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1 Executive Summary

This report summarises the results achieved in the following AQUACOMBINE project task:

- Task 5.4. – Extraction of protein by acidification by lactic acid bacteria.

Fresh but non-food grade (partly lignified) *Salicornia ramosissima* (commonly known as glasswort or sea asparagus) and *Tripolum pannonicum* (syn. *Aster tripoliumi,* commonly known as sea aster) were fractionated to green juice and fibre residue fraction. Green juice fractions were utilised for the production of protein-enriched concentrate (PEC), which can be further formulated into functional animal feed supplements. Screening of lactic acid bacteria (LAB) strains was performed beforehand in order to find a suitable strain to acidify the saline halophyte juice Out of the studied strains, *Lactiplantibacillus plantarum* and *Ligilactobacillus salivarius* exhibited the fastest growth and highest tolerance for acidic conditions and the presence of their metabolic product, lactic acid (product inhibition).

Besides fermentation with LAB, acidification with HCl and heat coagulation of proteins were tested as a reference. The obtained PEC was analysed for its dry matter (DM) and crude protein contents. Only acidification with HCl exhibited significantly different DM (p = .003) and crude protein (p =.004) recoveries to PEC compared to other treatments. *S. ramosissima* samples showed high crude protein recovery to PEC (> 54 % in all samples). In contrast, in *T. pannonicum* samples, the crude protein recovery to PEC was lower, indicating that the measured nitrogen could be present in compounds other than amino acids and protein. Lab-scale fermentations were carried out in *S. ramosissima* juice using *L. plantarum* due to its more widely reported probiotic properties. Generally, lower DM and crude protein recoveries were achieved in lab-scale bioreactor compared to flask trials. Fermentation to final pH 4.0 yielded a higher fraction of PEC and dry matter recovery compared to fermentation to final pH 3.5. However, there were no significant differences in the crude protein content of PEC and crude protein recovery.

Halophytes, such as *S. ramosissima*, could be seen as a potential feedstock for PEC production by acidification using LAB fermentation. However, further investigations and process development would be needed to improve the protein content and functionality of the product.

2 Introduction

Due to increased livestock, the demand for high-quality protein for feeding has increased rapidly. Nowadays, farmers in European Union are strongly dependent on imported protein sources, mainly soybean [1]. A potential alternative and locally produced source for protein is PECs from different agricultural residues and green forages processed according to the green biorefinery approach.

In green biorefinery, the biomass is first fractionated to green juice and fibre residue fractions. After fractionation, a significant amount of the protein of different forages would be water-soluble and present in the green juice fraction [1–3]. Water soluble proteins can be separated into PEC by coagulation using different treatments, such as acidification, heating, filtration, or adding flocculants [1,4]. Heat treatment has shown to be an efficient method for protein separation but has certain disadvantages related to operational expenses due to high-energy input, protein denaturation, and possible formation of Maillard reaction products from sugars and amino acids [4,5]. In acidification, which could be a gentler approach, the minimum protein solubility is achieved in the isoelectric point, which is usually between pH 3.2 – 4.5 for plant leaf biomasses [2,4]. Acidification can be done simply by adding acid or fermentation with LAB. Kiel et al. [6] developed a protein coagulation method, where the lactic acid fermentation of green juice from agricultural forages is performed. Inoculated LAB utilises the sugars present in the juice, and the produced lactic acid decreases the pH of the juice, allowing proteins to precipitate. This approach is also considered in the AQUACOMBINE project for halophyte green juice, and a simplified schematic is presented in Figure 1.

When fermentation is done using probiotic LAB strain, the functionality of the PEC could be enhanced. Probiotics are micro-organisms which provide several health benefits for the host. Probiotic bacteria can either colonise the gastrointestinal tract or create unfavourable conditions for pathogens [7]. Probiotic LAB could be used as a sustainable and safer replacement for antibiotic supplements in animal feed for aquacultures and terrestrial livestock. Probiotic properties of different LAB strains in animal feed were investigated in a comprehensive literature review, which is planned to be submitted in autumn 2022 [8]. In aquacultures, the LAB supplementation has shown a protective effect against several pathogens [9–16], but also attenuation of the effect of some toxins [9,17], a decrease of the accumulation of heavy metals to fish tissues [18], and improvement of the stress capacity in the acute exposure to low or high saline water [19,20]. Considering terrestrial livestock, such as chickens, LAB supplementation has shown an increased protective effect against *Salmonella* and *Escherichia coli* [21–25], enhanced growth performance, increased LAB colonisation in intestines, and an improved immune system [26,27].

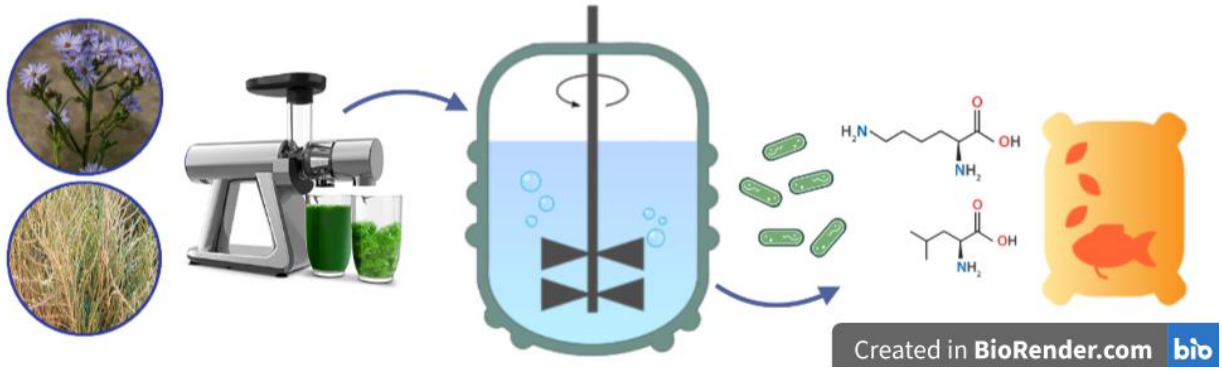

Figure 1: Simplified sketch of the green biorefinery concept targeting probiotic feed production.

AQUACOMBINE WP5 focuses on the green, partly lignified halophyte biomass and green biorefinery approach. This report considers the outcomings of the following project task:

- Task 5.4. – Extraction of protein by acidification by lactic acid bacteria.

3 Methodology

3.1 Raw Material

Biomass batches presented in Table 1 were used as raw material in the protein precipitation experiments. *S. ramosissima* (glasswort, sea asparagus) from RSR was fractionated to green juice and fibre residue fractions using a lab-scale single-auger, and *S. ramosissima* from LDM and *T. pannonicum* (sea aster) using a pilot-scale double-auger juicer. Juice fractions were immediately frozen and defrosted only prior to the analysis. *S. ramosissima* from RSR was received in two different development stages: after 12 weeks and 26 weeks of cultivation. This biomass has previously been characterised in AQUACOMBINE project deliverable D6.2. *S. ramosissima* harvested after 12 weeks was used only in the initial LAB screening, and green juice from 26-week-old plants was used in other experiments.

Table 1. Biomass used in protein precipitation experiments.

Species	Partner	Cultivation practise	Salinity
Salicornia ramosissima	RSR	Soil in greenhouse	Seawater irrigation
Salicornia ramosissima	LDM	Open-field in marsh	Seawater irrigation
Tripolium pannonicum	LUH	Hydroponics in greenhouse	7 g/l NaCl

Early on in the experiments, it was noticed that any of the selected LAB (see Section 3.3) was not able to ferment the *S. ramosissima* juice from LDM. This could be due to too high salt concentration for bacterial growth or a very low amount of free sugar monomers in the juice (< 0.5 g/100 g juice dry matter). Therefore, processing of *S. ramosissima* from LDM was not considered further, and all experiments were carried out using *S. ramosissima* from RSR.

3.2 Proximate Composition Analysis

The DM content of the fractions were determined according to the protocols by National Renewable Energy Laboratory (NREL) [28,29]. The crude protein content was determined by measuring the total nitrogen using an elemental analyser (and applying the Jones conversion factor of 6.00 [30]. Free sugar monomers and organic acids in green juice fractions were determined using the protocol by NREL [31]. Separated sugar monomers were glucose, xylose, and arabinose, and other separated compounds were lactic acid, acetic acid, and ethanol. Juice samples were analysed with high-performance liquid chromatography (1260 Infinity II, Agilent Technologies) using an organic acid column (Aminex HPX-87H), 0.005 M H_2SO_4 mobile phase, and a refractive index detector. The column temperature was 63 °C, the flow rate was 0.6 ml/min, and the detector operation temperature was 35 °C.

3.3 Screening of Lactic Acid Bacteria

3.3.1 Lactic Acid Bacteria in Saline Halophyte Juice

The initial selection of potential LAB strains was made by evaluating the existing literature about their tolerance for saline and acidic conditions, optimal cultivation temperature, and other properties described by Lübeck et al. [32]. Some of the properties are summarised in Table 2.

Table 2. Optimal incubation temperature, reported tolerance for acids and maximum NaCl concentration in cultivation media for selected lactic acid bacteria strains.

Strain	Temperature [°C]	Min. pH	Max. NaCl [%]	Ref.
Lactiplantibacillus pentosus	30 – 37	3.0	6.0 – 8.0	33–35
Lactiplantibacillus plantarum	30 – 37	3.0 – 4.5	6.0 – 10.0	35–37
Lactobacillus delbrueckii	37 – 42	4.4 – 4.5	3.5	38–40
Ligilactobacillus salivarius	37	2.5	~ 5.0	41–43
Lactococcus lactis	30	3.0 – 4.0	4.0	44,45
Tetragenococcus halophilus	30	4.0	25.0	46–48

In order to see if the inoculated LAB can acidify the juice instead of bacterial strain naturally present in the juice, two types of heat treatments were tested for *S. ramosissima* juice: vat pasteurisation, steam sterilisation, and one batch was left untreated (Figure 2). In vat pasteurisation, also known as low-temperature long-time pasteurisation, the sample was heated in a water bath until 63°C, kept there for 30 minutes, and cooled down in an ice bath. This type of pasteurisation is commonly used in the food industry for milk treatment [49]. Steam sterilisation was carried out in an autoclave, and samples were kept at 121 °C for 15 minutes. The pH of the juice was measured before heat treatments and after 4 v/v% LAB culture inoculation (914 pH/Conductometer, Metrohm AG). The experiment was carried out in screw-cap bottles, and 100 ml of juice was used for each sample. The bottles were incubated at 37 °C for 4 days. The pH of the fermented samples was measured, solids were separated with centrifugation, and concentrations of sugars and organic acids were measured from the supernatant, as described in Section 3.2. Due to the limited amount of juice available at the project's beginning, this test was carried out only once per sample.

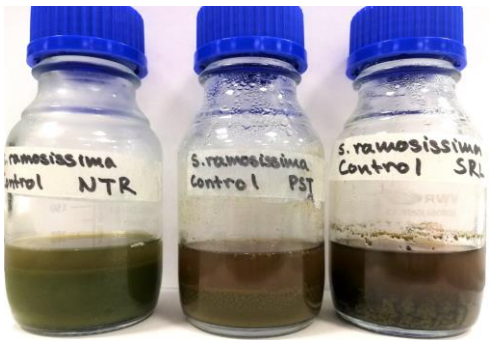

Figure 2: *S. ramosissima* juice (not inoculate) after different heat treatments. From left: no treatment, vat pasteurised, and steam sterilised.

3.3.2 Maximum Acidification in Optimised Media

Later in the project, it was decided to focus on LAB strains with probiotic properties to enhance the feed's functionality. Based on the initial study and reported probiotic effect on animals [8], the following strains were tested for their acidification potential: *L. plantarum, Lactiplantibacillus pentosus, L. salivarius*, and *Lactococcus lactis*. The acidification was carried out in sterilised MRS broth in a 1 litre fermenter (BioBench, Biostream) under anaerobic conditions and with constant measurement of pH, dissolved oxygen, and temperature. The temperature was set to 37 °C for all strains, except *L. lactis,* which had reported an optimal temperature of 30 °C. Media was inoculated with 10 v/v% LAB pre-culture in MRS broth, with the mean optical density (OD) at 600 nm being 0.819 (0.091). The bacterial growth in the fermenter was monitored by sampling and measuring the OD 600 nm hourly with a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies).

3.4 Protein Precipitation by Lactic Acid Fermentation

Fermentation trials were carried out in baffled shake flasks. Green juice volume of 180 ml (200 ml for control/natural fermentation samples) was measured, and samples were inoculated with 10 v/v% of LAB pre-culture and flushed with nitrogen. Flasks were kept in a shaking incubator at 37 °C for 48 h. The pH of the juice media was measured before and after fermentation. PEC, including probiotic LAB biomass, was separated by centrifugation at 5000 rpm for 20 min. Brown juice was decanted and discarded. The DM of obtained PEC was determined. Recovery of DM, recovery of crude protein, and lactic acid yield were calculated using the following equations:

$$DM\ recovery\ [\%] = \frac{DM\ in\ PEC\ [g]}{DM\ in\ green\ juice\ [g]} \times 100\ \%$$

$$Crude\ protein\ recovery\ [\%] = \frac{Crude\ protein\ in\ PEC\ [g]}{Crude\ protein\ in\ green\ juice\ [g]} \times 100\ \%$$

$$Lactic\ acid\ yield\ [\%] = \frac{Lactic\ acid\ produced\ [g]}{Glucose\ consumed\ [g]} \times 100\ \%$$

3.5 Protein Precipitation by Heat Treatment and Acidification

Heat coagulation was carried out as described by Christiansen et al. [50], where 200 ml of juice was heated in flasks in a water bath to 80 °C, kept there for an additional minute, and then cooled down in an ice batch. Acidification using HCl was performed at room temperature by slowly adding < 1 ml of 7.7 M HCl to a continuously stirring 200 ml green juice sample until the pH of 3.5 was reached. The final pH was selected based on the reported isoelectric point for plant biomass, where the protein has the minimum solubility to water [2,4]. The PEC separation and further processing were done for both treatments, as described earlier in section 3.4.

3.6 Fermentation in Lab-Scale Fermenter

According to Santamaría-Fernández and Lübeck [1], acidification to pH 3.5 – 4.0 has exhibited the highest PEC yields. Therefore, fermentation until pH 3.5 and pH 4.0 was tested in order to see if the final pH affects the PEC yield and crude protein recovery. Experiments were carried out in a 1 litre fermenter (BioBench, Biostream) flushed with nitrogen with a constant pH and dissolved oxygen measurement, and temperature control set at 37 °C (Figure 3). Juice (900 ml) was inoculated with LAB pre-culture in MRS broth with OD 600 nm of 1.2546 (0.1512). Levels of sugars and metabolites were determined by taking hourly samples, cooling samples down immediately to stop the bacterial growth, and measuring them for concentrations of sugars, lactic acid, acetic acid, and ethanol, as described in Section 3.2. Fermentation was stopped when the target pH was reached, and separation of PEC was separated as described earlier in Section 3.4.

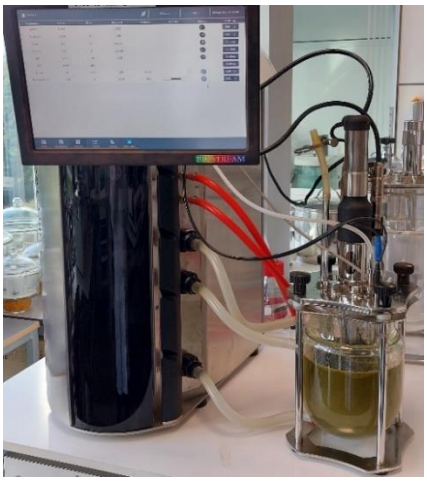

Figure 3: Lab-scale fermentation setup used at AAU.

3.7 Statistical Methods

Precipitation experiments were carried out in duplicates (n=2) unless stated otherwise. All analytical work was run in triplicates (n=3) unless stated otherwise. Results are given as mean values with standard deviation marked in brackets. One-way analysis of variance (ANOVA) combined with the Tukey honest significant difference (HSD) test was run to evaluate the significance of the differences between the results.

4 Results

4.1 Acidification Potential of Lactic Acid Bacteria

4.1.1 Acidification of Heat Treated *Salicornia* Juice

All tested LAB strains, except *T. halophilus*, were able to acidify the *S. ramosissima* green juice (Figure 4). In control samples, acidification was observed in non-treated samples but not in the samples with applied heat treatments. Therefore, in the non-treated *T. halophilus* inoculated sample, the drop in pH could be caused by a micro-organism natural to the juice rather than the added lactic acid bacteria. Plated colonies from control samples showed gram-negative bacillus in microscopic analysis. Therefore, sequencing of bacteria would be needed to confirm the results. During the fermentation period, the growth was fastest in the samples inoculated with *L. pentosus* and *L. plantarum,* as the cloudiness of the juice was clearly observed after 24 hours of fermentation. The mean lactic acid concentration in LAB inoculated samples after 4 days of fermentation was 5.61 (0.19) g/l, and > 80 % of the total available sugars were consumed. Considering the obtained PEC, on average only 6.14 (0.57) % of the total ash in the fresh juice ended up in the solid fraction.

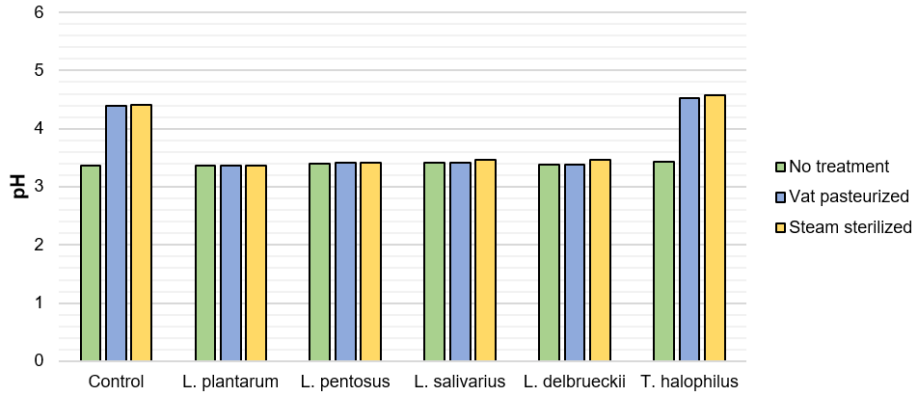

Figure 4: Final pH of S. ramosissima juice (harvested after 12 weeks) after 4 days of fermentation with different lactic acid bacteria after different heat treatments. The initial pH of juice before inoculation was 4.59 (0.07), and after inoculation, 4.29 (0.20).

4.1.2 Acidification of MRS broth

After seeing that selected strains could survive in saline halophyte juice, strains were tested in an optimised nutrient-rich medium to see how low pH could be achieved before the growth inhibition. During the fermentation in 1 litre fermenter, *L. salivarius* exhibited the most rapid growth and acidification of MRS media (Figure 5). For both *L. salivarius* and *L. plantarum,* the final reached pH was 3.8, and at that point, the growth got inhibited due to acidic conditions or product inhibition, as all available glucose in the media was not consumed. However, as lower pH was achieved in the LAB screening study in *S. ramosissima* juice, product inhibition could be the most plausible reason for inhibition. *L. pentosus* had a similar performance to *L. plantarum* but has less reported probiotic properties for aquacultures and terrestrial livestock in the reviewed literature [8]. *L. lactis* did not perform adequately in MRS broth, with slower growth rates and a final pH of 4.2.

Based on the screening experiments and literature review, the fermentation experiments were carried out using *L. plantarum* and *L. salivarius*. These LAB strains are robust, rapidly growing, and produce only lactic acid as their primary metabolite (homofermentative). Both stains were able to ferment the *Salicornia* juice to pH 3.5 – 4.0 and have several probiotic properties for aquaculture and terrestrial livestock [8].

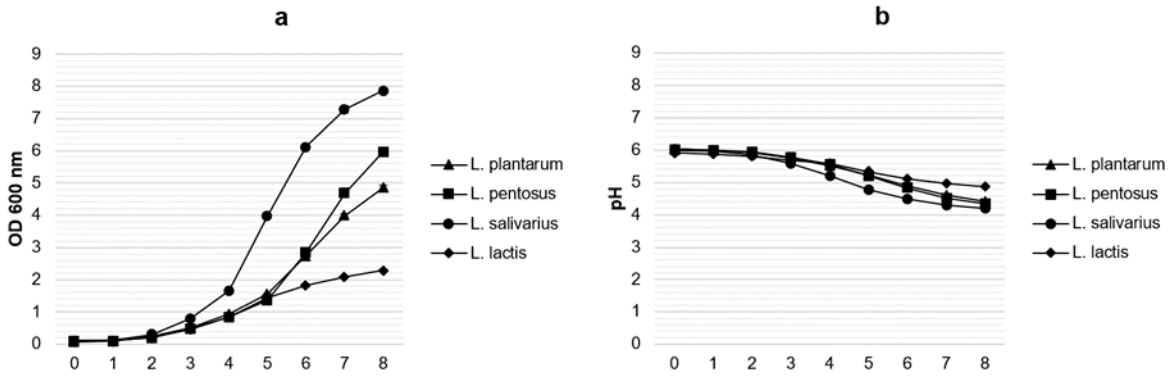

Figure 5: Optical density (OD) at 600 nm (a) and pH (b) of the bacterial culture in MRS broth during the first eight hours of fermentation.

4.2 Flask Fermentation and Green Juice Acidification

Both *L. plantarum* and *L. salivarius* were able to acidify the juices to the end pH of approximately 3.5 (Figure 6). A decrease in pH was also observed in the control sample of *T. pannonicum,* indicating the presence of a natural acidifier in the juice. Unlike in the LAB screening study with 12-week-old *S. ramosissima*, acidification of the control sample of 26-week-old *S. ramosissima* was not observed. During the fermentation, *L. plantarum* and *L. salivarius* consumed 97.70 (0.03) % and 83.41 (0.61) % of the available glucose in *S. ramosissima* juice, respectively, and 99.90 (0.06) % and 98.92 (0.37) % of available glucose in *T. pannonicum* juice, respectively. The lactic acid concentration in the fermented juice was 30.50 (0.08) g/l and 39.46 (0.21) g/l in *S. ramosissima* samples fermented with *L. plantarum* and *L. salivarius*, respectively. In *T. pannonicum* samples, the lactic acid concentration was 9.93 (0.24) g/l and 17.47 (2.99) g/l after fermentation with *L. plantarum* and *L. salivarius*, respectively. High lactic acid concentrations are due to the conversion of sugars from inoculation MRS media and possible sugars released from complex carbohydrates due to long retention time in elevated temperature. Lactic acid was not detected in fresh juice or control fermentation samples. Acetic acid and traces of ethanol were also detected in the fermented samples. It has been shown that the presence of oxygen and sugar deprivation can trigger the metabolic pathway of *L. plantarum* to co-produce lactic acid and acetic acid [51]. Production of these compounds can also be a sign of contamination, as the juice used in the study was not treated beforehand.

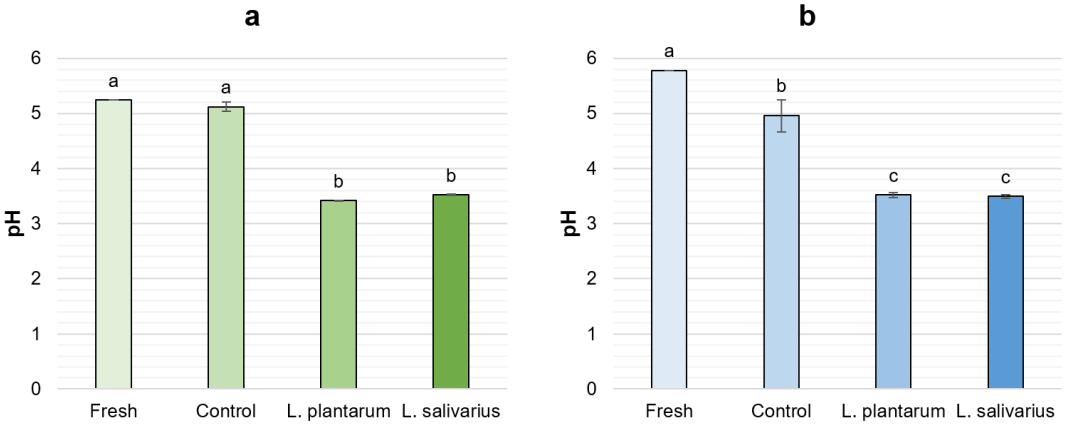

Figure 6: The final pH of S. ramosissima (a) and T. pannonicum (b) juice as fresh and after fermentation. Different letters denote significantly different results calculated individually for each species and for both p < .001.

4.3 The Yield of Protein-Enriched Concentrate

The fermented and heat-treated *T. pannonicum* juices did not form a stable solid cake during the centrifugation. Therefore, the concentrate after decanting had very low dry matter content. However, the fresh juice and the HCl acidified juice formed a less crumbly solid cake and were easier to decant. This issue was not observed in any of the *S. ramosissima* samples. The amount of PEC from different treatments and corresponding DM and crude protein contents are collected in Table 3.

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Table 3. Yield of protein enriched concentrate (PEC), PEC dry matter (DM), and crude protein (CP) content of PEC obtained from *S. ramosissima* and *T. pannonicum* juice fraction after different treatments. Results are mean values, standard deviations are marked in brackets, and different letters denote significantly different results calculated individually for each species.

Treatment	PEC [%]	PEC DM [%]	PEC DM CP [%]
Salicornia ramosissima			
Fresh (no treatment)	10.35 (0.94) ^{ab}	16.41 (2.13) ^{b}	20.78 (0.57) ^{ab}
Control fermentation	9.29 (0.41) ^{bc}	18.14 (0.27) ^{ab}	18.14 (2.01) ^{b}
L. plantarum	7.62 (0.25) ^{bc}	21.55 (0.32) ^{a}	21.64 (1.45) ^{ab}
L. salivarius	7.32 (0.22) ^{c}	21.41 (0.63) ^{a}	22.10 (0.93) ^{a}
HCl acidification	12.75 (1.49) ^{a}	17.95 (0.39) ^{ab}	20.10 (1.30) ^{ab}
Heat coagulation	9.78 (0.13) ^{abc}	18.80 (0.23) ^{ab}	18.98 (1.03) ^{ab}
p-values	.003	.008	.020
Tripolium pannonicum			
Fresh (no treatment)	12.76 (2.61) ^{ab}	6.19 (1.07) ^{b}	30.40 (0.80) ^{ab}
Control fermentation	7.53 (1.09) ^{b}	3.32 (0.18) ^{c}	31.66 (6.28) ^{ab}
L. plantarum	10.65 (0.89) ^{b}	4.61 (0.66) ^{bc}	31.36 (1.92) ^{ab}
L. salivarius	15.53 (6.75) ^{ab}	6.29 (0.12) ^{b}	33.92 (1.89) ^{a}
HCl acidification	11.33 (1.48) ^{b}	11.42 (1.11) ^{a}	29.86 (0.96) ^{ab}
Heat coagulation	28.11 (6.18) ^{a}	5.63 (0.19) ^{bc}	25.60 (0.93) ^{b}
p-values	.019	< .001	.067

The highest DM recovery to PEC in both *S. ramosissima* and *T. pannonicum* was achieved with HCl acidification (Figure 7). The DM and crude protein contents of fresh juice were 4.01 (0.04) % and 27.62 (1.65) g/100 gDM for *T. pannonicum*, respectively, and 6.47 (0.21) % and 8.68 (0.14) g/100 gDM for *S. ramosissima*, respectively. Recoveries are calculated based on these values. Considering DM and crude protein recovery to PEC, out of the *S. ramosissima* samples, only HCl acidified juice had significantly higher DM and crude protein recoveries, and differences between other results were non-significant, even when compared to fresh juice. All *S. ramosissima* samples, except the control fermentation sample, exhibited > 60 % crude protein recovery to PEC and in HCl acidified sample, the crude protein recovery was 81.8 (7.83) %. It must be noted that acidification with HCl was the only treatment where elevated temperatures were not applied. In *T. pannonicum* samples, DM recovery to PEC varied a lot. No significant differences were observed in crude protein recovery, and all samples exhibited recoveries < 40 %. This could indicate that the source of nitrogen measured in elemental analysis of fresh juice is not necessarily protein but also other water-soluble compounds like nitrate, which has been reported as a possible anti-nutrient factor for *T. pannonicum* [52]. However, analysis of amino acids would be required to evaluate the nutritional qualities of the samples. Due to slightly higher DM and crude protein recoveries, and more reported probiotic activities, *L. plantarum* was chosen for the fermentation of *S. ramosissima* juice in a lab-scale fermenter.

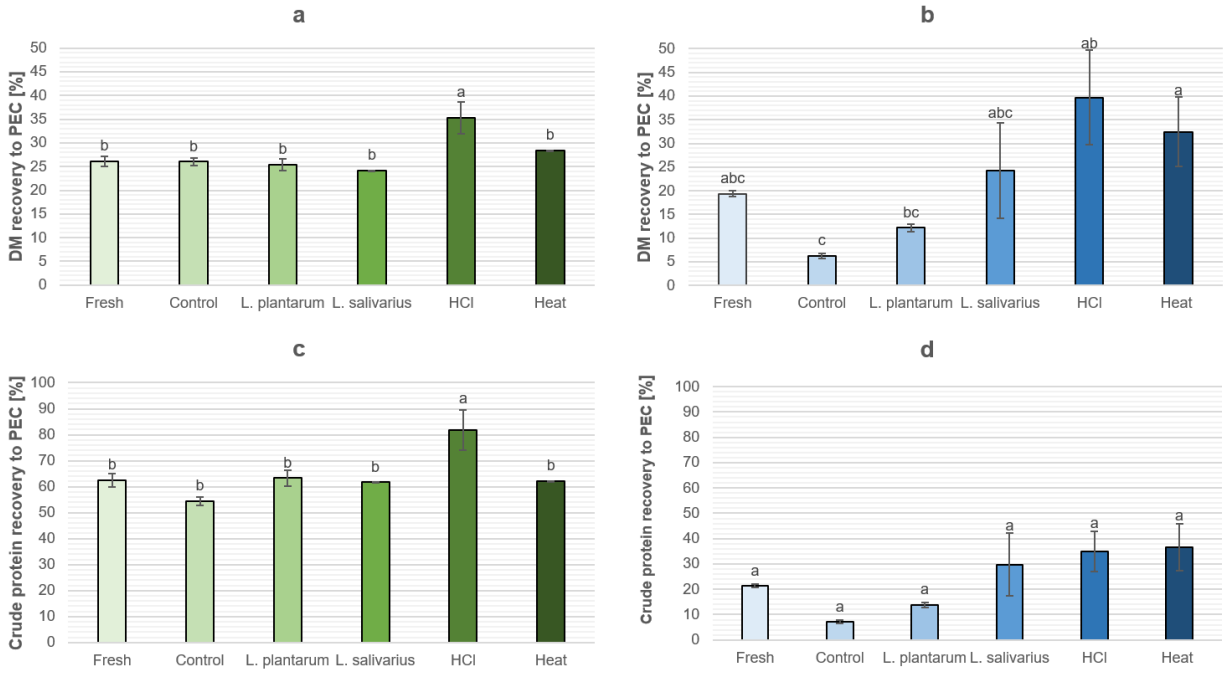

Figure 7: Dry matter (DM) recovery to protein-enriched concentrate (PEC) of S. ramosissima (a) and T. pannonicum (b) juices after different treatments, and corresponding crude protein recovery to S. ramosissima (c) and T. pannonicum (d) PEC. Different letters denote significantly different results calculated individually for each figure, and p-values were (a) p = .003, (b) p = .016, (c) p = .004, and (d) p = .029.

4.4 Effect of Final pH of Fermentation

In order to reach pH 3.5 and 4.0, the fermentation of *S. ramosissima* with *L. plantarum* was carried out on average for 9 h 30 min (± 40 min) and 5 h 30 min (± 15 min) hours, respectively. Compared to flask fermentation, acidification in a lab-scale fermenter yielded a lower amount of PEC. Fermentation to higher final pH 4.0 yielded a higher amount of PEC, with higher DM recovery to PEC (Table 4). Differences between the DM content of the obtained PEC were non-significant. Regardless of the higher DM recovery, there were no significant differences in the crude protein content of PEC and crude protein recovery. Crude protein content was also lower (< 40 %) compared to flask fermentation (63.28 % for *L. plantarum* fermented *S. ramosissima*).

Table 4. Yield of protein enriched concentrate (PEC), PEC dry matter (DM), and crude protein (CP) content of PEC and DM and CP recoveries to PEC obtained from *S. ramosissima* after fermentation with *L. plantarum* to different pH levels. Results are mean values, standard deviations are marked in brackets, and different letters denote significantly different results.

Fraction	Final pH 3.5	Final pH 4.0	p-values
Salicornia ramosissima			
PEC [%]	5.16 (0.26) ^{b}	6.10 (0.84) ^{a}	.009
PEC DM [%]	17.67 (0.83) ^{a}	16.44 (1.87) ^{a}	.112
PEC DM CP [%]	22.07 (1.67) ^{a}	20.90 (0.97) ^{a}	.112
DM recovery [%]	14.07 (0.49) ^{b}	15.32 (0.98) ^{a}	.006
CP recovery [%]	35.80 (3.41) ^{a}	36.86 (2.66) ^{a}	.496

As halophyte juice was not sterilised before fermentation, ethanol and acetic acid contents were also measured to indicate the presence of possible natural acidifiers and other micro-organisms. Contamination was not observed, as only traces of these compounds were found in samples, and acetic acid content slightly increased only at the end of

fermentation to a pH of 3.5. The concentrations of sugars and metabolites are presented in Figure 8. The high standard deviation in the glucose content of the fermentation media may be due to bacterial culture, as the LAB pre-culture was diluted with glucose-rich MRS broth before inoculation. To reach the pH of 3.5, 58.67 (10.40) % of the initial available glucose was consumed in the fermentation. Only 11.62 (9.67)% of the initially available glucose was consumed to achieve the pH of 4.0. The lactic acid yield from fermentations exceeded 100 %, based on the theoretical conversion of glucose to lactic acid, showing that LAB has also used other molecules as a substrate, or additional glucose has been released from the green juice matrix during the fermentation.

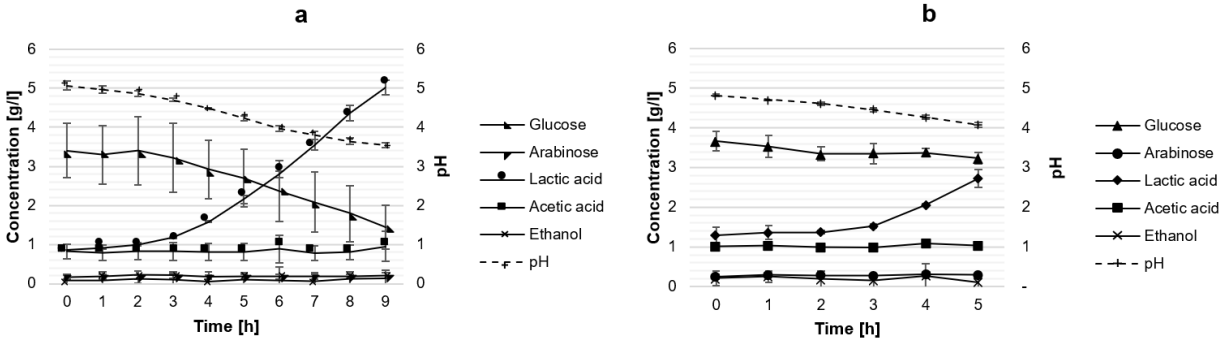

Figure 8: Concentration of sugars and metabolic products in S. ramosissima juice media inoculated with L. plantarum during the fermentation and acidification to pH 3.5 (a) and 4.0 (b).

5 Conclusions and Outlook

In the screening study, several LAB strains were found to be able to acidify the saline *S. ramosissima* juice. For further experiments, strains with reported probiotic effects on animals were selected, the most potential strain being *L. plantarum* and *L. salivarius*. Protein precipitation from fresh *S. ramosissima* and *T. pannonicum* juices was tested in flasks with lactic acid fermentation but also with heat coagulation and HCl acidification. Out of these methods, the highest DM and crude protein recovery to PEC was achieved with HCl acidification. Considering the obtained PECs from *S. ramosissima*, high crude protein recoveries were achieved (> 54 % in all samples), but changes between most treatments were non-significant. The results from *T. pannonicum* protein precipitation varied more than those from *S. ramosissima*, and lower crude protein recoveries were observed (< 40 % in all samples). Therefore, the production of green protein from *T. pannonicum* was not further considered.

In order to maximise protein precipitation and PEC yield from LAB fermented *S. ramosissima* juice, acidification to two different final pH were tested in 1 litre fermenters. This was done because the minimum solubility varies depending on the type of protein present in the juice. In general, fermentation in a larger-scale fermenter system yielded lower PEC than flask fermentations. However, the fermentation to the final pH of 4.0 yielded higher PEC and dry matter recovery compared to the final pH of 3.5. However, no significant changes were observed in crude protein recovery to PEC.

As the fermentation did not exhibit an increase of the crude protein recovery to PEC in flask experiments, the focus of the research is turned from precipitation of plant protein to maximising the production of probiotic LAB using green juice as fermentation media. The probiotic effect of *L. plantarum* fermented PEC will be tested for aquacultures. Following research activities related to Task 5.4. will be carried out:

- Feeding trials in collaboration with RSR, where PEC from *L. plantarum* fermented *S. ramosissima* juice will be incorporated with shrimp feed in order to see the possible probiotic effect of feed supplement for stress tolerance of shrimp juveniles.
- Determining the amino acid profile of PECs to evaluate the product's nutritional value.
- Fermentation of *S. ramosissima* juice in a pH-controlled fed-batch reactor in order to increase the growth and production of probiotic LAB biomass and improve the functionality of the feed product.
- Sequencing of the bacteria present in the fermented samples to detect possible pathogens or natural acidifiers.
- Scale-up and demonstration in a pilot-scale system with volume up to 100 litres is planned for the last year of the AQUACOMBIME project.

Halophytes could provide a source of sustainable, green protein for feed applications. However, further investigations need to be carried out to develop processes to extract plant protein and produce probiotic microbial biomass and analyse the product's nutritional qualities and functional properties.

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