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Original Research Article

Microbial safety and protein composition of birch sap

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ABSTRACT

Consumers' demand for birch sap, a nutritional juice tapped directly from birch trees, for human consumption is growing. This study aimed to investigate the time- and weather-related variability of the microbiota and protein content in birch sap throughout a complete tapping season, and the effect of birch sap freezing on its shelf life. Birch sap was collected daily during the 2018 season and harvested once a week during three consecutive weeks in 2019. Microbiota and protein content was 0.6–5.7 log(CFU/mL) and 3–60 μg/mL, respectively, with the highest content of both being in the end of the season. Daily temperatures correlated statistically with microbiota counts throughout the tapping season but not with protein concentration. The most prevalent bacteria was the genus *Pseudomonas*. Freezing birch sap for two weeks reduced the microbiota counts ~1 log unit but did not affect the shelf life and type of bacteria. Twenty proteins related to plant defence against pathogens and abiotic stress were identified. In conclusion, birch sap harvested in the beginning of the tapping season had a longer shelf life and contained less protein than at the end of the season, which is of importance when developing procedures for microbial safe collection of birch sap and for the collection of sap containing bioactive substances.

1. Introduction

Birch sap is a colourless liquid from birch trees that has been used for centuries all around the world, as a beverage or syrup (Maher et al., 2005; Salminen et al., 2005; Zhang and Shi, 2005; Svanberg et al., 2012), as a food ingredient or as a probiotic after fermentation (Semjonovs et al., 2014).

Birch sap, the xylem of birch trees, is harvested from different species of *Betula* in cooler regions of the Northern Hemisphere in a period of two to five weeks in early spring, from a drilled hole in the tree, a process named tapping (Drozdova et al., 1995; Jiang et al., 2001; Maher et al., 2005; Ozolinčius et al., 2016; Shaoquan et al., 1995; Zhang and Shi, 2005; Zyryanova et al., 2005). The sap yield depends on the birch species, location and seasonal weather. For instance, *B. pendula* tree exudes lower amount of sap than *B. pubescens* (Svanberg et al., 2012), and *B. platyphylla* sap exudation starts earlier as well as reaches a maximum flow rate before *B. verrucosa* (Jiang et al., 2001). The location of the tree and the soil nutrients also affect the birch sap quantity, eg. being higher from trees grown in well-aerated mineral soils than from trees grown in undrained or flooded soils (Mingaila et al., 2020). In addition, trees grown along the edge of the forests exude more sap than trees from the interior (Zajączkowska et al., 2019).

Birch sap contains bioactive substances, such as sugars, proteins, minerals and vitamins, rendering it is nutritional value (Kallio and Ahtonen, 1987; Kallio et al., 1985, 1989; Kūka et al., 2013; Ozolinčius et al., 2016; Shaoquan et al., 1995). The amount and composition of the bioactive substances vary between the different birch species, location and harvest day. Thus, glucose and fructose concentrations in *B. pubescens* sap are approximately 30% lower than in saps from *B. pendula* trees grown in Poland and Latvia (Grabek-Lejko et al., 2017). Dissimilar weather conditions between tapping seasons seem to affect the sap sugar composition, likely due to a rapid break of the dormancy,

Abbreviations: CFU, Colony-forming units; MRS, DeMan, Rogosa and Sharpe; DG, Dichloran-glycerol; MW, Molecular weight; RT, Room temperature; TSA, Tryptone soy agar.

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development of the buds or early sugar liberation in warmer years (Kallio and Ahtonen, 1987). The total content of amino acids may also vary from 100 to 500 mg/L depending on the exact tapping day (Ahtonen and Kallio, 1989). A few studies have described that birch sap contains proteins, and that the amount increases during the tapping season (Jiang et al., 2001; Kallio et al., 1995), but knowledge on the composition of the proteins is very limited (Bilek et al., 2019; Jiang et al., 2001; Kallio et al., 1995).

The self life of fresh birch sap, the length of time that it maintains the quality and safety according to taste and microbial load, is short, deteriorating rapidly at room temperature (RT), while it can be stored for three to five days in the refrigerator (Bilek et al., 2016; Nikolajeva and Zommere, 2018; Shaoquan et al., 1995). According to the Food safety authorities, the manufacturer is responsible for assigning food shelf life and needs to be aware of all factors affecting microbiota growth. The short shelf life of birch sap might be an issue for the manufacturer, and nutrition preserving techniques such as freezing, microfiltration, fermentation or pasteurisation might be advantageous in prolonging the shelf life (Bilek et al., 2016; Li and Gao, 1995; Salminen et al., 2005; Semjonovs et al., 2014). A study investigating the shelf life of defrosted birch sap, found that the yeast and total aerobic bacteria count increased two-fold after two days of storage in the refrigerator (Nikolajeva and Zommere, 2018).

The objectives of the present study were to investigate the time- and weather-related variability of the level and composition of microbiota and proteins in birch sap harvested daily throughout a 35 days tapping season in Denmark, and to determine the effect of freezing on the birch sap shelf life. We hypothesise that the specific seasonal harvest time has a significant impact on both microbiota and protein level as well as composition.

2. Materials and methods

2.1. Sampling and handling of birch sap

Birch sap from *B. pendula* was harvested each day, from a single tree, for a period of 35 days, the complete 2018 tapping season (from March 26th until April 29th, 2018) in the forest of Ravnsholt Skov (Birkerød, Denmark) (coordinates: 55°50′11.2″N 12°21′18.7″E). The daily harvest was frozen in small aliquots at −20 °C immediately after the tapping and defrosted at 4 °C for analysis. A tap hole was made on day 0 at a height of 1.10 m, and the tap hole was changed at day 18 and later at day 23. For shelf life studies, three defrosted birch sap aliquots from harvest day 1, 7, 14, 21, 28 and 35 were used and stored for seven days.

Birch sap harvested in the 2019 tapping season was used to compare the total microbiota counts of fresh and defrosted sap. The birch sap was harvested one day per week during three consecutive weeks (March 18th, March 25th and April 1st, 2019) in the same forest and from one single tree. Birch sap was either analysed for total microbiota counts immediately after harvest (fresh) or after freezing at −20 °C for two weeks. Furthermore, shelf life at 4 °C for triplicates of fresh and defrosted samples from week 2 were compared for seven days.

Weather data such as temperature, amount of rain and hours of sunshine, throughout the 2018 tapping season, were gathered from the Danish Meteorological Institute (DMI), 2020.

2.2. Microbiota counts

Birch sap harvested throughout the 2018 tapping season, was analysed for: (1) total counts of microbiota on tryptone soy agar (TSA) (Thermo Fisher Scientific), (2) lactic acid bacteria on DeMan, Rogosa and Sharpe (MRS) agar (Oxoid, Thermo Fisher, Scientific), and (3) yeast and fungus on dichloran-glycerol (DG18) agar (Sigma-Aldrich). Each sample was 10-fold serially diluted with 0.9% (w:v) NaCl, and 100 µL were spread on TSA, MRS and DG18 agar plates. Plates were incubated at RT for three to four days (on TSA) or seven days (on MRS and DG18 agar) to quantify the colony-forming units (CFU)/mL. Total microbiota counts in samples from 2019 were determined on TSA as described above.

2.3. Microbiota identification by mass spectrometry (MS)

Birch sap samples from the 2018 tapping season (day 1, 7, 14, 21, 28 and 35) were analysed for identification of bacteria at day 0 (initial), 4 and 7 of storage at 4 °C. Moreover, fresh and defrosted birch sap from the 2019 tapping season (week 2) after storage for 0, 7 and 14 days at 4 °C was also analysed. When TSA plates contained 10–15 colonies, all colonies were selected for microbiota identification, whereas plates with more than 15 colonies were divided into areas containing 10–15 colonies to obtain random selection. From the respective plates, 175 colonies were identified out of a total of 1225 colonies in 2018, whereas 137 colonies were identified out of a total of 399 colonies in 2019. The selected colonies were subcultivated on new TSA plates, incubated for three days at RT and thereafter frozen at −80 °C in 15% glycerol until analysis. Three days before bacterial identification, the frozen strains were inoculated onto TSA plates and incubated at RT.

Single colonies were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) using a Biotyper System (Bruker Daltonics, Bremen, Germany) as previously described (Madsen et al., 2016). MALDI-TOF MS analysis was performed on a Microflex LT mass spectrometer (Bruker Daltonics) using the Bruker Biotyper 3.1 software and the BDAL standard library. A bacterial test standard (Bruker Daltonics) was used to calibrate the instrument. Data are presented at species level.

2.4. Protein concentration

Birch sap harvested each day for a period of 35 days, the complete 2018 tapping season, was freeze dried in a pilot scale freeze dryer (Beta 1–8, Martin Christ GmbH, Osterode am Harz, Germany) under vacuum for 24 h. Samples were dissolved in Laemmli sample buffer (65.8 mM Tris−HCl, pH 6.8, 26.3% glycerol, 2.1% SDS, 0.01% bromphenol blue) (BioRad, Hercules, CA, US) and 2 M dithiothreitol (DTT) (Sigma-Aldrich, Darmstadt, Germany) by heating at 65 °C for 15 min as described previously (Cabanillas et al., 2014). The protein concentration was determined using the Pierce™ 660 nm Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, US), containing an ionic detergent compatibility reagent, following the manufacturer's instructions.

2.5. Protein separation by SDS-PAGE

SDS-PAGE of proteins dissolved in Laemmli sample buffer was performed using 4–20% Tris-Glycine Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad) according to the manufacturer's instructions. Proteins were visualised by Bio-Safe Coomassie Stain (BioRad) using the Imager ChemiDoc XRS+ (BioRad).

2.6. Protein separation by 2D electrophoresis

Birch sap harvested on days 7 and 28 in the 2018 tapping season was loaded onto a PD10 desalting column (Merck KGaA, Darmstadt, Germany) to remove low-molecular weight (MW) (< 5 kDa) compounds and further freeze dried as descried in section 2.4. Samples were dissolved in isoelectric focusing (IEF) immobilised pH gradient (IPG) strip rehydration buffer (8 M urea; 2% CHAPS; 50 mM DDT; 0.2% IPG buffer; 0.001% bromophenol blue) (BioRad) for 12 h. Linear IEF IPG strips (7 cm, BioRad), pH 3–10 or 4–7, were used as the first dimension in a PROTEAN® i12™ IEF System (BioRad) according to the manufacturer's instructions. IPG strips were soaked in the equilibration solution (6 M Urea, 2% SDS, 20% glycerol, 37.5 mM Tris−HCl, pH 8.8, BioRad) for 10 min with 2% (w:v) DTT and 10 min with 2.5% (w:v) iodoacetamide at RT. Mini-Protean TGX stain-free protein gels (4–20%) 7 cm IPG/prep

(BioRad) were used for the second dimension according to the manufacturer's instructions. Proteins were visualised after activation with UV light using the Imager ChemiDoc XRS+ (BioRad).

2.7. Protein identification by liquid chromatography-tandem mass spectrometry (LC–MS/MS)

Birch sap harvested on day 7 in the 2018 tapping season was loaded onto a PD10 desalting column (Merck KGaA) to remove low-MW (< 5 kDa) compounds and further freeze dried as described in section 2.4. Lysate preparation and digestion of the samples were performed using StageTips as previously described (Kulak et al., 2014; Rappsilber et al., 2007).

Peptides were separated on a 50 cm C18 reverse-phase analytical column (Thermo Scientific™ EASY-Spray™ LC Column, Thermo Fisher Scientific) using an EASY-nLC 1000 ultra-high pressure system coupled to the Q Exactive mass spectrometer (Thermo Fisher Scientific).

The MS/MS data were searched using Proteome Discoverer 2.2 against *Betula* protein data from the UniProtKB database, 2020.

2.8. Statistical analyses

Graphs and statistical analyses of the data were performed using GraphPrism version 7.0 (San Diego, CA, US). The nonparametric Spearman's rank correlation test was used to determine a possible correlation between the microbiota counts and the protein concentration in birch sap harvested throughout the 2018 tapping season, and the weather conditions throughout the same season. A p-value ≤ 0.05 was considered statistically significant.

Microbiota data are expressed as log(CFU/mL) and presented as mean of three birch sap aliquots from the batch of the day (batch replicates) ± standard deviation (SD). For comparison of the fresh and frozen birch sap only one sample from the batch was tested. One way ANOVA was performed to test if there was any significant difference in the microbial counts.

The DMFit tool from the program ComBase (https://browser.combase.cc/DMFit.aspx) was used to predict lag phase and the maximum rate for bacterial growth at 4 °C in fresh or defrosted birch sap harvested in 2019. The observed growth curves were fitted to the Baranyi and Roberts growth curve models with no asymptote for the defrosted sample and no lag phase for the fresh birch sap sample. From the maximum growth rate, the generation time was calculated.

3. Results

3.1. Initial microbiota concentration in birch sap from 2018

Overall, the later in the season the sap was harvested the higher the initial microbiota count (Fig. 1). The first 13 days of the season the aerobic bacteria counts were below 2 log(CFU/mL), after which they increased to a maximum of approximately 5.5 log(CFU/mL) in the end of the season (Fig. 1). The difference in the initial mean values of the microbiota from week 1 compared to week 5 was statistically significant (Fig. 1). Counts of yeast (DG18 and MRS agars), fungi (DG18 agar) and lactic acid bacteria (MRS agar) were constant from day 1 to day 23 but increased at the end of the season up to 2 log(CFU/mL) (Fig. 1).

On day 23, the tap hole in the birch tree was changed, before collecting the sap, due to visual changes with white deposits around the hole and a slight cloudiness of the sap. The counts were reduced from 5.5 to 2.5 log(CFU/mL) but did not reach the same low levels as that observed at the beginning of the season (Fig. 1).

The initial level of the microbiota grown on TSA, MRS and DG18 agar plates throughout the tapping season, showed a strong statistical positive correlation with the daily mean temperature (Table 1 and supplementary Table 1) as well as with the daily minimum and maximum temperature (data not shown) throughout the 2018 tapping season but not with other weather conditions such as rainfall and sunshine hours.

3.2. Shelf life and microbiota identification

The growth and composition of the microbiota were determined in the birch sap samples harvested once a week in 2018 and during storage at 4 °C for seven days (Fig. 2 and supplementary Fig. 1). The aerobic total microbial counts on TSA were around 1 and 2 log(CFU/mL) at day 1 and 7, respectively, and the counts only raised 0.5 log(CFU/mL) after seven days at 4 °C (Fig. 2). In these samples, Gram positive bacteria such as *Microbacterium*, *Staphylococcus* and *Rothia* species were mainly

Table 1
Spearman's correlation between birch sap protein concentration (mg/L) and bacteria count (log(CFU/mL)), and the temperature (°C), amount of rain (mm) and hours (h) of sunshine throughout the 2018 tapping season.

	Correlation coefficient (r)	P value
Protein vs Temperature	0.041	0.816
Protein vs Rain	0.107	0.540
Protein vs Sunshine	−0.039	0.826
Microbiota in TSA vs Temperature	0.641*	3.28 e-005
Microbiota in TSA vs Rain	−0.072	0.680
Microbiota in TSA vs Sunshine	0.194	0.264
Microbiota in TSA vs Protein	0.151	0.388
Microbiota in MRS vs Temperature	0.534*	0.001
Microbiota in MRS vs Rain	0.335*	0.048
Microbiota in MRS vs Sunshine	0.118	0.497
Microbiota in MRS vs Protein	0.289	0.092
Microbiota in G18 vs Temperature	0.578*	2.79 e-004
Microbiota in G18 vs Rain	0.265	0.122
Microbiota in G18 vs Sunshine	0.226	0.190
Microbiota in G18 vs Protein	0.291	0.088

* A p-value ≤ 0.05 was considered statistically significant.

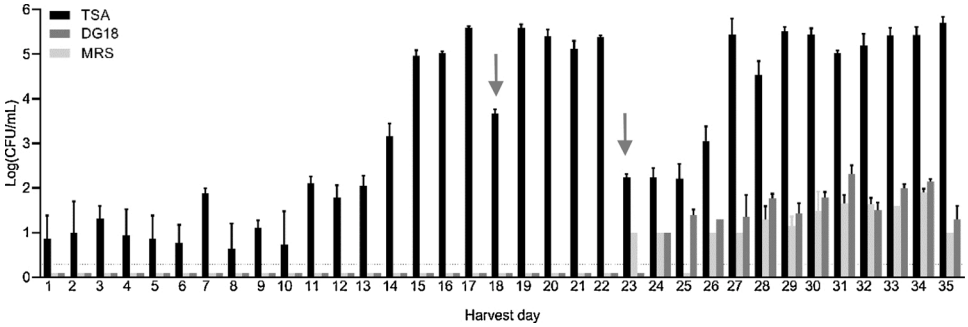

Fig. 1. Initial total microbiota count in birch sap harvested each day for a period of 35 days, the complete 2018 tapping season, after freezing at −20 °C and defrosting at 4 °C. The level of total aerobic bacteria, the lactic acid bacteria and the level of yeast and fungus were tested on TSA, MRS and DG18 agar, respectively. Arrows indicate change of tap hole. Data are presented as means of three individual aliquot samples ± SD.

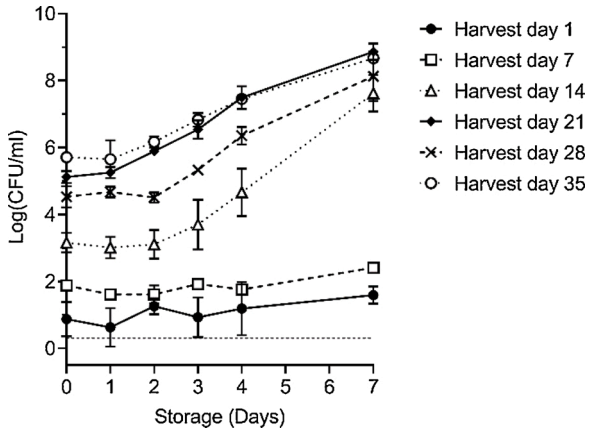

Fig. 2. Microbiota growth in defrosted birch sap samples harvested at day 1, 7, 14, 21, 28 and 35 in the 2018 tapping season analysed at day 0, 1, 2, 3, 4 and 7 of storage at 4 °C. Total counts were determined on TSA plates. Results are expressed as colony-forming units (CFU) per millilitre and $\log_{10}$-transformed (log(CFU/mL)). The dotted line represents the limit of detection at 0.3 log(CFU/mL). Data are presented as means of three individual aliquot samples ± SD.

identified (Table 2).

In the rest of the season, the aerobic total microbial counts were between 3 log(CFU/mL) and 6 log(CFU/mL) which increased after storage for one to two days (Fig. 2). Moreover, the microbiota counts reached around 7 log(CFU/mL) after seven days of storage at 4 °C in birch sap harvested from day 17 to 35 (Supplementary Fig. 1).

The type of bacteria shifted from Gram positive to Gram negative after harvest day 14. Besides one *Rahnella* species identified at harvest day 28 and 35, only *Pseudomonas* species were detected (Table 2). Particularly at day 14 and 21, colonies were classified as *Pseudomonas brenneri* whereas seven different *Pseudomonas* species were identified at day 28. At harvest day 35, colonies were classified as four different *Pseudomonas* species while only a single *Rahnella* species was detected.

3.3. Effect of freezing on microbiota growth and composition

The effect of freezing on microbiota counts was studied in birch sap harvested one day per week during three consecutive weeks of the 2019 tapping season (Fig. 3A). The effect of freezing was minimal for samples harvested in week 1, where the microbial count was the same in frozen and fresh samples (approximately 2.5 log(CFU/mL)). However, an effect of freezing was observed for samples harvested in week 2 and week 3, with reductions of around 1 and 1.5 log(CFU/mL), respectively (Fig. 3A).

Growth and composition of the microbiota in fresh and defrosted birch sap samples during storage at 4 °C up to seven days were tested in birch sap harvested in week 2 (Fig. 3B and Table 3). Even though there was a difference of 1 log units between the initial microbiota counts in fresh and defrosted birch sap there was no difference in shelf life. The growth kinetic analysis showed that in defrosted samples, the lag phase of the microbiota was 3.6 h longer but the generation time 1.3 h shorter. Both fresh and defrosted birch sap samples reached approximately 7 log (CFU/mL) after three days of storage at 4 °C (Fig. 3B). *Pseudomonas* species were the main species in both the fresh and defrosted birch sap as well as in the stored samples (Table 3).

3.4. Protein concentration

Birch sap harvested each day for a period of 35 days, the complete 2018 tapping season, contained a total protein content ranging from 3 to 60 mg/L, which increased in amount throughout the season (Fig. 4).

A potential effect of the weather conditions, during the season, on the total protein content was investigated. No statistically significant correlation between protein concentration and temperature, rainfall and sunshine hours, respectively, was observed (Table 1).

3.5. Protein profile

Birch sap showed a varying protein profile throughout the 2018 tapping season, as determined by SDS-PAGE, with two dominant proteins with sizes of approximately 25 and 30 kDa, respectively (Fig. 5A-D). While proteins in the beginning of the season primarily had sizes between 17 and 75 kDa (Fig. 5A and B), the proteins showed a range of sizes between 12 and 150 kDa by the end of the season (Fig. 5C and D). It is noticeable that a protein of around 17 kDa present in the beginning of the tapping season almost disappeared by the end, and two proteins of around 12 and 20 kDa seemed to emerge from day 27 (Fig. 5D).

To investigate the protein profile in more detail, samples from the beginning (day 7) and the end (day 28) of the tapping season 2018 were

Table 2
Bacterial species and number of colonies identified by MS in birch sap harvested on days 1, 7, 14, 21, 28 and 35 of the 2018 tapping season analysed at day 0, 4 or 7 of storage at 4 °C.

Harvest day	1			7			14			21			28			35		
Storage day	0	4	7	0	4	7	0	4	7	0	4	7	0	4	7	0	4	7
Kocuria palustris	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Microbacterium maritypicum	–	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Micrococcus luteus	–	–	1	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus warneri	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus pasteuri	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus hominis	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Rothia endophytica	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Rothia amarae	–	–	–	–	2	–	–	1	–	–	–	–	–	–	–	–	–	–
Sphingomonas aerolata	–	–	–	–	2	1	–	–	–	–	–	–	–	–	–	–	–	–
Moraxella osloensis	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–
Pseudomonas brenneri	–	–	–	–	–	–	12	8	9	14	10	10	9	2	–	3	–	–
Pseudomonas libanensis	–	–	–	–	–	–	–	2	–	–	–	–	1	–	–	–	–	–
Pseudomonas synxantha	–	–	–	–	–	–	–	1	–	–	–	–	1	–	–	–	–	–
Pseudomonas brassicacearum	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–
Pseudomonas chlororaphis	–	–	–	–	–	–	–	–	1	–	1	2	–	–	–	–	–	–
Pseudomonas fragi	–	–	–	–	–	–	–	–	–	–	–	–	1	1	1	2	5	2
Rahnella aquatilis	–	–	–	–	–	–	–	–	–	–	–	–	–	2	2	6	6	3
Pseudomonas tolaasii	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	1
Pseudomonas fluorescens	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–
Pseudomonas orientalis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–
Pseudomonas genus	–	–	–	–	–	–	–	–	–	–	–	–	–	2	6	–	–	–

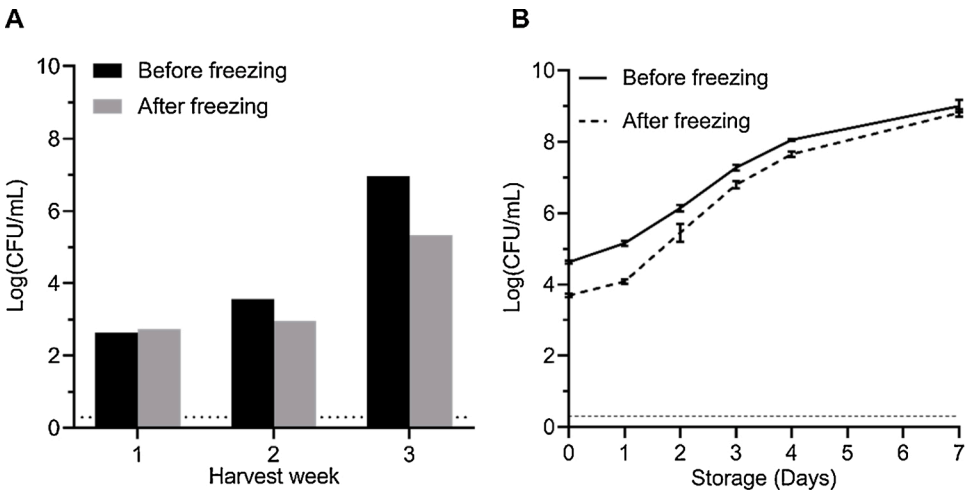

Fig. 3. (A) Microbiota count in birch sap harvested one day per week during three consecutive weeks of the 2019 tapping season, before (fresh sample) or after freezing at −20 °C for two weeks (defrosted sample). One sample of the respective batch was analysed. (B) Microbiota count in fresh and defrosted birch sap harvested in week 2 analysed at day 0, 1, 2, 3, 4 and 7 of storage at 4 °C. Total counts were determined on TSA and expressed as colony-forming units (CFU) per millilitre and $\log_{10}$-transformed (log(CFU/mL)). The dotted line represents the limit of detection at 0.3 log(CFU/mL). Data are presented as means of three individual aliquot samples ± SD.

Table 3
Bacterial species and number of colonies identified by MS in birch sap harvested in week 2 in 2019 analysed at day 0, 4 or 7 of storage at 4 °C.

Harvest day Storage day	Fresh 0	Frozen 0	Fresh 7	Frozen 7	Fresh 14	Frozen 14
Pseudomonas frederiksbergensis	9	7	–	–	–	–
Pseudomonas koreensis	5	2	1	1	4	3
Pseudomonas veronii	–	1	1	2	–	1
Pseudomonas fluorescens group	–	1	–	–	–	–
Pseudomonas brenneri	–	–	2	–	–	–
Pseudomonas antarctica	–	–	2	2	1	1
Pseudomonas extremorientalis	–	–	3	3	2	–
Pseudomonas chlororaphis	–	–	1	–	1	–
Pseudomonas agarici	–	–	1	–	–	–
Pseudomonas cedrina	–	–	–	–	–	–
Pseudomonas poae	–	–	–	1	–	–
Pseudomonas fluorescens	–	–	–	1	1	3
Pseudomonas brassicacearum	–	–	–	1	1	–
Brevibacterium celere	–	–	–	–	–	–
Pseudomonas azotoformans	–	–	–	–	2	–
Erwinia billingiae	–	–	–	–	–	1

selected and analysed by 2D electrophoresis (Fig. 5E-G). The detected proteins covered a pI range between 4 and 8 and a size range between 10 and 70 kDa. While the dominant proteins at day 7 revealed sizes between 20 and 37 kDa (Fig. 5E and F), the most dominant proteins at day 28 had sizes between 20 and 25 kDa (Fig. 5G).

3.6. Protein identification

Birch sap harvested on day 7 in the 2018 tapping season and analysed by LC–MS/MS revealed a great complexity in the protein content (Supplementary Fig. 2). Of the 20 proteins identified as corresponding to either *B. pendula* or *B. platyphylla* (due to similarities in proteins form these two Birch species) proteins by LC–MS/MS in combination with *in silico* analyses, 17 corresponded to proteins related to the defence of the plant against pathogens and abiotic stress (Table 4). In addition, proteins involved in plant functions and biological processes were identified.

4. Discussion

Birch sap has been consumed since antiquity due to its nutritional value and health benefits, and currently, its popularity is increasing. Therefore, it is relevant to consider its nutritional aspects to better utilise birch sap as beverage or as an ingredient in foods. Several studies have described the mineral or carbohydrate composition of birch sap, however, information regarding the composition of proteins, substances with biological activity, is scarce. Moreover, whether and how protein and microbiota composition is affected by harvest day and weather conditions is unknown. The present study is the first report of an in-depth characterisation of the microbiota and protein composition, as well as shelf life of birch sap from *B. pendula* in a complete tapping season.

Weather conditions during the tapping season in 2018 seemed to have a major effect on the microbiota, according to the strong

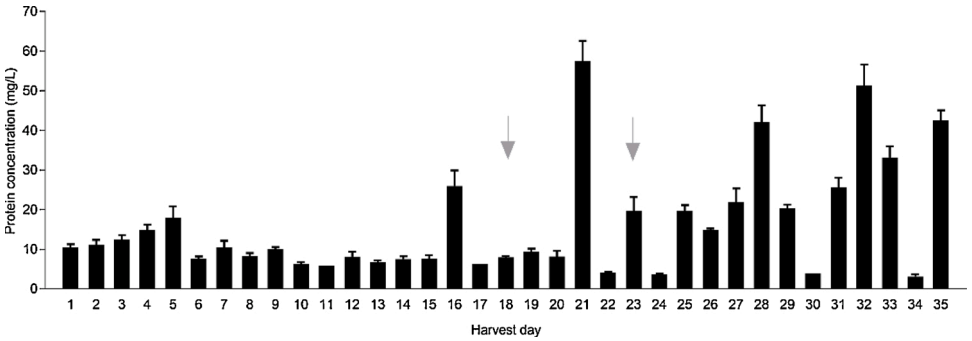

Fig. 4. (A) Protein concentration (mg/L) in birch sap harvested each day for a period of 35 consecutive days, the complete 2018 tapping season, after freezing at −20 °C and defrosting at 4 °C. Arrows indicate change of tap hole.

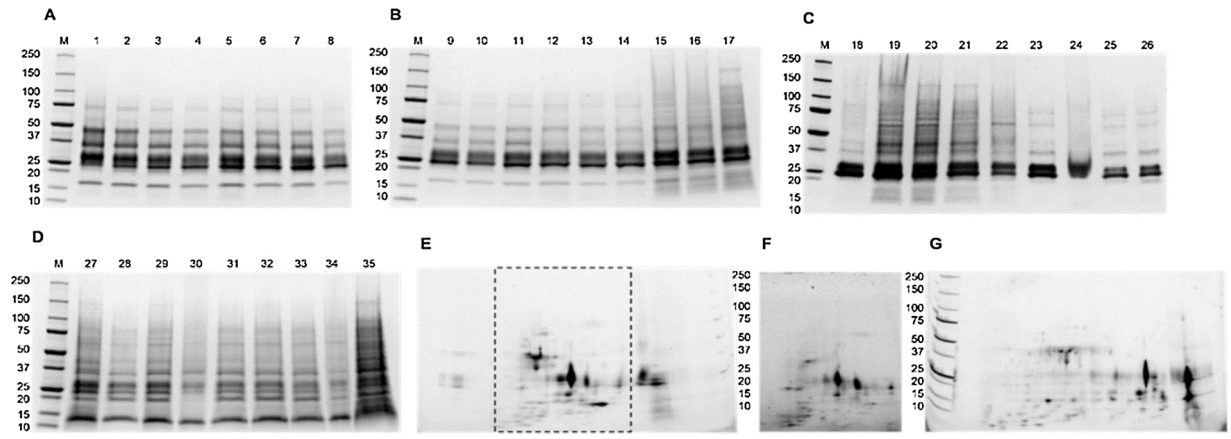

Fig. 5. Electrophoretic analysis of proteins from birch sap frozen at −20 °C and defrosted at 4 °C. (A–D) SDS-PAGE analysis of birch sap harvested each day for a period of 35 days, the complete 2018 tapping season, and visualised by Coomassie staining. (A) lane 1: MW marker, lane 2-9: day 1-8. (B) lane 1: MW marker, lane 2-10: day 9-17. (C) lane 1: MW marker, lane 2-10: day 18-26. (D) lane 1: MW marker, lane 2-10: day 27-35. (E–G) 2D gel electrophoresis analysis of proteins from birch sap harvested on day 7 (E, G) or day 28 (F) of the 2018 season. (E) Box area indicates region from day 7 chosen for comparison to birch sap harvested on day 28 of the 2018 season (F). Proteins were separated using 7 cm linear isoelectric focusing (IEF) immobilised pH gradient (IPG) strips with an IEF gradient pH 3–10 (E, F) or pH 4–7 (G) for the first dimension, and with 4–20% Mini-Protean TGX stain-free protein gels for the second dimension.

Table 4
List of identified proteins in birch sap harvested from one tree on day 7 of the 2018 tapping season by LC–MS/MS and *in silico* analyses.

Protein name	Organism	Accession number	Coverage (%)	Peptides No.	Unique Peptides No.	Length (calc.) (aa)	MW (calc.) (kDa)	pI (calc.)	PEP Score	Score Sequest HT	Function
Beta-galactosidase	*Betula platyphylla*	A0A4D6C821	44	22	22	836	92.0	6.90	109.58	171.47	CWM
Germin-like protein 1 (Fragment)	*Betula pendula*	P85336	100	1	1	13	1.3	9.99	2.49	7.11	PD
Germin-like protein 2 (Fragment)	*Betula pendula*	P85352	100	1	1	14	1.6	4.50	5.07	8.41	PD
Glutathione reductase	*Betula pendula*	Q9M3T6	15	3	3	358	38.9	6.34	6.59	9.50	OS
Pectin lyase-like protein	*Betula platyphylla*	A0A5B9FYX5	7	2	2	513	54.6	6.62	4.22	4.69	CWM
Peroxidase	*Betula platyphylla*	A0A0H4CPS8	64	20	20	321	34.8	5.57	101.94	210.23	OS
Peroxidase	*Betula platyphylla*	A0A0H4CNW3	68	19	14	318	33.5	6.77	90.94	223.71	OS
Peroxidase	*Betula platyphylla*	A0A0H4CNW8	52	12	12	272	29.7	6.35	58.14	123.22	OS
Peroxidase	*Betula platyphylla*	A0A0H4CRM9	47	10	7	322	33.8	8.24	50.16	100.64	OS
Peroxidase	*Betula platyphylla*	A0A0H4CRM3	23	7	2	569	59.8	5.08	24.61	89.01	OS
Peroxidase	*Betula platyphylla*	A0A0U2TQ78	21	4	4	315	3.4	6.40	10.08	22.86	OS
Peroxidase	*Betula platyphylla*	A0A0H4CVY2	3	1	1	326	35.6	8.41	1.17	2.40	OS
Peroxidase 3 (Fragment)	*Betula pendula*	P85334	100	1	1	19	2.2	4.50	7.71	16.20	OS
Polcalcin	*Betula pendula*	Q39419	36	3	3	85	9.4	4.94			CB
PR protein	*Betula pendula*	P43176	21	1	1	160	17.5	5.87			PD
PR protein 1 (Fragment)	*Betula pendula*	Q9M4Y4	11	1	1	102	10.8	6.51	4.21	7.74	PD
PR protein 1 (Fragment)	*Betula pendula*	Q9M3T1	56	1	1	57	6.0	5.17	3.25	3.99	PD
PR protein 1	*Betula platyphylla*	A0A4D6C8J9	6	1	1	176	19.3	8.65	1.43	1.77	PD
Superoxide dismutase [Cu-Zn]	*Betula platyphylla*	A0A0H4CPR2	29	3	3	220	22.5	6.65	10.11	13.36	OS
Superoxide dismutase 1	*Betula platyphylla*	A0A0H4CNV4	17	2	2	157	15.9	6.51	5.93	16.05	OS

PR: Pathogenesis-related; CWM: cell wall metabolism; CB: calcium binding; OS: oxidative stress; PD: pathogen defence. No.: number.

correlation observed between bacteria, yeast and fungi counts and daily temperature. It might also explain the changes in microbiota levels throughout the season. The temperature had a larger impact on the microbiota than did the amount of rain or hours of sunshine and should be considered when choosing harvest day. However, our study could not establish any correlation between protein concentration and the daily weather conditions that could explain the changes in protein concentration throughout the season. To our knowledge, there are no studies relating temperature measurements with either microbiota or protein concentration or composition.

Our observation of the initial count of the microbiota in birch sap harvested daily in the 2018 tapping season, ranging from 0.6 log(CFU/mL) to 5.7 log(CFU/mL), is in line with another study where the initial microbiota count was 3.5 log(CFU/mL) from the same birch species in Latvia, although the specific harvest day was not noted (Nikolajeva and Zommere, 2018). Due to the transparency of the birch sap, turbidity is often used as a measure of the microbiota content instead of growth on general agar media (Bilek et al., 2016, 2018). In our study, visual changes around the tap hole and unclear sap led to a change of the hole twice, as it could be indication of microbial contamination, and indeed it correlated with high counts of total microbiota and protein concentrations on both days. A tap hole contamination study during harvest of maple sap also showed that the microbiota contamination was low in the beginning of the tapping season and higher in the end (Lagacé et al., 2004). Changing the tap hole is a strategy that seems to have an influence on the microbiota level since a decrease in counts was observed upon a change. However, the bacterial count did not reach the same low levels as observed in the beginning of the season, probably related to the increase in air temperature observed throughout the season. These observations suggest that frequent changes of the tap hole could minimise the level of the initial microbiota count and thereby prolong the shelf life, and thus, the implications of good practice during the tapping procedure.

Shelf life of birch sap at 4 °C depended on the initial count of microbiota and most likely also the type of microbiota present. We observed a predominant presence of bacteria compared to yeast and fungi, similar to what has been seen in other products such as maple sap (Lagacé et al., 2002). Thus, the low microbiota growth in birch sap harvested in the beginning of the season and stored at 4 °C might be due to the low initial microbiota level and the presence of mesophilic genera such as *Kocuria*, *Micrococcus* and *Staphylococcus* (Vos et al., 2009; Whitman et al., 2009). In contrast, the shorter shelf life observed for samples harvested at the end of the season may be due to an initial microbial count above 5 log(CFU/mL) and the presence of different psychrotrophic *Pseudomonas* species. A shelf life of three to four days for birch sap was confirmed by other studies (Bilek et al., 2016; Nikolajeva and Zommere, 2018). However in these studies, the identified microbiota differed by the absence of *Pseudomonas* species, while other products such as maple sap was shown to contain this species (Lagacé et al., 2004). Yet, it should be noted that the identified bacteria species depend very much on selection strategy and in this study, colonies to be identified were randomly selected. *Pseudomonas* species is generally considered as a food spoilage bacteria and can dominate and outgrow other bacteria at low temperatures (Nikolajeva and Zommere, 2018) and while a count of more than 7 log(CFU/mL) might be safe to ingest it might be organoleptic unacceptable for the consumer.

Since fresh birch sap has a shelf life of three to four days at 4 °C, it is often preserved by freezing (Li and Gao, 1995). In our study, freezing of birch sap for two weeks did not noticeable change the shelf life at subsequent storage at 4 °C. Prolonged freezing might have an impact on the cells so they are not only reduced in numbers but also damaged by the freezing process and thereby requiring time to recover before they can grow (Ray and Speck, 1973). A shelf life of 15–20 days after storage at 4 °C, was observed for defrosted Korean *B. platyphylla* sap where the microbiota counts only increased 1 log unit (Jeong et al., 2013). However, enumeration of the microbiota was performed on agar plates incubated at 37 °C for 24 h, which might reduce the number of psychotropic bacteria.

With values between 3 and 60 mg/L, the concentration of proteins in birch sap from *B. pendula* harvested daily throughout the season, was in line with other studies reporting concentrations of 15 mg/L in Japan in 1998 (Jiang et al., 2001), 12.9 mg/L in Latvia in 2018 (Nikolajeva and Zommere, 2018), or 40 mg/mL and 70 mg/L, respectively, from an average of several *B. pendula* trees in two different locations in Finland in 1995 (Kallio et al., 1995). Higher protein concentrations have been described in other studies, for instance, a mean value of 127 mg/L in birch sap harvested in Latvia in the first week of the tapping season in 2010 (Kūka et al., 2013) or 277 mg/L in birch sap harvested from four to five trees grown at three different locations in Poland at the end of March and at the beginning of April 2015 (Grabek-Lejko et al., 2017). The protein concentration of the xylem from other plants such as broccoli, oilseed rape, pumpkin and cucumber is between 50–100 g/L (Buhtz et al., 2004) whereas it has been shown to be 300 g/L and 100 g/L of apple and pear xylem saps, respectively (Biles and Abeles, 1991), and hence much higher than that of birch sap.

The fivefold increase in the protein content from the beginning to the end of the season observed in the present study was higher than the twofold increase previously reported for several *Betula* species, i.e. *B. pendula, B pubescens*, *B. platyphylla* variety *japonica* and *B. verrucosa* (Jiang et al., 2001; Kallio et al., 1995). This increase during the tapping season might be related to higher nitrogen concentration in the xylem sap by the end of the season as previously reported (Kallio et al., 1995). The nitrogen reserves of plants are located in organelles named protein bodies used to produce proteins. During dormancy, this is the major source of nitrogen which is mobilised into the xylem during spring. The xylem, then, distributes the nitrogen compounds from the roots to the shoots, which might explain the availability of proteins at the end of the harvest period (Sauter and van Cleve, 1992).

The protein profile also changed throughout the 2018 season, thus not only the concentration but also the composition was affected by the specific harvesting time. Whereas the major changes of the protein profile occurred in the low sizes region, the most abundant proteins seemed to be steady across the complete tapping season, a pattern also observed by Kallio et al. (1995). Whether the changes observed are a result of protein proteolysis or a result of new proteins expressed during the season, requires further investigations.

To our knowledge, only one study has previously identified proteins in birch sap but no information on the sequences was available (Bilek et al., 2019). The 20 proteins identified in our samples are involved in the metabolism and functionality of plants, as well as in antifungal activities (e.g. pathogenesis-related proteins (PR)) and abiotic stress signals (e.g. peroxidases), emphasising a diverse role of sap in the defence of various stress factors (Dafoe and Constabel, 2009). This is in line with reports of the sap proteome in several other plant species such as maize, rice or poplar characterised by 2D electrophoresis, LC–MS/MS techniques and *in silico* analysis (Dafoe and Constabel, 2009; Rodríguez-Celma et al., 2016). When plants are exposed to biotic or abiotic stress conditions such as climate alterations, chemical pollution, wounding and pathogen attacks, cell responses are activated to combat those conditions. Exposure length, severity, plant tissue, age and genotype are some of the factors that influence the type of the plant responses which may manifest in minutes as in the case of air temperature stress (De Freitas Bueno et al., 2019). Peroxidases play a role in abiotic stress reactions by generating reactive oxygen species (ROS) that accumulate in the plant as a result of oxidative stress (Demidchik, 2015), however, peroxidases may have other biological functions such as lignin biosynthesis (Hiraga et al., 2001). The expression of proteins such as PR proteins gives the plant resistance against infection. PR proteins comprise 17 protein families according to their shared sequence homology that are ubiquitous in the plant kingdom and found in almost all plant compartments (Jain and Khurana, 2018). But up to date they have not been identified in sap. Germin and germin-like proteins are also

associated with defence against several biotic and abiotic stresses in some plant species (Ilyas et al., 2016). Expression of these proteins suggests an established defence system that creates a hostile environment to prevent pathogen infection.

5. Conclusion

In conclusion, we have provided essential information on key elements in birch sap by performing the first detailed study of the level and composition of the microbiota and protein of birch sap harvested daily throughout a whole tapping season. Our results indicated that the initial level of bacteria, yeast, fungi, and proteins was lower in the beginning of the tapping season compared to the end of the season. Not only the microbiota and protein level but also their composition changed throughout the season. The initial level of the microbiota as well as the bacteria species, harvest time and storage influenced the growth in birch sap and hence shelf life.

Freezing the birch sap for two weeks reduced the microbial level approximately 1 log units. However, no effect on the shelf life was observed since storage of fresh and defrosted birch sap at 4 °C for 7 days resulted in similar microbial load, with *Pseudomonas* being the main species in both saps. Finally, the best time to harvest birch sap due to a low microbiota load and optimal shelf life is the first 14 days of the season. Thus, the present study clearly demonstrates that the specific seasonal harvest day and temperature have a great impact on the microbiota level and composition, which influence the shelf life at subsequent storage at 4 °C.

Data availability

Data available on request from the authors.

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Authors contributions

All: All authors made substantial intellectual contributions, reviewed the manuscript critically, and approved the final version of the manuscript. **A.I.S**: conceived and conducted the 2D electrophoresis experiments and conceived the SDS-PAGE experiments, drafted the manuscript and prepared Figs. 4, 5, S2 and Tables 1, 4 and S1. **A.M.M**: conducted the MALDI-TOF experiments. **J.M.G.**: conducted some of the microbiology experiments. **K.L.B.**: conceived the study and revised the manuscript. **S.E.H.**: conducted the protein quantification and SDS-PAGE experiments. **T.B.**: conceived and conducted some of the microbiology experiments, reviewed the manuscript and prepared Figs. 1, 2, 3, S1 and Tables 2 and 3. **T.R.**: harvested and delivered the birch sap.

Declaration of Competing Interest

TR is the owner of Birkesaft.dk Aps. The rest of the authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2021.104347.

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