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Quality and safety aspects in fermentation of winged kelp (*Alaria esculenta*) and sugar kelp (*Saccharina latissima*) by the natural microbiota with or without addition of a *Lactiplantibacillus plantarum* starter culture

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ABSTRACT

Nourishment of the growing human population requires new and alternative food sources, preferable produced without occupying new land areas. Cultivation of seaweed presents an opportunity, however, a major obstacle is sustainable preservation. Fermentation has been used for centuries to preserve vegetables, e.g., to produce kimchi based on cabbage. This study investigated changes in the microbiota, characteristics (pH, organic acids and water soluble carbohydrates) and food safety of raw shredded *Alaria esculenta* and *Saccharina latissima* during fermentation by the natural microbiota with or without addition of a *Lactiplantibacillus plantarum* starter culture. The *Lb. plantarum* fermented products retained a high Shannon diversity index, indicating a partially unsuccessful fermentation. *Lb. plantarum* performed better in *A. esculenta* causing pH to drop to below 4.6, a critical limit for control of growth of *Clostridium botulinum*, within 2 days compared to 7 days for *S. latissima*. Natural fermentation by the endogenous microbiota resulted in unsafe products with high final pH values (4.8–5.2), presence of unwanted organic acids, such as butyric acid, and in the case of *A. esculenta* sustenance of inoculated *Listeria monocytogenes*. Fermentation of *A. esculenta* and *S. latissima* by *Lb. plantarum* is a promising preservation method. However, future work is needed to optimise the process, by investigation of the use of different starter cultures, seaweed pre-treatments (blanching, freezing, etc.) and adjuvants (i.e., addition of sugars, minerals and similar) to promote growth of the starter culture and ensure the fermented products are safe to eat.

1. Introduction

The growing human population constitutes a significant challenge for global food production and security. The arable area available for food production has not increased since 1992 (FAO, 2020). Seaweed cultivation has the benefit of not requiring the use of a terrestrial land area, freshwater, fertilisers and feed. From 2005 to 2015, the annual global seaweed production increased from 13.5 to 30.4 million tons per year, making it one of the fastest-growing food sectors with a projected global seaweed production in 2050 of 500 million tons (FAO, 2018; World Bank Group, 2016). In the Nordic region, Norway has assessed its potential for seaweed cultivation to be between 70 and 140 tons seaweed per hectare of ocean cultivation area, and by 2050 it is expected that in Mid-Norway alone the annual production will reach 20 million tons (Olafsen, Winther, Olsen, & Skjermo, 2012). Today the seaweeds are utilised for human consumption (Mahadevan, 2015), food additives (Bixler & Porse, 2011), animal feed (Rajauria, 2015) and biofuels (Marquez et al., 2015).

In temperate and arctic climates, seaweeds are a highly seasonal crop with an annual high-quality harvest in the late spring or early summer. Since fresh seaweed has a short refrigerated shelf-life of 3–14 days (Liot, Colin, & Mabeau, 1993; Nayyar & Skonberg, 2019), it is necessary to stabilise the seaweed to avoid spoilage and food losses. Currently, the post-harvest treatment consists primarily of drying. However, with the expected significant increase in production, drying may not be the most sustainable method to stabilise the seaweed biomass due to the high-energy consumption and the requirement for specialised drying facilities, which are only used during a short period of the year. Interestingly, fermented seaweed has been an important part of the traditional East Asian food tradition, however, research in the fermentation process did not begin until the 1970 s with the first food related fermentation studies appearing in 1998 (Uchida & Miyoshi, 2013).

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Previous research has reported that fermentation of seaweed with *Lactiplantibacillus plantarum,* a common lactic acid bacteria (LAB), was improved after heating the seaweed for 15 min at 95 °C (Gupta, Abu-Ghannam, & Scannell, 2011). The use of heat treatment to obtain a rapid pH reduction in fermenting sugar kelp was also observed in the study conducted by Bruhn et al. (2019). Recently, lactic acid bacteria fermentation of raw seaweed was shown to be viable in recipes using mixtures of white cabbage to sugar or winged kelp ratios of 1:1, however, higher proportions of seaweed resulted in a less successful fermentation (Skonberg, Fader, Perkins, & Perry, 2021). Fermented seaweed would have prolonged shelf-life, altered the sensory properties to a milder taste and reduced the iodine content (Bruhn et al., 2019). Moreover, fermented seaweed may have other value-added attributes, including being a potential source of non-dairy probiotic food (Gupta & Abu-Ghannam, 2012). However, not much is known about seaweed as a substrate for fermentation and what constitutes suitable starter cultures for cultivated seaweed species aimed for human consumption, currently primarily *Saccharina latissima* and *Alaria esculenta* in the Nordic countries. Nor is it known how starter culture would interact with the raw seaweeds' commensal microbiota, since the composition of the endogenous and commensal microbiota on these brown microalgae species is largely unknown. With a fundamental understanding of the fermentation process, food manufacturers would be able to control the process and ensure a high-quality product with good food safety attributes.

Listeria monocytogenes and *Clostridium botulinum* are pathogenic bacteria found ubiquitously in the environment and associated with illness due to consumption of ready-to-eat or lightly preserved seafood (Huss, 1993). Controlling the pH value during the fermentation can be used to control these (and other) foodborne pathogens. Research has shown that the lower growth boundary in terms of pH is 4.97 for *L. monocytogenes* (Dalgaard & Mejlholm, 2016), 5.14 for non-proteolytic *C. botulinum* (Koukou, Mejlholm, & Dalgaard, 2021) and 4.6 for proteolytic *C. botulinum* (Peck, 2014). The non-proteolytic *C. botulinum* type E is the predominant toxin type found in seafood (Huss, 1993).

The objective of the present study was to determine the fermentability of two brown seaweed species, *S. latissima* and *A. esculenta*, and evaluate the metabolite formation and food safety including the growth inhibition of *L. monocytogenes*. Firstly, chemical and microbial changes were studied during a batch fermentation of raw, shredded *S. latissima* and *A. esculenta* performed by the endogenous microbiota with or without addition of a lactic acid bacteria (*Lactiplantibacillus plantarum*). Secondly, food safety was assessed based on pH reduction and the ability *L. monocytogenes* to grow during fermentation of the seaweed.

2. Materials and methods

2.1. Seaweed and pre-processing

Samples of cultivated brown seaweed of *A. esculenta* and *S. latissima* were collected mid-July in Maniitsoq, Greenland, and shipped to Denmark by air in 80L plastic containers sealed with a lid. Each container held 20 kg of wet seaweed. The transportation took 30 h at ambient temperatures (5–15 °C). After arrival at our laboratory, the seaweed was stored at 2 °C until processing on the same day (approximately 36 h after harvest). In preparation for the fermentation, the seaweed was cut into 2x2 cm pieces with a knife. The endogenous NaCl content in raw *A. esculenta* and *S. latissima* was 1.6 and 1.7%, respectively, as described in the "Chemical changes during fermentation" section. Additional NaCl was mixed with the shredded seaweed, as is customary in kimchi and sauerkraut preparation to reduce propagating of spoilage bacteria and extract fermentable nutrients (Fan & Truelstrup Hansen, 2012), to achieve final concentrations of 2.5 and 2.9% in *A. esculenta* and *S. latissimi*, respectively. The pH level of the raw seaweed were 6.4 ± 0.1 SD and 6.3 ± 0.3 SD (Fig. 1) in *A. esculenta* and *S. latissimi*, respectively. Water-soluble carbohydrate (WCS) and organic acids concentrations are shown in Table 2.

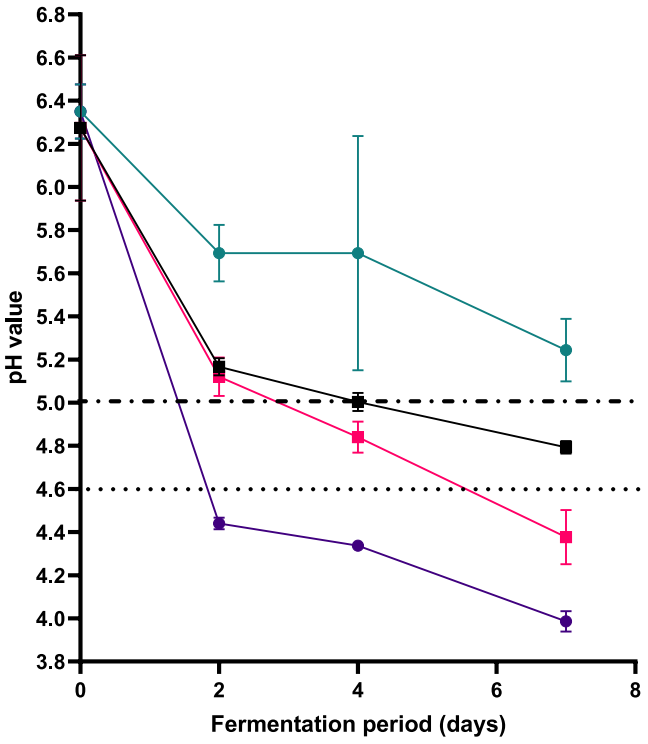

Fig. 1. pH change during the fermentation process of seaweeds. : (■) Naturally fermented *S. latissima,* (■) LAB fermented *S. latissima*, (●) naturally fermented *A. esculenta* and (●) LAB fermented *A. esculenta*. The dotted line (• • • •) represents the growth boundary for proteolytic *C. botulinum* and the mixed line (• — • —) represents a shared boundary for non-proteolytic *C. botulinum* and *L. monocytogens.* Symbols and error bars indicate average values ± SD.

Table 1
Overview of microbial genes of interest related to degradation of carbohydrate found in the water-soluble phase of sugar and winged kelp.

Carbohydrate targeted for degradation	Gene name
Galactose	*galM, gale, galK, galT, galU, cps2D* and *cps4D*
Glucose	*pts11A, pts23A, pts32A, glkA, pgmB, galM, pgi, pgm, scrB* and *gluP*
Mannitol	*pts2CB, ptsH, ptsI, pts G, ptsN, pts2A, mtlR, mtlA, mtlF* and *mtlD.*
Cellubiose	*celA, celB, chbC, chbG, bglA, bglC, bglF, bglG, bglH* and *bglK*
Maltose	*mapA, mapB, map, malG, malH, malK, malL, malP, malX, malY, malZ, ganC, kojP, maa, glvB, glvC* and *mak.*
Fucose	*fcsk, fuk, fuca1, fuca2, fucI, fucP* and *fcl.*
Fucoidan	*Fda1, Fda2m FcnA*, SVI_0379, *FFA2, FFA1, Fdl1A, Fdl1B*, *fct*114 and *fud.*

2.2. Preparation of the inoculum

A novel strain of *Lb. plantarum* was isolated from sour dough and named strain DK22. To prepare the inoculum, DK22 was revived from the freezing tube by streaking out on de Man, Rogosa, Sharpe (MRS) agar (CM0361, Oxoid, Fisher Scientic, Denmark) followed by incubation at 30 °C for 48 h. An overnight activated culture was subsequently prepared by the transfer of one colony into 40 mL MRS broth (Oxoid, Fisher Scientic, Denmark) and incubation at 30 °C for 24 h. The overnight activated culture was distributed into 50 mL tubes and cells were harvested by centrifugation for 5 min at 2100*g* and 20 °C (Sigma 4-16KS, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and removal of the supernatant. The cells in pellet was resuspended in 20 mL sterile 0.9% w/v NaCl water and used as the starter inoculum. To gain information

Table 2
Water-soluble carbohydrates and organic acids profile during the fermentation process of seaweeds.

Product	Water soluble carbohydrates (ppm ± SD)							Alcohols (ppm ± SD)
	Fucoidan	Fucose	Cellobiose	Maltose	Glucose	Galatose	Mannitol	Ethanol
DK22 Starter culture								
API® 50 CHL Medium test[a]	na	–	++	++	++	++	++	
Genes detected[b]	0/10	0/7	8/10	5/19	4/10	3/7	6/10	
S. latissima								
Raw material	32,468 ± 5,331^{A}	17 ± 10^{AB}	215 ± 27^{A}	454 ± 390^{A}	781 ± 585^{A}	263 ± 41^{A}	21,085 ± 9,938^{A}	nd^{A}
Day 2 naturally fermented	54,970 ± 2,271^{B}	29 ± 4^{B}	190 ± 4^{BC}	670 ± 10^{A}	521 ± 35^{A}	242 ± 13^{A}	16,623 ± 799^{A}	244 ± 10^{AB}
Day 4 naturally fermented	48,038 ± 6,942^{B}	12 ± 14^{AB}	153 ± 3^{D}	614 ± 109^{A}	406 ± 62^{A}	208 ± 22^{A}	14,699 ± 3,126^{A}	233 ± 107^{AB}
Day 7 naturally fermented	53,503 ± 2,472^{B}	6 ± 4^{A}	169 ± 4^{BCD}	684 ± 62^{A}	556 ± 124^{A}	245 ± 12^{A}	17,129 ± 1,012^{A}	344 ± 51^{B}
Day 2 LAB fermented	54,067 ± 2,793^{B}	7 ± 0^{A}	198 ± 14^{AB}	682 ± 59^{A}	455 ± 71^{A}	231 ± 3^{A}	14,420 ± 1,052^{A}	76 ± 131^{A}
Day 4 LAB fermented	56,165 ± 1,143^{B}	nd^{A}	190 ± 7^{AC}	626 ± 47^{A}	400 ± 56^{A}	235 ± 2^{A}	14,048 ± 188^{A}	157 ± 155^{AB}
Day 7 LAB fermented	53,609 ± 6,926^{B}	5 ± 4^{A}	162 ± 10^{CD}	532 ± 40^{A}	365 ± 52^{A}	205 ± 43^{A}	13,919 ± 1,645^{A}	346 ± 36^{B}
A. esculenta								
Raw material	30,796 ± 5,799^{A}	7 ± 11^{A}	223 ± 53^{ABC}	430 ± 146^{AB}	1,009 ± 517^{AB}	327 ± 41^{AB}	13,303 ± 4,983^{A}	nd^{A}
Day 2 naturally fermented	49,364 ± 9,317^{BC}	nd^{A}	237 ± 19^{AC}	722 ± 33^{BC}	1,746 ± 330^{B}	298 ± 19^{B}	10,398 ± 3,571^{A}	295 ± 267^{A}
Day 4 naturally fermented	51,532 ± 1,292^{B}	3 ± 5^{A}	282 ± 51^{A}	1,049 ± 264^{C}	3,441 ± 548^{C}	417 ± 58^{C}	13,759 ± 2,049^{A}	473 ± 229^{A}
Day 7 naturally fermented	35,644 ± 4,358^{AC}	0 ± 1^{A}	142 ± 22^{BCD}	226 ± 218^{A}	722 ± 119^{A}	230 ± 5^{BD}	6,989 ± 3,921^{A}	4,191 ± 2,420^{B}
Day 2 LAB fermented	53,712 ± 1,602^{B}	1 ± 2^{A}	212 ± 13^{ACD}	188 ± 56^{A}	528 ± 47^{A}	219 ± 37^{BD}	9,781 ± 1,797^{A}	310 ± 44^{A}
Day 4 LAB fermented	52,655 ± 2,045^{B}	nd^{A}	165 ± 15^{CD}	157 ± 23^{A}	379 ± 50^{A}	223 ± 15^{BD}	7,902 ± 169^{A}	429 ± 59^{A}
Day 7 LAB fermented	42,975 ± 7,404^{ABC}	nd^{A}	129 ± 12^{D}	158 ± 23^{A}	296 ± 38^{A}	199 ± 28^{D}	6,004 ± 629^{A}	423 ± 196^{A}

nd: Not detected.
na: Not analysed.
[a]Test score for the API® 50 CHL Medium test, "–" symbolise no utilisation, "+" weak utilisation and "++" strong utilisation of the carbohydrate.
[b]Number of detected genes that are associated with catabolism of the carbohydrate in the whole genome by Prokka/Number of genes that are associated with catabolism of the carbohydrate as per the NCBI data base.
[A–D]Capital letters denote significant differences ($p < 0.05$) within the column and species of seaweed.

Product	Organic acids (ppm ± SD)						
	Citric acid	Lactic acid	Acetic acid	Butyric acid	Succinic acid	Formic acid	Propionic acid
S. latissima							
Raw material	604 ± 173^{A}	157 ± 84^{A}	141 ± 114^{A}	nd^{A}	nd^{A}	nd^{A}	nd^{A}
Day 2 naturally fermented	515 ± 36^{A}	371 ± 7^{AB}	164 ± 6^{A}	nd^{A}	115 ± 20^{B}	204 ± 15^{B}	nd^{A}
Day 4 naturally fermented	451 ± 76^{A}	548 ± 92^{BC}	180 ± 42^{A}	nd^{A}	100 ± 36^{B}	215 ± 49^{B}	nd^{A}
Day 7 naturally fermented	525 ± 35^{A}	764 ± 60^{C}	185 ± 35^{A}	nd^{A}	103 ± 40^{B}	189 ± 39^{B}	nd^{A}
Day 2 LAB fermented	446 ± 16^{A}	384 ± 27^{AB}	247 ± 83^{A}	nd^{A}	77 ± 6^{B}	104 ± 91^{ABC}	nd^{A}
Day 4 LAB fermented	456 ± 6^{A}	739 ± 43^{C}	182 ± 7^{A}	nd^{A}	70 ± 13^{AB}	244 ± 15^{BC}	nd^{A}
Day 7 LAB fermented	413 ± 62^{A}	1,235 ± 252^{D}	127 ± 20^{A}	nd^{A}	65 ± 32^{AB}	231 ± 58^{B}	nd^{A}
A. esculenta							
Raw material	1,184 ± 210^{AB}	249 ± 76^{A}	508 ± 306^{ABC}	nd^{A}	277 ± 182^{A}	nd^{A}	nd^{A}
Day 2 naturally fermented	1,040 ± 100^{AB}	806 ± 75^{A}	609 ± 145^{ABC}	nd^{A}	354 ± 103^{B}	nd^{A}	nd^{A}
Day 4 naturally fermented	1,262 ± 48^{A}	836 ± 285^{A}	654 ± 250^{BC}	nd^{A}	405 ± 95^{B}	130 ± 226^{A}	nd^{A}
Day 7 naturally fermented	883 ± 165^{BC}	350 ± 382^{A}	916 ± 344^{C}	569 ± 986^{A}	91 ± 158^{A}	408 ± 91^{B}	1,237 ± 818^{B}
Day 2 LAB fermented	713 ± 118^{BC}	4,455 ± 436^{B}	210 ± 84^{AB}	nd^{A}	259 ± 81^{B}	nd^{A}	nd^{A}
Day 4 LAB fermented	695 ± 51^{BC}	5,788 ± 752^{B}	97 ± 29^{AB}	nd^{A}	276 ± 48^{B}	nd^{A}	nd^{A}
Day 7 LAB fermented	607 ± 120^{C}	5,894 ± 1,516^{B}	52 ± 22^{A}	nd^{A}	219 ± 70^{AB}	nd^{A}	150 ± 261^{A}

nd: Not detected.
[A–C]Capital letters denote significant differences ($p < 0.05$) within the column and species of seaweed.

about the initial starting concentration after inoculation, the cell concentration was determined by plating of serial diluted aliquots on MRS agar plates and enumeration after incubation for 3 days at 25 °C.

A cocktail of four *L. monocytogenes* isolates from various marine seafoods (Giménez & Dalgaard, 2004; Jørgensen & Huss, 1998) was used for the challenge tests. The isolates were individually grown in BHI broth for 18 h at 25 °C and diluted in fresh BHI tubes immediately prior to the start of the challenge test. The four isolates were mixed in equal proportions based on $OD_{540\ nm}$ measurements (UV3100PC spectrophotometer, VWR, Radnor, Pennsylvania, USA), and direct microscopy (phase contrast, BX51, Olympus Life Science Solution, Tokyo, Japan), to estimate the cell concentration in the final cocktail. Serial diluted aliquots were also plated on Palcam agar plates (SR0150, Oxoid) and incubated for 2 days at 35 °C for enumeration of CFU/ml of the cocktail.

2.3. Seaweed fermentation

Inspired by traditional vegetable fermentations, the prepared seaweed (Section 2.1) were prepared into two types of fermentation, where (a) relied solely on the endogenous microbiota found on the blades of the seaweed and (b) involved the addition of *Lb. plantarum* (3% w/w to provide an inoculated concentration of 8.4 log(CFU/g)) to the endogenous microbiota for the purpose of increasing lactic formation during the fermentation. For both seaweeds and types of fermentations, the potential for growth of *L. monocytogenes* during the fermentation was tested by spiking one half of the samples with 3.8 log(CFU/g) of the

L. monocytogenes cocktail. The seaweed samples (*A. esculenta* or *S. latissimi*, ±*Lb. plantarum*, ±*L. monocytogenes*) were subsequently distributed into 96 100-mL polypropylene plastic containers (Sarstedt, Nümbrecht, Germany). The containers were completely filled to avoid a large headspace of air and sealed with a lid. The containers were left to ferment at 19.2 °C ± 0.1 SD, and on days 2, 4 and 7, three containers from each of the eight treatments were withdrawn for further analyses.

2.4. Characterisation of the starter culture based on its carbohydrate utilization pattern and whole genome sequence

To assess the DK22 strain's ability to degrade common carbohydrates found in brown seaweed, an API® 50 CHL Medium (BioMérieux, Marcy-l'Étoile, France) test was performed. The strain was grown overnight at 30 °C in sterile MRS media followed by measurement of $OD_{600\ nm}$ (UV3100PC spectrophotometer). The assay was carried out according to instructions from the manufacturer and recording of results after incubation for 48 h at 30 °C. A strong colour change from purple to yellow was noted as "++" (strong use of the carbohydrate), a weak colour change was noted as "+" (weak use), and no colour change was noted as "-" (no use).

Lb. plantarum was identified using MALDI-ToF (Biotyper® sirius System, Bruker, Germany) following the instructions from the manufacturer.

For whole genome sequencing of *Lb. plantarum* DK22, extraction of its DNA was done with the Qiagen 96well HT Kit using the standard protocol with DNeasy kit buffers (Qiagen, Germany). DNA was quantified using the Qubit 3.0 (Invitrogen, Carlsbad, CA, USA) and the Qubit DNA HS assay kit (Invitrogen). The DNA concentration was normalized prior to library generation. Libraries for sequencing was prepared using the Nextera kit (Illumina, San Diego, CA, USA) according to the manufacturer's industructions and sequenced on a Nextseq 500 platform (Illumina). The full genome sequence of DK22 is available on NCBI, PRJNA716165. The DNA sequence had a quality score of 92.39%, and 1,593,550 reads. KmerFinder was used to predict the species from the genomic sequence, which was compared to the MALDI-TOF identification. From the assembly, known sequences of microbial genes (see Table 1) related to the degradation of the common sugars found in brown seaweed were compared against the DK22 genome to find possible matches, using a Prokka (version 1.14.0) command line software in the BioPerl (2019, Bioperl, program).

2.5. Chemical changes during fermentation

The pH of the fermenting seaweed was measured in a mixture of 5 g of seaweed and 20 mL of distilled water. The mixture was stabilised by magnetic stirring for 15 min before measuring the pH with a PHC805 universal electrode (Hach, Brønshøj, Denmark). Measurements were performed in duplicate for each sample container.

Frozen samples of fermented seaweed were thawed overnight at 5 °C and used for determination of water activity (Aqua Lab model CX-2, Decagon devices Inc., Pullman, US) and concentrations of NaCl by automated potentiometric titration of a seaweed-water mix (1:5) (785 DMP Titrino, Metrohm, Hesisau, Switzerland).

For determination of WSC and organic acids, samples were prepared by adding 1 g of wet seaweed sample to 5 mL of 5 mM sulphuric acid. The samples were stored at 4 °C overnight followed by addition of an additional 5 mL of 5 mM sulphuric acid. Samples were inverted 5 times to mix, then centrifuged at 2,795*g* for 8 min, and the supernatant was then filtered through a 0.22 μm syringe filter (Labsolute, Th. Geyer GmbH & Co. KG, Renningen, Germany). The HPLC analysis was performed on an Aminex HPX-87H (Bio-Rad, Hercules, USA) column in an Ultimate HPLC (Dionex, Thermo Fisher Scientific, USA) system equipped with a Shodex RI-101 refractive index detector (Showa Denko K.K., Tokyo, Japan). Sulphuric acid (5 mM) was used as the mobile phase. The column oven temperature was set to 60 °C, and a flow rate of 0.5 mL/min was used for all samples. Standards for each carbohydrate were prepared in concentrations of 50, 100, 500 and 1,000 μg/mL (ppm) in 5 mM sulphuric acid and filtered through a 0.22 μm syringe filter. Results were analysed using the Chromeleon 2.0 software (Thermo Fisher Scientific, USA).

2.6. Microbial changes during fermentation

On each sampling day, the products were tested for pH, culture-dependent microbiology and DNA extracted for culture-independent analysis. The remaining sample materials were stored at −20 °C for later analyses of the content of water-soluble carbohydrates, organic acids, NaCl, dry matter, and water activity.

For microbiological analyses, aliquots of 20 g of seaweed were mixed with 180 g of peptone saline (PS, peptone 1 g/L, NaCl, 9 g/L) in a stomacher bag, followed by stomaching for 1 min and further ten-fold dilutions in PS. The aerobic viable count was determined by spread plating suitable dilutions on a modified Long and Hammer (LH) agar with 1% NaCl (7 d at 25 °C) (NMKL, 2006). Lactic acid bacteria were enumerated by plating on MRS agar (3 d at 25 °C) and *L. monocytogenes* was determined by spread plating onto Palcam agar (2 d at 37 °C) prepared with the Palcam selective supplement (SR0150, Oxoid). To lower the detection limit for *L. monocytogenes* to 1 log(CFU/g) two times 0.5 mL from the stomacher bag were spread plated on two Palcam agar plates (0.5 mL on each plate).

Culture-independent analysis of the microbiota was performed on DNA extracts prepared from the fermenting seaweeds. Briefly on days 2 and 7, microbial cells were harvested by centrifugation (10 min at 2,370*g* at 4 °C) of volumes of 10 mL from the stomacher bags (10^{-1} dilution). DNA was subsequently extracted from the resulting pellet using the DNeasy PowerSoil® Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Total bacterial load from *16S rRNA* gene copy numbers were estimated by quantitative PCR (qPCR) with the specific primers for V8-V9 region of the *16S rRNA* gene (forward: CGGTGAATACGTTCYCGG, reverse: GGWTACCTTGTTACGACTT (Suzuki, Taylor, & DeLong, 2000)). Each reaction was composed of 9.5 μL nuclease free water, 12.5 μL Type-it HRM PCR Master Mix (Mainz, Qiagen, Germany), 1 μL of each primer with a concentration of 10 μM and 1 μL of the sample DNA, to a total reaction volume of 25 μL. The qPCR was performed on a Stratagene Mx3005P qPCR System (Agilent Technologies, City, Country) with four phases; (i) initial denaturation for 5 min at 68 °C, (ii) 40 cycles of 30 s at 95 °C, 30 s at 50 °C and 30 s at 68 °C, (iii) 5 min at 68 °C, (iv) melting curve analysis with 1 cycle of 30 s at 45 °C following by 0.1 °C increments to the final temperature of 95 °C. Gene copies per gram seaweed were calculated based on a standard curve that was constructed using 10-fold dilutions of a plasmid DNA extracted from an *Escherichia coli* DH5α culture containing a positive control plasmid (pCR2.1, TOPO TA PCR 2.1) with the *16S rRNA* fragment (gift from Dr. C. Yost, University of Regina, Canada). The standard curve was made from triplicate measurements of standards containing from 10^0 to 10^9 gene copies/reaction. The qPCR efficiency was calculated as 113%, with R^2 value of 0.993. The limit of quantification and limit of detection (LOD) were 10^4 and 10^3 gene copies/reaction, respectively. In the seaweed samples, this corresponded to a LOD of 10^5 gene copies/g.

The qualitative characterization of the microbiota during the fermentation process was performed after day 2 and 7 from the production day by 16S rRNA amplicon sequencing (Illumina, 2021). DNA amplicon sequences from the V3-V4 region were obtained from triplicate samples from each treatment and fermentation day, together with a negative control (DNA extracted from clean PS) and a positive control consisting of the American Type Culture Collection (ATCC) Even mixed mock sample (ATCC® MSA-1000™, LGC Standards GmbH, Wesel, Germany). Sample libraries were prepared according to the standard 16S Metagenomic Sequencing Library Preparation protocol (Illumina, 2021) and sequenced on an Illumina Miseq. Quantitative Insights Into

Microbial Ecology 2 (QIIME2) (Bolyen et al., 2019) using the DADA2 pipeline (Callahan et al., 2016) and following the standard operating procedure (https://github.com/LangilleLab/microbiome_helper/wiki/Amplicon-SOP-v2-(qiime2-2020.8)) were used to assign Amplicon Sequence Variants (ASV) from reads. To minimize sequencing carry-over contamination between MiSeq runs, ASVs with an abundance of less than 0.1% of the total observations were filtered out, and the sampling depth of the analysis was based on the number of reads in the sample with fewest reads. The SILVA 138.1 SSU Ref NR 99 database (Quast, Pruesse, Yilmaz, Gerken, Schweer, & Yarza, 2013) was used to process, filter and trim reads with amplicon region primers (forward: CCTACGGGNGGCWGCAG, reverse: GACTACHVGGGTATCTAATCC) using RESCRIPt (Robeson II et al., 2020). The taxonomy was assigned to each ASV using a Naïve-Bayers model. Reads were deposited at the NIH NCBI Sequence Read Archive with the accession number PRJNA716165.

The total number of ASVs for each treatment were used to represent the species richness, while the Shannon diversity index (Shannon, 1948) was used as a measure of phylogenetic differences within a treatment. Phylogenetic beta-diversities were calculated using the weighted UniFrac matrix and used for pairwise comparison of microbial communities between treatments with values ranging from 0.0 for complete similarity to 1.0 for complete dissimilarity (Lozupone & Knight, 2005).

2.7. Statistical analysis and graphical software

Graphical representation of pH level and microbial growth and statistical analyses for the differences in pH, WSC, organic acids and ethanol among the fermentation treatments were performed using GraphPad Prism 9.2.0 (GraphPad Software, California, USA). The statistical analyses were using one-way ANOVA followed by a Tukey's multiple comparisons test in GraphPad Prism (9.2.0) to identify significant differences within each treatment (i.e., naturally fermented *S. latissima*, LAB fermented *S. latissima*, natural fermented *A. esculenta* and LAB fermented *A. esculenta*). Analyses related to the amplicon sequencing analysis and visualisation of the taxonomic abundance, were performed with the built-in tools of QIIME2. Graphical representation of the beta-diversity was performed with Excel 365 (Microsoft Corp., Washington, USA). The graphical abstract was made using the Adobe Creative Cloud applications (Adobe, California, USA).

3. Results

3.1. Characterisation of the starter culture

Screening of *Lb. plantarum* strain, DK22, showed that of the carbohydrates found in the two species of seaweed, the bacterium was able to utilise galactose, glucose, mannitol, cellobiose, and maltose (Table 2). In contrast, it could not utilise fucose in neither the L- nor D-fucose structures. Sequencing of the genome resulted in high base quality of 92.39%, 1,593,550 reads, and identification of the bacterium as *Lb. plantarum* using the KmerFinder prediction, which was identical to the MALDI-TOF identification. The Prokka comparison between the genome and known genes from sugar degradation pathways confirmed its ability to use galactose, glucose, mannitol, cellobiose, and maltose. At the same time, no matches were found for genes predicted to be involved in fucoidan and fucose degradation (Table 2).

3.2. Chemical changes during fermentation

After two days of fermentation, a significant ($p < 0.05$) drop in pH was observed for both naturally and LAB fermented samples of seaweed from both species. The addition of starter culture to *A. esculenta* resulted in a significantly ($p < 0.001$) larger and more rapid pH drop compared to *A. esculenta* undergoing natural fermentation. In contrast, there was no significant ($p > 0.05$) difference between naturally and LAB fermented *S. latissima* (Fig. 1).

The available WSC phase were dominated by fucoidan and mannitol for both seaweed species (Table 2). The utilisation of the carbohydrates depended on the species of seaweed and the addition of DK22. The mannitol content was significantly ($p < 0.05$) reduced during fermentation of both seaweed species, and to a higher degree when DK22 starter culture was added (Table 2). The concentration of fucoidan increased in both seaweed species after the initial two days of fermentation. A drop in fucoidan was observed in both *A. esculenta* fermentations after 7 days, while it remain at the same high level in *S. latissima* (Table 2).

The content of lactic acid in *A. esculenta* rose from an initial concentration of 249 ppm to 4,455 ± 436 ppm after two days in *A. esculenta* fermented by DK22 with additional increases to 5,894 ± 1,516 ppm on day seven (Table 2). In contrast, fermentation of the same seaweed by its endogenous microbiota yielded an undesirable acid profile with a low content of lactic acid and appearance of a range of unwanted acids, such as acetic, butyric, formic, and propionic acids as well as considerable amounts of ethanol (Table 2). For *S. latissima,* the DK22 addition did not affect the acid profile, except for the lactic acid concentration which rose from 157 ± 84 to 1,235 ± 252 ppm after seven days (Table 2). *S. latissima* contained regardless of the fermentation type no measurable concentrations of butyric or propionic acid. Moreover, there was no acetic acid formation compared to the raw material (Table 2).

3.3. Quantitative measurement of microorganisms

High numbers of 16S rRNA gene copies (gc)/g of 10^{10}–10^{11} were constantly present in all four products throughout the fermentation (Fig. 2A–D). It should be noted that the qPCR method would also detect algal chloroplasts, however, analysis of 16S rRNA amplicon sequencing results showed that this would account for less than 8% of assembled reads (data not shown). Enumeration of aerobic viable count on LH agar with 1% NaCl detected 7 log(CFU/g) for naturally fermented *A. esculenta* (Fig. 2A) while the corresponding molecular qPCR count amounted to 11.2 log(gc/g). LAB, as determined on MRS agar, occurred in initial levels of 10^4 CFU/g in the naturally fermented *A. esculenta* and reached levels of 6×10^8 CFU/g after seven days of fermentation (Fig. 2A). Inoculation of *A. esculenta* with DK22 resulted in stable LAB levels of >9.0 log(CFU/g) during the whole fermentation process (Fig. 2B). The bacteria detected by qPCR in naturally fermented *S. latissima* did not grow on LH agar plates (below detection limit of 10^6 CFU/g, Fig. 2C) and no LAB were naturally present in *S. latissima*. Inoculation of *S. latissima* with DK22 was less successful as the seaweed after two days contained just 6.5 log(CFU/g) LAB, which then stabilised to levels of 7.5 log(CFU/g) after 4 days (Fig. 2D).

3.4. Food safety aspects

Simulated contamination of *L. monocytogenes* at initial levels of 3.8 log(CFU/g) were reduced to below the detection limit of 1 log(CFU/g) in all treatments, except for in naturally fermented *A. esculenta* where the pathogen maintained viable levels of 3.7–4.2 log(CFU/g) throughout the seven day period (Fig. 2). In terms of the risk of growth of *C. botulinum* a safe pH level (<4.6), i.e., lower than the growth boundary for proteolytic *C. botulinum*, was obtained within two days of fermentation of A. *esculenta* with DK22 (Fig. 1), while it took seven days for *S. latissima* with DK22. Naturally fermented *S. latissima* reached a safe pH level after seven days with regards to *L. monocytogenes* and non-proteolytic *C. botulinum.* However, the pH level remained unsafe (pH > 5) for naturally fermented *A. esculenta* and therefore it was classified as an unsuccessful fermentation (Fig. 1).

3.5. The microbiota of fermented seaweed

Analysis of the beta diversity of the microbial communities in the four products revealed marked differences among the products (Fig. 3).

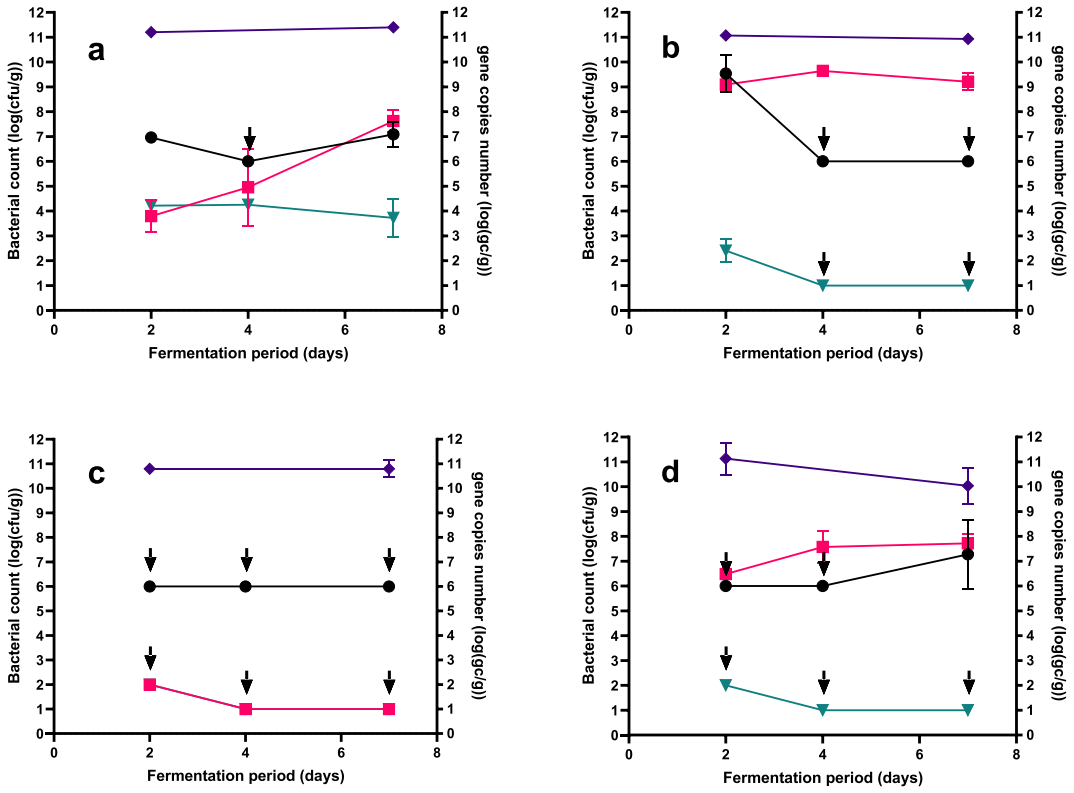

Fig. 2. Microbial changes during the fermentation process of seaweeds: Naturally fermented *A. esculenta* (A), LAB fermented *A. esculenta* (B), naturally fermented *S. latissima* (C) and LAB fermented *S. latissima* (D). (♦) Gene copy number of 16S rRNA determined by qPCR, (●) aerobic viable count determined on Long and Hammer agar, (■) lactic acid bacteria determined on De Man, Rogosa and Sharpe agar and (▼) *Listeria* spp. determined on Palcam agar. Symbols and error bars indicate Avg. ± SD. The arrows indicate bacterial counts below the detection limit for the specific agar and sampling day. Numbers of lactic acid bacteria and *Listeria* spp. are identical in Fig. 2C.

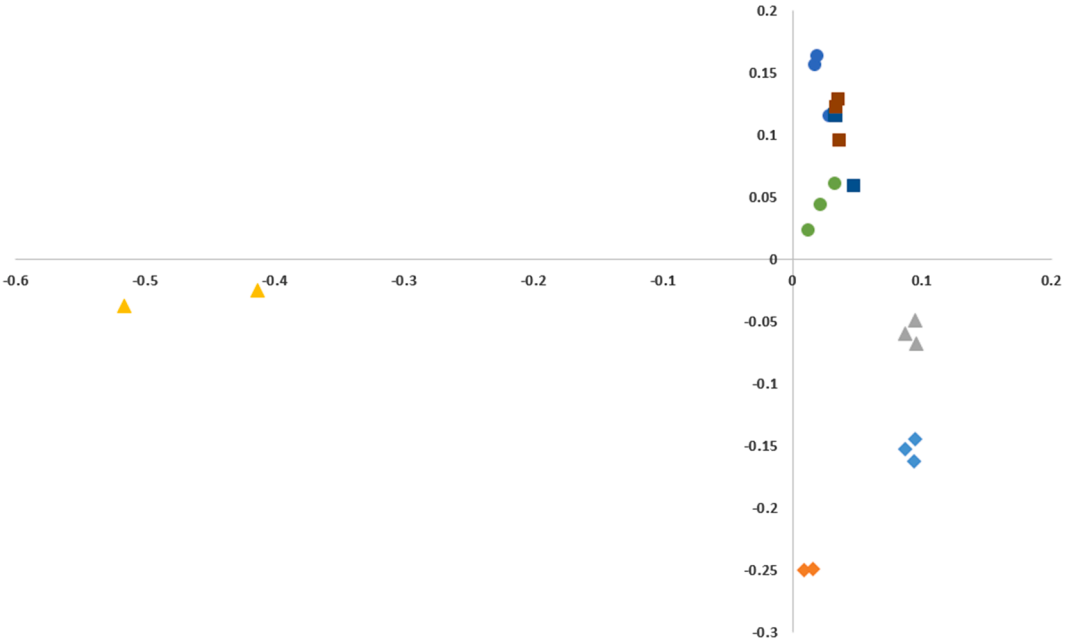

Fig. 3. The weighted UniFrac beta diversity of fermented seaweeds. PCoA illustrated with PC1 (47.27%) and PC2 (32.74%). The scores were obtained for naturally fermented *A. esculenta* sampled after two days (▲) and seven days (▲), LAB fermented *A. esculenta* sampled after two days (♦) and seven days (♦), naturally fermented *S. latissima* sampled after two days (■) and seven days (■) and LAB fermented *S. latissima* sampled after two days (●) and seven days (●).

A. esculenta fermented by the naturally occurring microbiota showed a large change between day two and day seven resulting in an average weighted UniFrac distance of 0.60. Similar changes were not observed for naturally fermented *S. latissima* where an average weighted UniFrac distance of 0.04 was observed over time. The two seaweed products with added DK22, exhibited minor changes and had an average weighted UniFrac distances of 0.16–0.19.

The total number of observed amplicon sequencing variance (ASV) remained high >100 for all four products. For *A. esculenta* fermented with and without DK22 and naturally fermented *S. latissima* there was a decreasing trend in observed ASVs and the Shannon diversity index during the fermentation process (Table 3). For LAB fermented *S. latissima*, the Shannon index were significantly ($p < 0.05$) higher after the seven-day fermentation period (Table 3).

In naturally fermented *A. esculenta*, *Photobacterium* dominated after two days of fermentation, however, it almost disappeared after seven days where *Fusobacterium* and *Sphaerochaeta* became dominant (Fig. 4A), with low levels of *Clostridium* spp. being detected after seven days (1.6 ± 0.8%). Inoculation of *A. esculenta* with DK22 led to formation of a mixed microbiota after two days, which consisted of *Aliivibrio, Lactiplantibacillus, Photobacterium,* and *Pseudoalteromonas.* Seven days of fermentation with DK22 resulted in a microbiota of *Lactiplantibacillus* (35.0%) mixed with *Aliivibrio* (37.4%) (Fig. 4A).

In contrast, the microbiota of the two fermented *S. latissima* products were similar and consisted primarily of *Psychromonas, Marinomonas, Cobetia* and *Aliivibrio* (Fig. 4B). Inoculating *S. latissima* with DK22 resulted in low relative abundances of *Lactiplantibacillus* comprising 2.0 and 7.9% of the microbiota after two and seven days of fermentation, respectively.

4. Discussion

Lactic acid bacteria fermentation of seaweed relies on the availability of sugars for growth. The fermentation requirements of any crop has been suggested to have to fulfil a fermentation coefficient (FC) of 45 or above (Weissbach & Honig, 1996). The parameters for FC include WSC, buffering capacity, and dry matter (DM). Previously, the FC of *S. latissima* has been proven to be higher than the recommended level (Herrmann et al., 2015). No FC information is available for *A. esculenta*, however, the FC of other brown seaweed species (*Ascophyllum nodosum*, *Laminaria digitate* and *Saccorihiza polyschides*) were reported to be below the recommended level (Herrmann et al., 2015). The buffering capacity of *A. esculenta* used in this study is unknown and the mannitol content is at a lower level compared to *S. latissima*, 10.4 ± 3.9 and 18.0 ± 8.5% of DM, respectively, which are comparable to levels previously reported (Schiener, Black, Stanley, & Green, 2015). It could be hypothesed that the FC of *A. esculenta* is lower compared to *S. latissima* and might be close to the FC 45 boundary, since the natural fermentation of *A. esculenta* was unsuccessful with its end-point pH > 5, thus making it unsafe for proteolytic *C. botulinum* and *L. monocytogenes*. The increase in fucoidan and mannitol after two days of fermentation could be an indication of a breakdown of cell walls by enzymatic or bacterial processes and release intracellular fucoidan and mannitol to the water-soluble phase. The low amount of mannitol present was utilised in DK22 fermented *A. esculenta*, similar to what was seen in fermentation of previously frozen *S. latissima* with *Lb. plantarum* (Bruhn et al., 2019). The lower utilisation of mannitol observed for *S. latissima* inoculated with DK22 (Table 2), could be due to lower concentration of *Lb. plantarum* observed by enumeration on MRS agar (Fig. 2D) and 16S rRNA amplicon sequencing (Fig. 4), indicating that the environmental conditions were not optimal for growth of the *Lb. plantarum* strain used in this study.

The slow reduction of pH in fresh naturally fermented *S. latissima* was observed in another study (Herrmann et al., 2015) and even the addition of starter culture in our study did not result in a faster pH reduction, likely due to the limited growth of DK22 in the *S. latissima*. In our study, only LAB fermented *A. esculenta* met the pH-requirements for safe storage based on inhibition of proteolytic *C. botulinum* (Peck, 2014) after two days of fermentation, while for *S. latissima* with DK22 it took seven days. Fermentation of *A. esculenta* by the naturally occurring microbiota failed to reach pH < 5 meaning that pH alone would not inhibit *C. botulinum* or *L. monocytogenes*, a finding that was supported by the observed survival of *L. monocytogenes.* In addition, the product would not meet the FDA requirements for low-acid food (FDA, 2020a, 2020b).

Freezing *S. latissima* before fermentation with a starter culture has previously been shown to enable successful colonisation of LAB and accelerate the pH reduction to be achieved within 40 h (Bruhn et al., 2019). The freezing process may inactivate part of the naturally occurring microbiota; similar to observations in salmon, where it was shown that different species of bacteria are inactivated by freezing at different rates (Emborg, Laursen, Rathjen, & Dalgaard, 2002). Our study's observations suggest that a pre-treatment step such as freezing or addition of adjuvants, i.e., additives which promote growth of the starter culture (s), should be investigated as means to ensure a successful seaweed fermentation process by improving the colonization of the starter culture(s).

Fermentation of seaweeds is an emerging platform based on the traditions for LAB fermentation of plant-based materials (Uchida & Miyoshi, 2013). Two widely-known platforms are the Korean kimchi and the European sauerkraut, and a recent review published in 2021 focus on application of different omics technics to develop and optimise fermentation (Di Cagno, Filannino, Acín-Albiac, & Gobbetti, 2021). For example, amplicon sequencing analysis of kimchi has shown the successful fermentation is characterised by a rapid reduction of the Shannon diversity index. In a study of different batch fermented plant ingredients, all salted, sequencing revealed that initial Shannon indices of 4–5 decreased to 1 already after one day of fermentation at 20 °C, a level that remained constant throughout of the fermentation process (Zabat, Sano, Cabral, Wurster, & Belenky, 2018). In our fermentation of seaweed, regardless of species or addition of a LAB culture, a Shannon index lower than 4.6 was not reached (Table 2). The result indicated that some naturally occurring bacteria dominated and suppressed the LAB in the raw seaweed, which is in contrast to the complete dominance by LAB observed in kimchi after a short fermentation process. Our results showed that the endogenous microbiota of *A. esculenta* belonged to the *Photobacterium*, *Aliivibrio* and *Psychromonas* genera, and for *S. latissima* mainly belonged to the *Psychromonas* and *Marinomonas* (Fig. 4). All genera, except *Marinomonas*, are facultative anaerobes (Brenner, Krieg, & Staley, 2005) and may be better adapted to grow on the raw seaweed and hence would outcompete the starter culture to dominate the microbiota.

In the naturally fermented *A. esculenta* at day seven, undesirably genera were observed in the microbiota, i.e., *Fusobacterium, Sphaerochaeta, Bacteroides* and *Clostridium* spp. Together they made up 64.7 ±

Table 3
Alpha diversity of fermented seaweed.

Products	Species richness Average ± SD (n of ASV)	Shannon index Entropy ± SD (average)
A. esculenta		
Day 2 naturally fermented	281.3 ± 23.1^{A}	5.4 ± 0.1^{A}
Day 7 naturally fermented	173.5 ± 109.6^{A}	3.6 ± 0.9^{A}
Day 2 LAB fermented	203.7 ± 20.8^{A}	4.9 ± 0.1^{A}
Day 7 LAB fermented	194.0 ± 1.4^{A}	4.2 ± 0.1^{A}
S. latissima		
Day 2 naturally fermented	215.3 ± 38.6^{A}	4.8 ± 0.5^{A}
Day 7 naturally fermented	216.7 ± 25.7^{A}	4.5 ± 0.3^{A}
Day 2 LAB fermented	132.7 ± 35.9^{B}	4.0 ± 0.4^{A}
Day 7 LAB fermented	248.7 ± 61.9^{A}	5.1 ± 0.1^{B}

$^{A–B}$Capital letters denote significant differences ($p < 0.05$) within the column and species of seaweed.

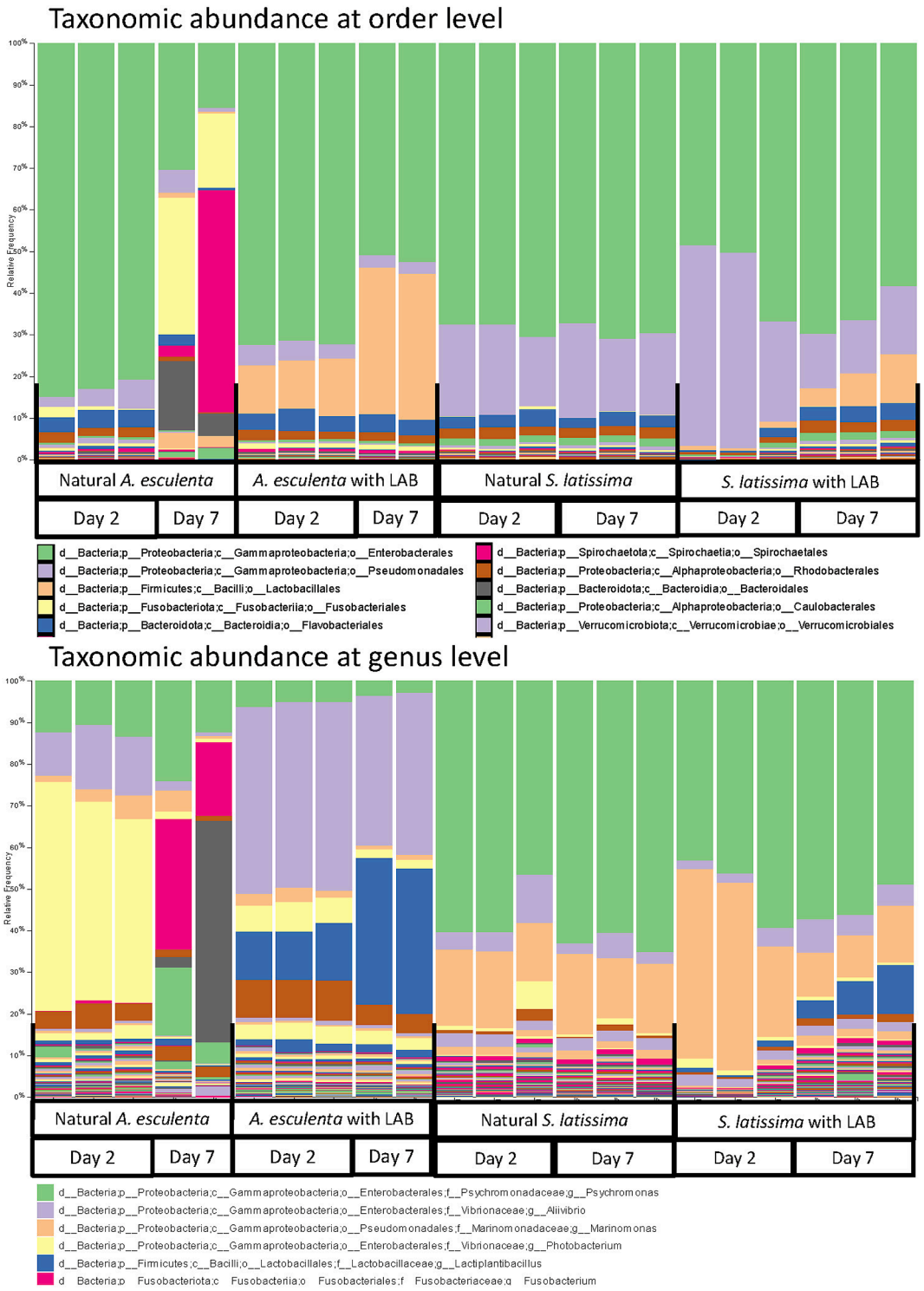

Fig. 4. Taxonomic bar charts of bacterial communities in fermented seaweeds. Bacterial community profiles in *A. esculenta* and *S. latissima* fermented with or without addition of a starter culture, *Lb. plantarum*. Relative abundance of the bacterial 16S rRNA gene amplicon sequences at the order and genus levels are shown for seaweed samples fermented for 2 and 7 days. The ten and six most abundance ASV at order and genus levels, respectively, are shown in the legends.

19.0% of the relative abundance and are characterised by being common in human faecal matter (Duncan, Hold, Harmsen, Stewart, & Flint, 2002; Wexler, 2007) and in marine environments (Miyazaki, Sakai, Ritalahti, Saito, Yamanaka, Saito, & Imachi, 2014; Wu, Zheng, Wu, Yang, & Liu, 2014). The finding of *Clostridium* is supported by a study that isolated *C. algifaecis* from a decomposed algal scum (Wu et al., 2014). A fermentation driven by *Clostridium* spp. results in the production of carbon dioxide and a range of organic acids (Herrmann et al., 2015), and an acetone-butanol-ethanol (ABE) fermentation could occur. The ABE fermentation occurs in two steps, with initial formation of acids, mainly butyric and acetic acid and in the later cell cycle phase the *Clostridium* spp. form ethanol (Patakova et al., 2018). The ABE fermentation could potentially have happened in the naturally fermented *A. esculenta*, with formation of both butyric and acetic acids in addition to ethanol after seven days of fermentation (Table 1). A closely related bacteria, *Fusobacterium prauznitzii* from the human faecal biota is

known to ferment acetic acid to produce butyrate, formic and lactic acids (Duncan et al., 2002).

While inactivation of the natural microbiota of seaweed before fermentation would remove unwanted spoilage bacteria, it may also induce food safety concerns since the lack of competition makes it easier for contaminants from the production environment to grow. In the case of inadequate hygiene in production facilities and contamination with *L. monocytogenes*, it was shown that the pathogen could potentially grow in the untreated seaweed if there is a limited reduction in pH (Fig. 1). *C. botulinum* type E is found widespread in the marine and arctic environment (Horowitz, 2010; Huss, 1993) and could be a potential hazard. Predictive models for growth of the two foodborne pathogens in seaweed may be developed or calibrated based on complete fermentation and product characterization, with off-set in known models for *L. monocytogens* (Martinez-Rios, Gkogka, & Dalgaard, 2020) in cheese and lightly preserved seafood for *C. botulinum* (Koukou et al., 2021).

5. Conclusion

Fermentation of raw shredded *A. esculenta* and *S. latissima* using the endogenous microbiota with or without the addition of a *Lb. plantarum* was only partially successful. While *Lb. plantarum* grew during fermentation of *A. esculenta* and resulted in pH values dropping below 4.6 after 2 days, the same was not the case for *S. latissima* where *Lb. plantarum* was less successful in colonisation of the seaweed. Fermentation by the endogenous microbiota failed to reduce to pH values below 4.6 and led to the development of undesirable organic acids (e.g., butyric) and ethanol as well as harboured a high alpha diversity, indicating the lack of suitable naturally occurring starter cultures in the seaweed.

Further work should aim at selecting the most optimal LAB strain(s) to secure a faster pH drop and increase food safety. The selection of LAB should be based on their genomic profile as well as composite analysis of the two seaweed species to elucidate their potential to sustain growth of LAB with or without adjuvants. Improved fermentations could also be achieved through studies of pre-treatment induced changes in the bioavailability of nutrients, anti-nutrients and endogenous microbiota.

CRediT authorship contribution statement

Jonas Steenholdt Sørensen: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Sanne Kjærulf Madsen:** Investigation, Writing – review & editing. **Claus Heiner Bang-Berthelsen:** Conceptualization, Resources, Writing – review & editing. **Lisbeth Truelstrup Hansen:** Conceptualization, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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