± 1.5%. These values were significantly higher than the cut samples at 4°C, with the lowest values of ash (average content of 53.3% ash) (as analyzed by ANOVA, $p < 0.05$). A possible explanation

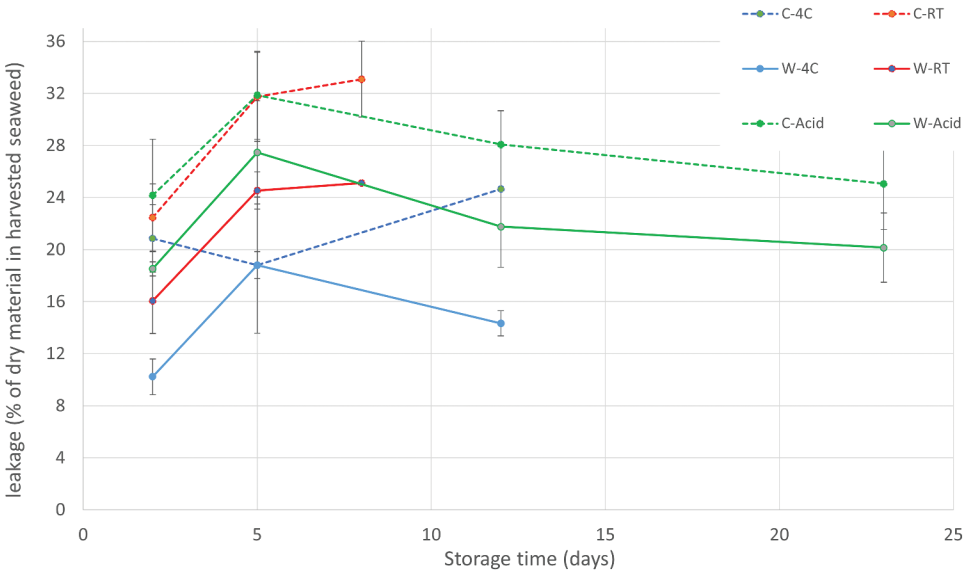

Figure 3. Dry material obtained by the rapid water extraction of *S. latissima* of whole and cut seaweed (as % of dry material in initial seaweed). The extraction was performed to illustrate changes in leakage of components during the storage. W-RT: whole in room temperature, C-RT: cut in room temperature, W-Acid: whole with acid addition, W-4C: whole at 4°C and C-4C: cut at 4°C, C-Acid: cut with acid addition.

Table 2. Ash content (% of dw) of water extracts sampled at day 5 and day 12.

	day 5	day 12
W-RT	78 ± 2	
C-RT	66 ± 7	
W-Acid	61 ± 6	57 ± 7
C-Acid	57 ± 4	56 ± 6
W-4°C	58 ± 17	65 ± 9
C-4°C	53 ± 4	62 ± 3

for the higher ash content in the water extract from the W-RT is that the consumption of soluble sugars by bacteria forming volatile compounds lead to a reduced content of organic constituents in the extract. The results are in accordance with the drastic reduction of mannitol from day 2 to day 5, as shown in the ^{1}H NMR results section. On the other hand, leakage of organic components from cut samples (C- 4°C) could increase the percentage of organic components in the water extract and thereby explain the low ash value of this water extract.

Total amino acids in seaweed residue

Total amino acid content (which includes both free amino acids and protein-bound amino acids) expressed as weight % of dry weight of the seaweed residue ranged from 13.9 to 11.4% for the different treatments on day 2 (Figure 4). For seaweed stored at natural pH, the content of total amino acids increased during storage. In example, for W-RT the total AA content increased from 13.9 ± 1.2% at day 2 to 19.3 ± 0.5% at day 8. This apparent increase in total AA is somewhat unexpected and contrary to a recent study, where it was found that both free and protein-bound amino acids, as a function of crude protein (total nitrogen content x 5), decreased during 4 and 12 weeks of ensiling (Larsen et al. 2021). The findings by Larsen et al. (2021) were explained by either possible acidic degradation of certain amino acids or the insufficient reduction of pH and degradation of proteins and amino acids by proteolytic bacteria. However, in the current study, we analyzed the remaining seaweed after water extraction that was applied to collect easily soluble metabolites that would be lost in drip loss, and the apparent increase in total amino acids may therefore be explained by a relative higher leakage (and/or consumption) of other components and thereby 'up-concentration' of amino acids in the remaining seaweed. In general, whole seaweed had higher levels of total AA compared to cut seaweed for both temperatures at all storage times, and a possible explanation is that the degree of washing out/consumption of other components was smaller for whole seaweed. The composition of water extracts for seaweed without acid addition is discussed in the ^{1}H NMR section.

The amount of total AA in acid preserved seaweed (W-Acid and C-Acid), calculated as percentage of dry weight, did not show the above-mentioned apparent increase but seems to be more stable

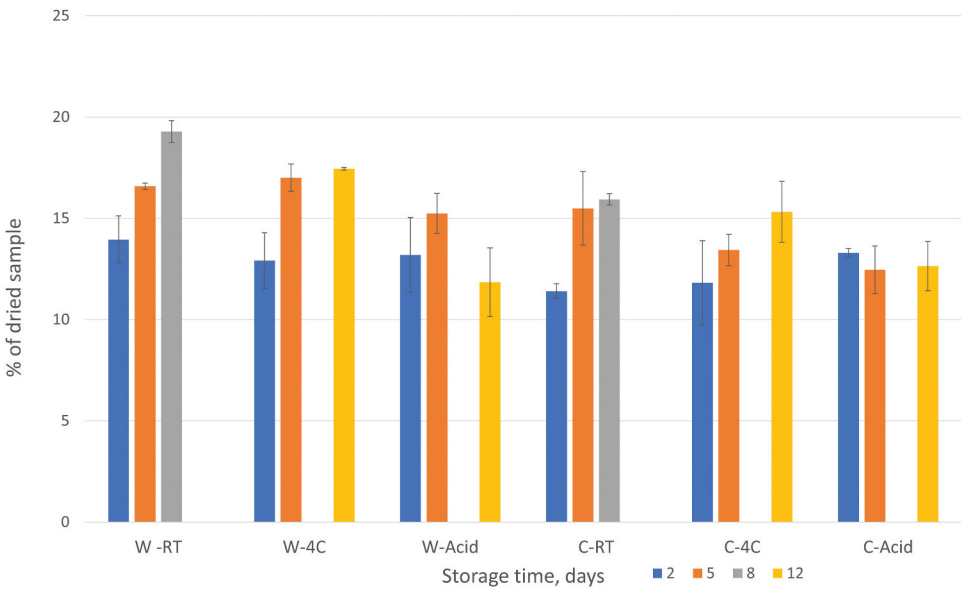

Figure 4. Content of total amino acids in the seaweed residue (after removal of easily soluble components) during storage at different conditions, reported as mean of three replicate samples with standard deviation. W-RT: whole in room temperature, C-RT: cut in room temperature, W-Acid: whole with acid addition, W-4C: whole at 4°C and C-4C: cut at 4°C, C-Acid: cut with acid addition.

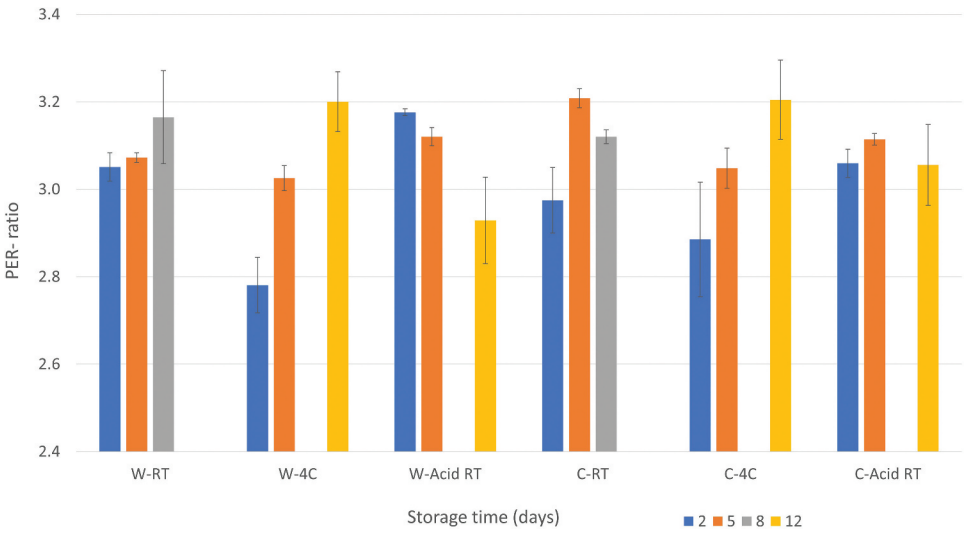

Figure 5. Protein efficiency ratio of the seaweed residue (after removal of easily soluble components) during storage at different conditions. W-RT: whole in room temperature, C-RT: cut in room temperature, W-Acid: whole with acid addition, W-4C: whole at 4°C and C-4C: cut at 4°C, C-Acid: cut with acid addition.

during the whole storage time. This indicates that by treating the seaweed with acid, the profile of components removed by water extraction does not change remarkably during the current storage time, which is also confirmed by ^{1}H NMR data.

PER value of proteins increased for whole and cut algae at natural pH, especially for samples stored at 4°C (i.e. W-4C, C-4C), as shown in Figure 5. As above, a possible explanation is that leakage (or consumption) of non-essential amino acids (NEAA) was higher than that of essential AA (EAA), leading to 'up concentration' of the EAA. On the other hand, PER for *acid preserved* samples shows a different trend, where PER was stable in the cut sugar kelp, while for whole kelp there seems to be a slight decrease in PER ratio during storage (significant decrease from day 2 to day 23 as evaluated by ANOVA). Even though it cannot be excluded that acid addition may have had a slight negative influence on essential amino acids, the results indicate that any influence is small. Overall, the total amino acids seem to be well-preserved during storage at low pH up to 23 days. Some studies of fish silage and hydrolysates have shown a decrease of essential amino acids during storage (van 't Land et al. 2017), as explained by autolysis leading to deamination of amide – N groups in amino acids and production of NH_3 (Sajib et al. 2022; van 't Land et al. 2017). However, the results in literature differ between studies and type of biomass and are not conclusive, as discussed by van 't Land et al. (2017). As discussed in the ^{1}H NMR results section, in the present study, the results showed that free amino acids are degraded by bacteria when stored at natural pH.

Analysis of water extract by ^{1}H NMR metabolomics

^{1}H NMR spectroscopy was used as a non-targeted approach to determine and quantify water soluble metabolites in the seaweed water extract for each sampling. The extraction was performed to give a profile of easily extractable metabolites that could be released to the drip loss during storage. The compounds quantified included soluble carbohydrates, organic acids and alcohols, free amino acids, nucleobases, and other metabolites. During storage, both changes in extractability and bacterial or enzymatic activity may influence the content of compounds present in this water extract.

Peaks from mannitol were the overall dominating peaks in early samplings, as exemplified by a representative ^{1}H NMR spectrum at day 2 (Figure 6), in accordance with previous studies of sugar kelp water extracts (Kirkholt et al. 2019). For samples with acid addition, the mannitol content in the

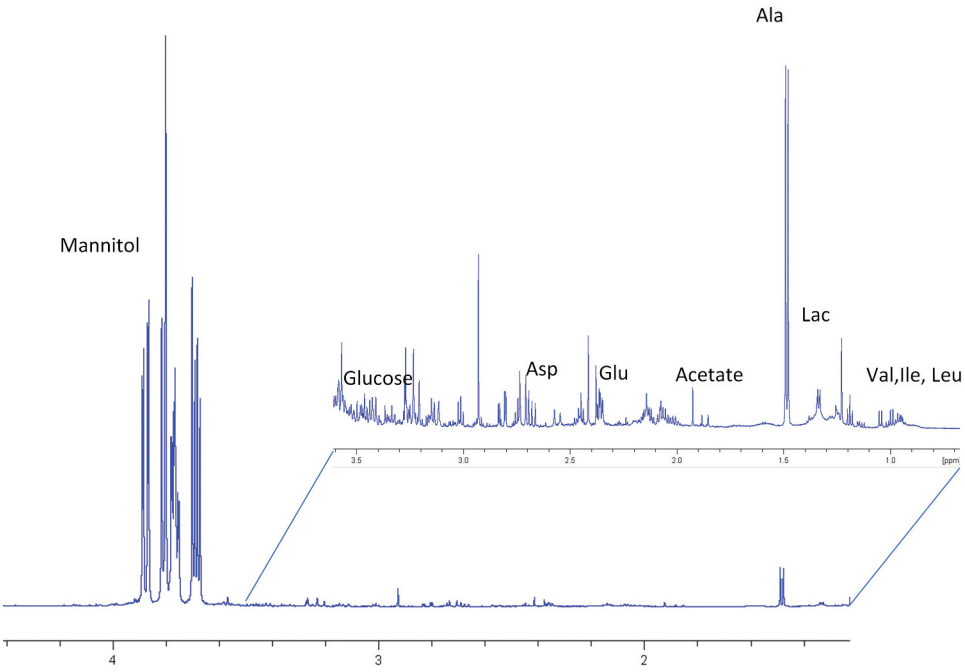

Figure 6. Representative 300 K ^{1}H NMR spectra of seaweed water extracts at day 2. The example is from cut seaweed stored at 4°C (C-4C).

extracts was stable during the storage (592 ± 37 mg/dL for W-Acid and 420 ± 45 mg/dL for C-Acid). Even though there were detectable signals from free amino acids such as Ala, Asp, Glu/Gln, Thr, Val, the intensity of such peaks were low, and no measurable change could be observed during the 23 days storage (results not shown).

However, large changes in the profile of water-soluble components were observed during storage for samples where pH was not adjusted to pH < 4. The results from PCA in Figure 7 illustrate these changes. Mannitol was the main constituent in the early samplings, and samples with high mannitol levels are positioned to the upper/left quadrant (day 2 samples). Lowest values of mannitol were observed for samples stored for the whole several days at room temperature (W-RT, day 8 - positioned in the lower left quadrant). During storage, mannitol levels decreased, and other compounds, such as ethanol, lactate, and other organic acids, and free amino acids increased. Samples of cut seaweed stored at room temperature (C-RT) after 5 and 8 days storage were closely positioned and located far away from the other samples in the PCA score plot, illustrating that they had a different composition of metabolites than the other samples. In Table 3 and the section below, quantitative information on the changes and differences in selected metabolites is provided.

Mannitol is an easily solubilized and fermentable carbohydrate (Sandbakken et al. 2018). Figure 8 shows the mannitol concentration in the seaweed extracts. The levels of mannitol between replicate samples showed large variations, which could be attributed to the limited sample size for the extraction and the variation in composition and bacterial community in different parts of the seaweed specimen. On day 2, extracts from seaweed stored at 4°C had higher mannitol levels than extracts of samples stored at room temperature, which can be explained by reduced bacterial degradation at lowered temperature. A trend with reduction in mannitol levels in the extracts from day 2 to day 5 was observed for all storage conditions at natural pH. The reduction was largest for whole algae stored at room temperature, and almost all mannitol was degraded at day 5. The decrease in mannitol is not reflected in the amounts of secondary metabolites detected. From Figure 9, it is seen that cut samples at room temperatures (C-RT) had the highest sum of acids in the extract and were higher than the whole algae stored at the same temperature.

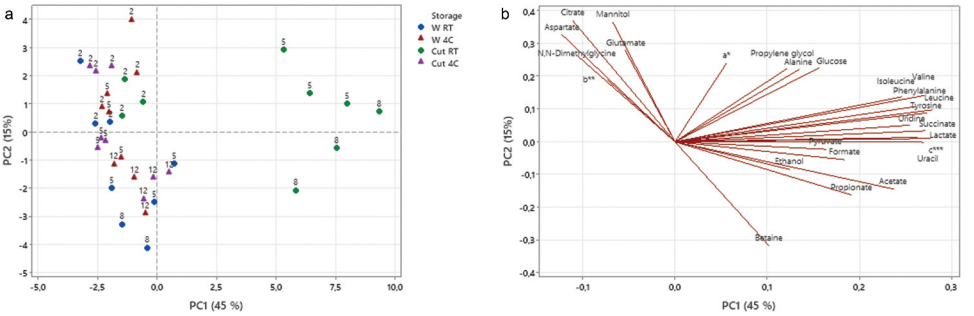

Figure 7. PCA scores (a) and loadings plot (b) of quantified metabolites in water extracts of *S. latissima* samples stored at different conditions and temperature. W-RT : whole in room temperature, W-4C: whole at 4°C, C- RT: cut in room temperatures and C-4C: cut at 4°C. The sample labels indicate sampling day (2, 5, 8 or 12). See footnote in table 3 for more information on tentative assigned peaks (a*, b**, c***).

Table 3. Low molecular weight metabolites quantified (mg/dL) in ^{1}H NMR spectra of water extract of *S. latissima* stored at different conditions. W-RT: whole stored at room temperature, W-4C: whole stored at 4°C, C-RT: cut stored at room temperature, and C-4C: cut stored at 4°C. Results are reported as average values with standard deviation ($n = 3$).

	Time	W- RT	W-4°C	C- RT	C-4°C
Acetate	T02	1.2 ± 0.60	0.5 ± 0.4	3.2 ± 0.8	1.4 ± 1.6
	T05	10 ± 7	2 ± 2	14 ± 3	0.5 ± 0.2
	T08	4.7 ± 0.3	7 ± 5	17 ± 3	13 ± 6
Alanine	T02	6 ± 2	8 ± 5	9 ± 3	6 ± 1
	T05	5 ± 3	10 ± 4	13 ± 2	4.8 ± 0.5
	T08	1 ± 1	5 ± 2	9 ± 4	8 ± 3
a *4.5 ppm (d)	T02	0.1 ± 0.2	10 ± 7	11 ± 6	7 ± 5
	T05	0.2 ± 0.3	2 ± 3	6 ± 4	2.1 ± 0.7
	T08	0.1 ± 0.1	2 ± 2	8 ± 7	3 ± 2
Aspartate	T02	6 ± 1	4 ± 1	3 ± 1	4.2 ± 0.6
	T05	3 ± 1	4 ± 2	1.8 ± 0.2	1.0 ± 0.2
	T08	0.6 ± 0.5	1.3 ± 0.6	1 ± 1	0.23 ± 0.07
Betaine	T02	0.5 ± 0.2	0.6 ± 0.3	0.5 ± 0.1	0.3 ± 0.1
	T05	1.1 ± 0.6	0.23 ± 0.2	0.6 ± 0.1	0.3 ± 0.1
	T08	1.3 ± 0.1	0.9 ± 0.3	1.2 ± 0.7	1.13 ± 0.04
Choline	T02	0.24 ± 0.04	0.18 ± 0.05	0.17 ± 0.04	0.20 ± 0.01
	T05	0.30 ± 0.30	0.10 ± 0.02	0.9 ± 0.1	0.14 ± 0.03
	T08	0.05 ± 0.05	0.07 ± 0.02	0.7 ± 0.3	0.05 ± 0.04
Citrate	T02	0.8 ± 0.5	0.7 ± 0.7	0.9 ± 0.6	1.5 ± 0.1
	T05	0.30 ± 0.0	1.1 ± 0.4	0.8 ± 0.1	1.1 ± 0.1
	T08	0.20 ± 0.2	0.3 ± 0.3	0 ± 0	0.3 ± 0.2
c*** 5.87ppm (d)	T02	0 ± 0	0 ± 0	0.1 ± 0.1	0 ± 0
	T05	0.1 ± 0.2	0 ± 0	6 ± 3	0 ± 0
	T08	0.1 ± 0.1	0.6 ± 0	10 ± 6	2.9 ± 0.7
Ethanol	T02	0.7 ± 0.3	0.7 ± 0.2	0.70 ± 0.1	0.3 ± 0.1
	T05	4 ± 2	4 ± 4	2.6 ± 0.2	1 ± 0.3
	T08	0.34 ± 0.07	0.4 ± 0.2	5 ± 4	1 ± 0.2
Formate	T02	0.09 ± 0.07	0.09 ± 0.04	0.28 ± 0.07	0.11 ± 0.07
	T05	2 ± 1	0.04 ± 0.00	3 ± 1	0.06 ± 0.01
	T08	1.5 ± 0.8	0.6 ± 0.9	1 ± 2	0.08 ± 0.03
Glucose	T02	0.4 ± 0.1	11 ± 13	7 ± 4	4 ± 1
	T05	0.4 ± 0.7	2 ± 2	27 ± 20	2 ± 1
	T08	0 ± 0	2 ± 2	12 ± 9	4 ± 2
Glutamate	T02	6.3 ± 0.6	4 ± 1	4.3 ± 0.8	5 ± 2
	T05	5 ± 2	2.9 ± 0.7	4 ± 1	2.0 ± 0.4
	T08	2.3 ± 0.3	2 ± 1	2 ± 1	1.6 ± 0.2
Isoleucine	T02	0.3 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
	T05	0.3 ± 0.1	0.26 ± 0.05	1.1 ± 0.3	0.18 ± 0.03
	T08	0.2 ± 0.1	0.2 ± 0.1	1.0 ± 0.9	0.21 ± 0.03
Lactate	T02	0.32 ± 0.05	0.6 ± 0.3	1.0 ± 0.5	0.55 ± 0.05

(*Continued*)

Table 3. (Continued).

	Time	W- RT	W-4°C	C- RT	C-4°C
	T05	0.4 ± 0.2	0.26 ± 0.04	17 ± 3	0.3 ± 0.1
	T08	0.2 ± 0.1	1 ± 1	22 ± 1	7.5 ± 0.1
Leucine	T02	0.24 ± 0.04	0.7 ± 0.40	0.4 ± 0.2	0.37 ± 0.08
	T05	0.5 ± 0.4	0.27 ± 0.01	2.3 ± 0.2	0.26 ± 0.09
	T08	0.3 ± 0.1	0.46 ± 0.08	2.3 ± 0.9	0.5 ± 0.2
Maleate	T02	0.11 ± 0.03	0.2 ± 0.2	0.18 ± 0.03	0.3 ± 0.2
	T05	0.06 ± 0.04	0.10 ± 0.04	0.18 ± 0.02	0.31 ± 0.09
	T08	0.01 ± 0.02	0.16 ± 0.06	0.13 ± 0.03	0.22 ± 0.01
Mannitol	T02	198 ± 230	327 ± 164	300 ± 53	485 ± 165
	T05	11 ± 6	219 ± 284	179 ± 89	183 ± 66
	T08	1 ± 2	71 ± 71	135 ± 65	157 ± 57
N.N-Dimethylglycine	T02	0.4 ± 0.3	0.6 ± 0.5	0.14 ± 0.04	0.4 ± 0.4
	T05	0.04 ± 0.01	0.2 ± 0.2	0.04 ± 0.01	0.3 ± 0.2
	T08	0.01 ± 0.00	0.12 ± 0.06	0.06 ± 0.06	0.09 ± 0.07
Phenylalanine	T02	0.5 ± 0.2	0.5 ± 0.3	0.5 ± 0.2	0.35 ± 0.04
	T05	0.8 ± 0.5	0.39 ± 0.00	1.7 ± 0.2	0.25 ± 0.10
	T08	0.2 ± 0.1	0.5 ± 0.2	1.5 ± 0.5	0.25 ± 0.08
Propionate	T02	0.01 ± 0.00	0.02 ± 0.02	0.03 ± 0.05	0 ± 0
	T05	0.04 ± 0.07	0 ± 0	0.24 ± 0.07	0 ± 0
	T08	0.4 ± 0.4	0 ± 0	0.4 ± 0.1	0.08 ± 0.03
Propylene glycol	T02	0.03 ± 0.02	0.5 ± 0.5	0.21 ± 0.03	0.2 ± 0.2
	T05	0.04 ± 0.01	0.1 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
	T08	0.04 ± 0.02	0.10 ± 0.07	0.4 ± 0.2	0.1 ± 0.1
Pyruvate	T02	0.01 ± 0.01	0 ± 0	5 ± 1	0.5 ± 0.1
	T05	0.1 ± 0.10	0 ± 0	1.5 ± 0.7	0.5 ± 0.2
	T08	0.16 ± 0.08	0.4 ± 0.7	8 ± 4	1.5 ± 0.3
Succinate	T02	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.8 ± 0.3
	T05	0.5 ± 0.3	0.4 ± 0.2	3.0 ± 0.7	0.5 ± 0.1
	T08	1.0 ± 0.6	0.2 ± 0.1	3.0 ± 0.4	0.7 ± 0.4
Thymidine	T02	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	T05	0 ± 0	0 ± 0	0.14 ± 0.24	0 ± 0
	T08	0 ± 0	0 ± 0	0.24 ± 0.25	0 ± 0
Tyrosine	T02	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.25 ± 0.06
	T05	0.6 ± 0.6	0.36 ± 0.01	1.5 ± 0.2	0.18 ± 0.09
	T08	0.14 ± 0.04	0.4 ± 0.1	1.4 ± 0.5	0.3 ± 0.1
Uracil	T02	0 ± 0	0 ± 0	0.01 ± 0.02	0 ± 0
	T05	0 ± 0	0 ± 0	0.7 ± 0.3	0 ± 0
	T08	0.04 ± 0.06	0.02 ± 0.03	1.2 ± 0.3	0.16 ± 0.05
Uridine	T02	0 ± 0	0 ± 0	0.06 ± 0.1	0 ± 0
	T05	0.1 ± 0.2	0 ± 0	1.7 ± 0.5	0 ± 0
	T08	0.1 ± 0.1	0.27 ± 0.07	0.9 ± 0.4	0.4 ± 0.2
Valine	T02	0.5 ± 0.2	0.8 ± 0.4	0.7 ± 0.2	0.6 ± 0.2
	T05	0.5 ± 0.2	0.52 ± 0.05	1.9 ± 0.3	0.33 ± 0.09
	T08	0.14 ± 0.04	0.5 ± 0.1	2.3 ± 0.3	0.4 ± 0.2
b** 6.6. ppm (s)	T02	1 ± 1	1.3 ± 0.8	2 ± 1	1.0 ± 0.4
	T05	0.3 ± 0.1	1.1 ± 0.4	0.6 ± 0.4	0.45 ± 0.03
	T08	0 ± 0	0.4 ± 0.3	0.4 ± 0.3	2 ± 1

Some peaks were not unambiguously assigned but to illustrate the changes during storage; quantifications were performed based on tentative assignments. a**unidentified carbohydrate peak with doublet at 4.5 ppm (tentatively identified and quantified as arabinose) c***unidentified compound at 5.87ppm (d) (and 5.75ppm (m)) – tentatively identified as nucleobases or related compounds (quantified as cytidine). b** – singlet at 6.6 ppm – not identified – but tentatively quantified as trans-aconitate.

The results are in accordance with the pH reduction in cut seaweed at room temperature. It is likely that for whole algae stored at room temperature (W-RT), volatile compounds are formed during the spoilage and are thereby not quantified in the extracts. For example, the decrease of acetate from day 5 to day 8 in W-RT samples may be due to its conversion to methane by methanogenic bacteria, as shown in a previous study on aerob degradation of brown algae (Moen et al. 1997). The formation of volatile compounds may also be a possible explanation for the high ash content of the extracts from whole algae stored at room temperature at day 5, compared to the other treatments at this time point (Table 2).

For whole algae stored at room temperature (W-RT), acetate was the main secondary metabolite in the water extract, followed by ethanol and formate. Notably, for cut samples, lactate and succinate were present

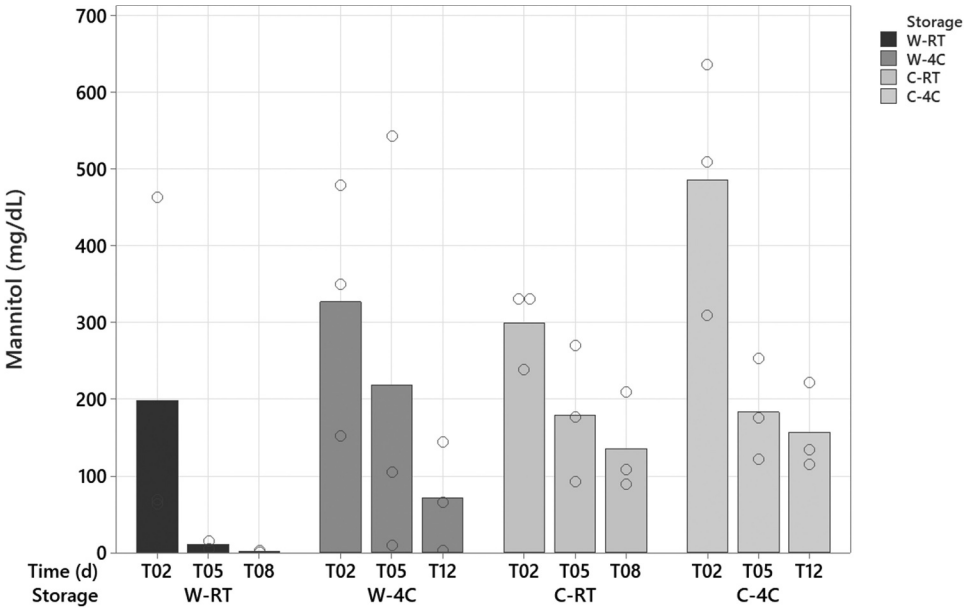

Figure 8. Mannitol levels in water extracts from *S. latissima* stored at different conditions. W-RT: whole in room temperature, W-4C: whole stored at 4°C, C-RT: cut in room temperature and C-4C: cut stored at 4°C.

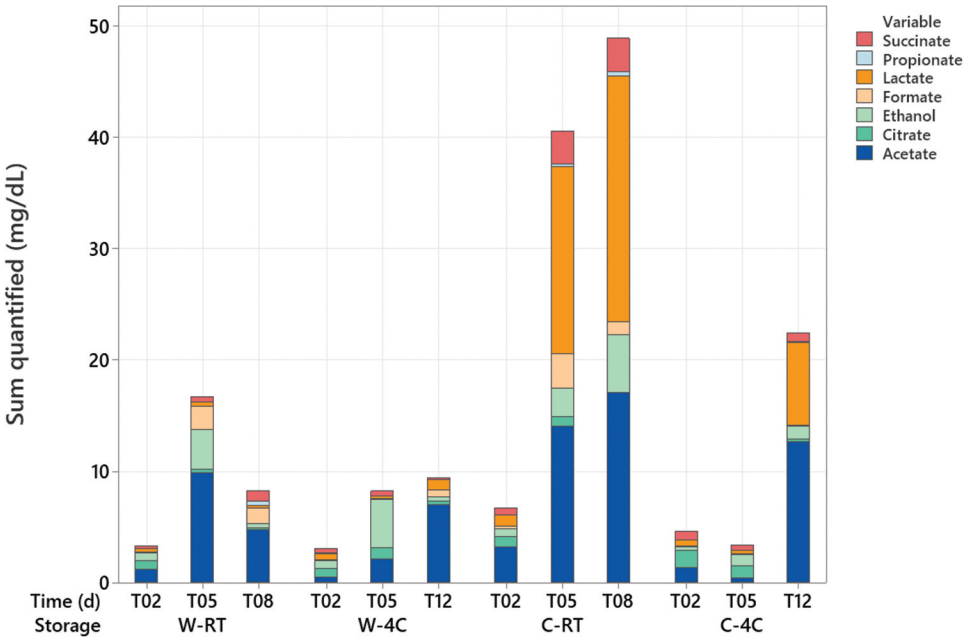

Figure 9. Sum of organic acids and ethanol, quantified in the water extracts of *S. latissima.*

also in the extract, implying that different conditions favor different microbial activity. Lactate and succinate are typical metabolic products from heterofermentative fermentation, and the results indicate that cutting may be favorable for lactic acid fermentation of *S. latissima*. The presence of different bacteria in *S. latissima* and *A. escuelenta* has recently been reported (Blikra et al. 2019). In the current study, the

seaweed was stored in plastic boxes, meaning that oxygen was available for aerobic spoilage bacteria, and the conditions were not optimal for fermentation by lactic acid bacteria.

In previous ensiling studies, different pre-treatments have been applied, such as washing, wilting/drying, heating, or freezing/thawing (Bruhn et al. 2019; Cabrita et al. 2017; Gallagher et al. 2021; Larsen et al. 2021; Yen et al. 2022). Bruhn et al. (2019) used heat-treated algae and added lactic acid bacteria inoculum to suppress the growth of yeasts or unwanted bacteria. Cabrita et al. (2017) used wilted seaweed and found that *S. latissima* promoted a homofermentative fermentation. Recently, Yen et al. (2022) studied the effect of pre-wilting and different fermentation additives during ensiling of seaweed. No fermentation of mannitol was seen during the ensiling of *S. latissima* in the latter study, which is contradictory to other ensiling studies (Bruhn et al. 2019). However, the effect of silage and fermentation characteristics depends on the composition of the biomass and the microorganisms present, which may vary from batch to batch, and are also influenced by pre-treatments such as washing or wilting. For example, soluble carbohydrates and dry matter are important parameters, which are known to vary significantly during the season (Cabrita et al. 2017; Novoa-Garrido et al. 2020) and by washing (Yen et al. 2022), which also reduces the salt content. The current study shows that cutting also impacts the microbial activity.

Results show that initial glucose was degraded at day 2 in samples from whole algae stored at room temperature (W-RT) (Table 3). Glucose levels were higher when stored at 4°C, and this can be explained by a lower bacterial activity at lowered temperature and thereby better conservation of glucose. Interestingly, in cut samples at room temperature (C-RT), an increase in glucose levels was seen after day 2. For cut algae, two opposite processes may influence the levels of glucose in the extracts. Better preservation of initial values by cutting, due to the lower bacterial activity as shown previously, but also increased leakage/extractability of this monosaccharide during storage, due to possible hydrolysis of more complex carbohydrates (e.g. laminarin) as suggested in previous studies (Sandbakken et al. 2018).

Ala, Glu, and Asp were the dominant free amino acids (FAA) in the extracts. For most treatments (except C-RT), the sum of FAA in extracts decreased during storage; a possible explanation is bacterial consumption of FAA (Figure 10). This is in accordance with previous studies that showed a decrease in total amino acid content in seaweed during storage due to bacterial degradation (Larsen et al. 2021; Wirenfeldt et al. 2022). However, the decrease in content of the sum and non-essential FAA (Figure 11a) was not observed for the essential FAA (Figure 11b). Even though a large variation between replicate samples was observed, there was a significant decrease in non-essential FAA from day 2 (19.7 ± 1.4 mg/dL) to day 8 (4.35 ± 2 mg/dL) in W-RT samples, implying bacterial degradation. For essential FAA, however, the content was lower but quite stable in the extracts from most treatments, except for a clear increase from day 2 to day 5 and day 8 for the C-RT treatment. The latter may be explained by a higher extractability of free amino acids in the cut samples at room temperature due to enzymatic protein hydrolysis during the storage. Cutting of the sugar kelp may have promoted autolysis by the release of cellular content and digestive enzymes. This increase was not seen in the cut samples stored at 4°C, and a likely explanation is that proteolytic enzymes exhibit lower enzymatic activity with decreased temperature (Kim et al. 2013). As discussed previously, an apparent increase in total amino acids (on a dry weight basis) was seen in the residual seaweed (after water extraction) during storage of seaweed without pH adjustment. The reason for the total amino acid content not reflecting the consumption of FAA as observed by ^{1}H NMR data is probably that the consumption of other components (such as mannitol) overshadowed these changes, leading to an apparent increase in total amino acids when reporting it as % of dry seaweed.

Several peaks from nucleobases or their derivatives were observed in the later storage times for cut seaweed at both storage temperatures (Figure 12). Previous studies have quantified the content of nucleosides and nucleobases in seaweed (Cao et al. 2014; Yuan et al. 2008), but also nucleotide sugars are reported in seaweed (Goulard et al. 1999). In the present study, uracil and uridine were the dominant nucleoside-related components identified, which is in accordance with a previous study on brown seaweed (Cao et al. 2014). But there were several unidentified peaks in this region; for example, two unidentified peaks at 5.87 ppm and 5.76 ppm and several triplets at 6.32, 6.48, and 6.50 ppm. New but more crowded peaks also arose at 5.2–5.5 ppm during the storage, a region where several carbohydrates may give signals amongst the

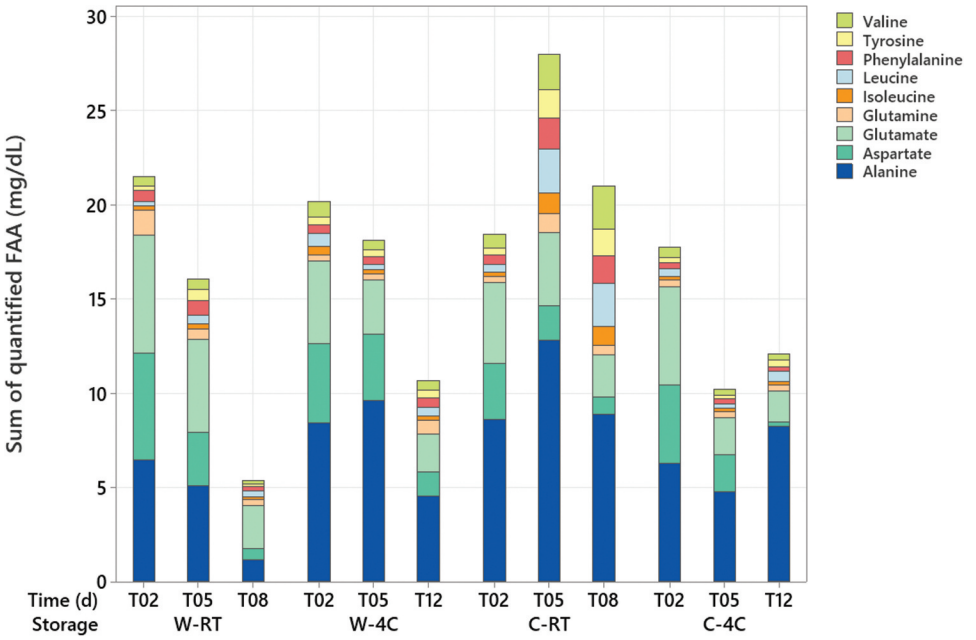

Figure 10. Sum of free amino acids (FAA) quantified in extracts from *S. latissima* stored at different conditions W-RT: whole in room temperature, W-4C: whole stored at 4°C, C-RT: cut stored at room temperature and C- 4C: cut stored at 4°C.

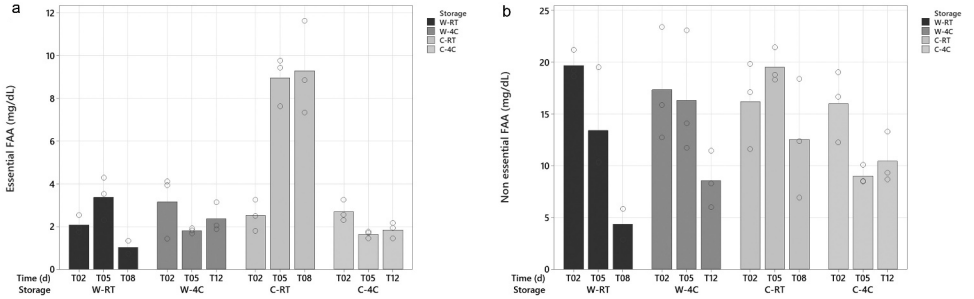

Figure 11. Sum of non-essential (a) and essential (b) FAA quantified in water extracts from *S. latissima* stored at different conditions W-RT: whole in room temperature, W-4C: whole stored at 4°C, C-RT: cut in room temperature. and C-4C: cut at 4°C.

water-soluble polysaccharides fucoidans (Bilan et al. 2010). However, a detailed assignment and characterization of polysaccharides by NMR would require isolation of such compounds.

To sum up, ^{1}H NMR analysis provided valuable information on a wide range of low molecular metabolites in seaweed water extracts. ^{1}H NMR analysis of easily extractable metabolites indicated that FAA were consumed by bacteria in seaweed stored at natural pH. However, the remaining, residual seaweed showed an apparent increase in total AA during the storage (on a dry weight basis). This apparent increase in total AA may be explained by a relative higher leakage/degradation of other components (in example mannitol). The total AA content in acid-treated seaweed did not show the apparent increase as the other treatments, and even though a slight decrease in PER ratio was observed in whole seaweed, this was not seen in the cut seaweed. Studies in the literature on the possible degradation of amino acids by acid addition are not conclusive. Since only minor changes were observed in AA composition in acid-treated seaweed in this study, the results indicate that acid preservation did not have a significant negative effect on amino acids up to 23 days storage. The ^{1}H NMR profile of water extracts from *S. latissima* stored at natural pH showed that mannitol, as the

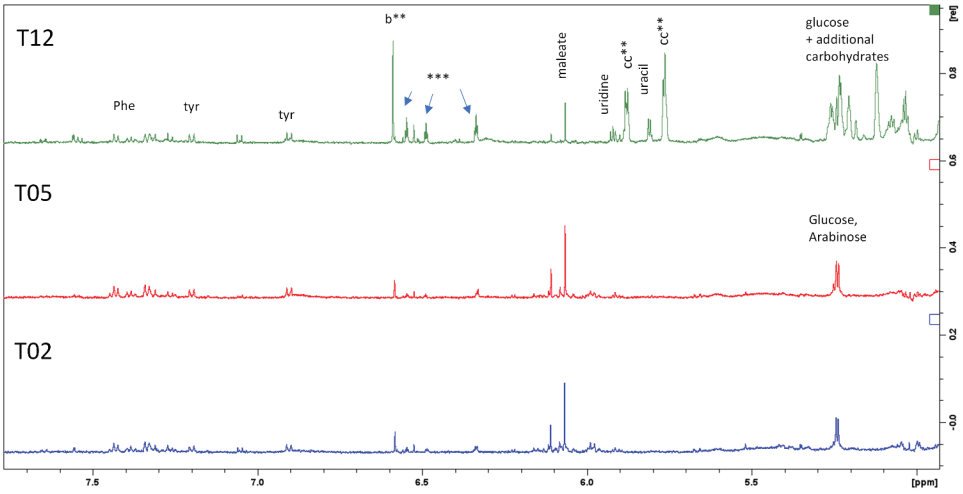

Figure 12. ^{1}H NMR region between 5.5–8 ppm of representative samples of cut seaweed stored in cold room (C-4°C) at day 2, day 5, and day 12 – showing an increase in uracil, uridine, and several unidentified components (b** (quantified as trans-aconitate), c** (quantified as cytidine), *** (not quantified) in addition to carbohydrate peaks.

overall dominant metabolite extracted, was easily available for bacterial consumption in *S. latissima*. In accordance with the lower bacterial counts in cut seaweed at room temperature, the ^{1}H NMR results showed that cutting preserved mannitol better than storage of whole seaweed. However, the results also indicated that cutting makes proteins and complex carbohydrates more susceptible for enzymatic hydrolysis, as increasing levels of glucose and free amino acids were seen in extracts during storage of cut seaweed at room temperature. Also, levels of nucleoside-related compounds, such as uridine and uracil, were higher in extracts of cut seaweed compared to whole seaweed at later storage times. The ^{1}H NMR results showed that other organic acids are formed during the storage of cut seaweed; i.e., the highest lactate value was observed in cut seaweed, indicating that cutting may be favorable when applying microbial fermentation as a preservation method of seaweed.

Conclusion

The quality of seaweed changes markedly during storage due to autolysis and the presence of microorganisms. This study highlights the importance of efficient preservation of seaweed shortly after harvest and provides knowledge on quality changes during relevant storage conditions for use of *S. latissimi* as a feed or food ingredient. Acidification of seaweed is proposed to be a relevant preservation method for wet seaweed, enabling cost and energy efficient preservation. Acid addition hinders bacterial growth, without having significant effects on amino acid composition during short term storage. However, further studies should investigate the effect of longer storage times and, depending on the final use of the seaweed, the effect on other valuable components (such as pigments or phenols) should be evaluated.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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