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Changes of physicochemical properties and predominant microbiota during storage of birch sap

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Abstract

Birch sap derived from silver birch Betula pendula is the most common spring sap. It is usually used as a fresh drink during the first days or as beverage during summer. It is important for it to be safe. The aim of this study was to identify predominant culturable microorganisms and changes in the composition of microbiota as well as some physicochemical parameters during storage. Fresh frozen sap gathered in Latvia was investigated. Defrosted sap underwent physicochemical and microbiological changes during two months' storage at temperatures of 4°C and 20°C. The pH value decreased from 5.52 to 3.41. Electrical conductivity was 0.453 mS cm^{-1} and protein content was 12.19 mg l^{-1} in the beginning and increased during storage. Total counts of colony-forming units (CFU) of aerobic bacteria ranged from log 3.5 in the fresh defrosted sap to log 11.3 after 38 days of storage at 4°C. The number of CFU of microorganisms showed a rapid increase during the first week of storage without a significant difference regardless of the temperature. Afterwards, the number of microorganisms tended to decrease at 20°C. The highest counts were detected after storage of the sap at 4°C. Predominant culturable bacteria belonged to the *Alpha-, Beta-*, and *Gamma-Proteobacteria*, to the *Actinobacteria, Flavobacteria* and *Sphingobacteria*. Indicators of faecal pollution were not found. Three species of yeasts and 14 species of bacteria were identified. Attention must be paid to the presence and growth of pathogenic bacteria and facultative pathogens.

Introduction

Spring sap, i.e. xylem sap of trees transfers nutrients from the roots to the opening buds. The sap contains carbohydrates, lipids and proteins as well as mineral substances (Harms and Sauter, 1992). The most common juice used since ancient times in Europe is derived from the silver birch *Betula pendula,* the downy birch *B. pubescens* and the Norway maple *Acer platanoides* (Svanberg *et al*., 2012). The sap is used as a fresh drink during the first days, but a range of fermented beverages is prepared for drinking during summer or the entire year. In this case, a variety of additives are added to the sap, such as rye, oats, rye flour, bread crust, dried fruits, black currant twigs, cloves, lemon or raisins. Semjonovs *et al.* (2014) studied opportunities to develop a probiotic-containing functional fermented beverage. Today, frozen sap and wine are also produced from the fresh sap. Birch sap is used for cosmetic applications (Svanberg *et al*., 2012) and in folk medicine against hepatitis, intestinal worms, scurvy (Brondegaard, 1979, quoted by Svanberg *et al*., 2012), colds, anaemia, constipation, fungal skin disease, abscess, herpes (Papp *et al*., 2014), rheumatism and infections, and as a diuretic (Peev *et al*., 2010) etc. Kūka *et al*. (2013) studied the chemical composition of birch sap gathered in Latvia. The predominant mineral substances were found to be calcium, potassium and sulphate ions. Their content is determined by the soil where the birch grows. The concentration of proteins, fructose, glucose and sucrose was 127 mg l^{-1}, 5.39 g/l, 4.46 g l^{-1} and 0.58 g l^{-1}, respectively.

Sap is an ideal growth medium for microorganisms because it contains enough available organic compounds and minerals. Therefore, birch sap quickly becomes colonised by microorganisms, especially yeasts and filamentous fungi. Most fungi survive in the soil or epiphytically on tree trunks (Weber, 2006). The presence and growth of microorganisms affect sap quality. Fresh birch sap for use without special processing can be stored for up to two months. For this purpose, the best temperature is from 0°C to 2°C. It is possible to increase the duration of storage by using a lower temperature, i.e. to -1.4°C because the freezing point of the sap is below that of water (Viškelis and Rubinskiene, 2012). Processing methods have not developed much over time as usually the sap is

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consumed in a short time after gathering. However, the food industry is increasingly being used for other types of processing instead of pasteurisation. In order to reduce this impact of living microorganisms, there are several techniques, such as different types of filtration, high pressure processing, ultrasound treatment, CO_2 treatment, UV treatment, pulsed light or pulsed electric field sterilization, ozone treatment etc. and combinations thereof (Beland and Barbeau, 2013). Microbiota inhabited birch sap has not been richly explored. There are no studies on the Latvian birch sap microbiota. The aim of this study was to identify predominant culturable microorganisms and changes in the composition of microbiota as well as some physicochemical parameters during storage of the sap at temperature of 4°C and 20°C.

Materials and Methods

Origin of birch sap

Non-commercial fresh frozen sap gathered from silver birch *Betula pendula* in Latvia and obtained from The Latvian Forestry Service („Latvijas Meža dienests" JSC) (Latvia) was investigated. Frozen sap was stored in 1 l polypropylene bottles at a temperature of -18°C and quickly thawed. Fifty milliliters of sap was poured into sterile glass bottles and stored at 4°C or 20°C in darkness for 58 days. Some parameters were compared with commercial bottled birch sap from Nordic Koivu Ltd. (Finland). According to the manufacturer's description, Nordic Koivu sap has not been heat-treated *(www.nordickoivu.com)* but the precise details have not been published.

Microbiological analyses

The number of culturable microorganisms was analysed by inoculation of sap samples in Petri plates with four microbiological media. In total, 0.1 ml of the serial dilutions of sap was plated on the following media: universal medium for bacteria R2A (Sifin, Germany) for aerobic bacterial plate count or total number of culturable bacteria; malt extract agar (ME, Biolife, Italy) for count of yeasts and filamentous fungi; MRS Agar with Tween 80 (Biolife, Italy) for lactic acid bacteria; *Acetobacter* medium (glucose 100 g l^{-1}, yeast extract 10 g l^{-1}, $CaCO_3$ 20 gl^{-1} and agar 20 g l^{-1}) for acetic acid bacteria, and Endo agar (Becton & Dickinson, France) for coliforms. Plates with Endo agar were incubated at 37°C for 24 h but the other plates were incubated at 20°C for 1-2 weeks. Bacteria with a clear area around colonies on the *Acetobacter* medium were considered as acetic acid bacteria. The tests were performed in duplicate. The number of microorganisms was expressed as logarithms of colony-forming units (CFU) per ml. Predominant culturable microorganisms were isolated from the highest dilutions of sap and purified. Identification of bacteria was performed with the BBL® Crystal™ Gram-positive ID kit and Enteric/ Nonfermenter ID kit (Becton & Dickinson, USA). Yeasts were identified using the API 20C Aux or API ID 32C (bioMerieux, France) kit.

Physical and chemical analyses

Electrical conductivity of the sap was determined with PWT HI98308 (Hanna Instruments, Mauritius). The pH value was measured with pH-meter AD-1405 (Adrona, Latvia). The browning index, turbidity, concentration of proteins and UV-spectrum (260 nm, 280 nm) were determined with spectrophotometer Ultrospec 3100 pro (Amersham Biosciences, UK). The browning index was measured at 420 nm, and turbidity at 560 nm (Jeong *et al*., 2013). The concentration of proteins was calculated according to Kalckar (1947): protein in mg $ml^{-1} = 1.45 \bullet A_{280} - 0.74 \bullet A_{260}$, where A_{280} and A_{260} are the absorbances of UV at 280 and 260 nm.

Statistical analyses

Analysis of variance and the Student t-test were used to test differences among groups. $P < 0.05$ was considered statistically significant. Program R.2.12.1. was used for Pearson correlation analyses.

Results and Discussion

Microbiota of birch sap

Birch sap microbiota has been little studied. According to our data, it consisted of bacteria and yeasts. They were also recovered from the defrosted sap. Species content underwent a change during the storage of the sap (Table 1). Fermentation of fresh sap always begins relatively quickly and the sap becomes leavened, but that does not mean that it can no longer be consumed. Our results demonstrate a rapid change of all of the investigated parameters during the first days of storage of the sap at 20°C as well as 4°C. Only bacterium *Burkholderia cepacia* was predominant both before and after the period of storage. *B. cepacia* is the name for a group of nine genetically distinct but closely related species that have useful properties in the natural environment as antagonists of plant pathogenic fungi and nematodes, as plant growth promoters and degradative agents of toxic substances but it is also a human opportunistic pathogen (Chiarini *et al*., 2006).

Birch sap is usually used as a drink, so it is important for it to be safe. It is not possible to obtain

Table 1. Predominant species isolated from fresh frozen birch sap immediately after defrosting and after 38 days of storage

Group	Class	Name	After defrosting	After storage
Yeasts	*Tremellomycetes*	*Cryptococcus laurentii*	X	
		Cryptococcus uniguttulatus	X	
	Urediniomycetes	*Rhodotorula mucilaginosa*	X	
Gram-positive bacteria	*Actinobacteria*	*Micrococcus luteus*		X
	Bacilli	*Bacillus cereus*		X
		Brevibacillus brevis		X
		Paenibacillus macerans		X
Gram-negative bacteria	*Alphaproteobacteria*	*Brevundimonas vesicularis*		X
		Sphingomonas paucimobilis	X	
	Betaproteobacteria	*Burkholderia cepacia*	X	X
	Gammaproteobac-teria	*Aeromonas hydrophila*	X	
		Kluyvera ascorbata		X
		Kluyvera cryocrescens		X
		Pantoea agglomerans		X
		Pseudomonas luteola		X
	Flavobacteria	*Chryseobacterium indologenes*	X	
	Sphingobacteria	*Sphingobacterium multivorum*	X	

sterile sap during gathering. Therefore, growth of autochthonous microorganisms determines the change of sap quality and shelf life. Microbial contamination is a significant aspect that affects sap quality (Filteau *et al.*, 2012). Legislative acts do not define the quality requirements of birch sap. However, the manufacturer is responsible for sap quality and safety (for example, Republic of Latvia Cabinet Regulation No 499 "Hygiene Requirements for the Primary Production of Products of Plant Origin and Direct Supply in Small Quantities to a Final Consumer"). Ciekure *et al*. (2015) compared the microbial count in birch sap with drinking water standards (Republic of Latvia Cabinet Regulation No. 235 "Mandatory Safety and Quality Requirements for Drinking Water, and the Procedures for Monitoring and Control thereof") and showed that the mean CFU of microorganisms of birch sap was higher than legally allowed for drinking water in Latvia. They also found high counts of coliform bacteria which points to the possible contamination by the environment or humans. Although we did not find coliforms or other indicators of faecal pollution, the total count of aerobic bacterial CFU ml^{-1} ranged from log 3.5 in the fresh defrosted sap to log 11.3 after 38 days of storage at 4°C (Figure 1). At the same time and under the same conditions, the count reached log 10.3 and log 10.7 for acetic acid bacteria and yeasts, respectively. Storage duration had a greater impact on the microbial concentration than storage temperature.

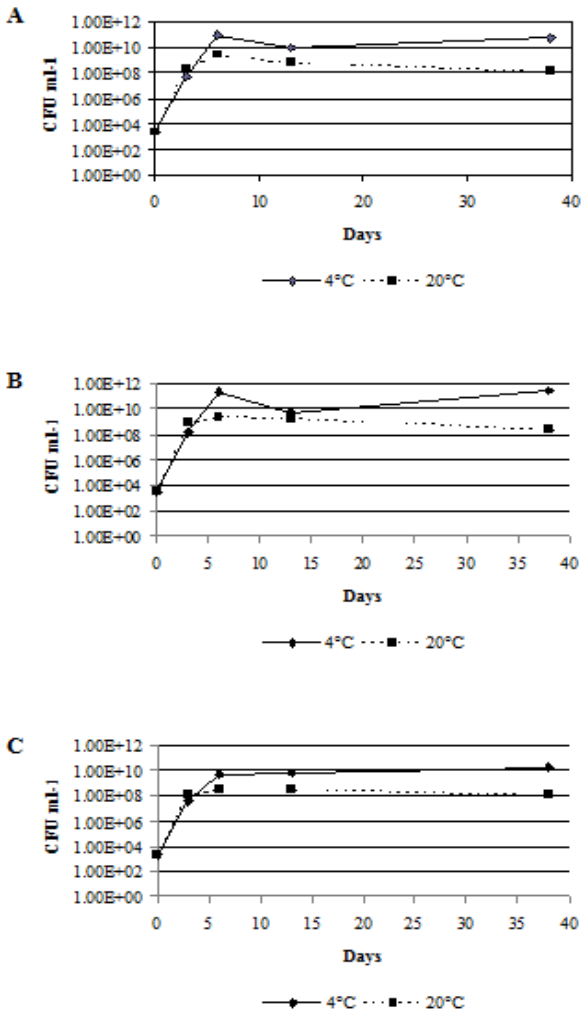

Figure 1. Number of CFU of yeasts (A), aerobic bacterial plate count (B) and acetic acid bacteria (C) during 38 days of storage of the sap at 4°C and 20°C, CFU ml^{-1}

Predominant microorganisms were isolated in pure cultures from fresh frozen birch sap immediately after defrosting and from the sap that had been stored for 38 days. Species identification was conducted with commercial biochemical kits. Three species of yeasts and 14 species of bacteria were identified (Table 1). Filamentous fungi were not found. The bacteria belonged to the *Alpha-, Beta-,* and *Gamma-Proteobacteria,* to the *Actinobacteria, Flavobacteria* and *Sphingobacteria.* Representatives of *Bacilli (Bacillus cereus, Brevibacillus brevis* and *Paenibacillus macerans*) were in a predominant position only in the stored sap. Attention must be paid to heat-stable emetic toxin and heat-labile diarrheagenic enterotoxins producing and heat-resistant endospore forming food pathogen *B. cereus* (Todar, 2012). Bacterium *Aeromonas hydrophila* which is common in water may also cause gastroenteritis (Janda and Abbott, 2010). The number of predominant *Gamma-Proteobacteria* also increased during storage. 57% of the isolated bacterial species

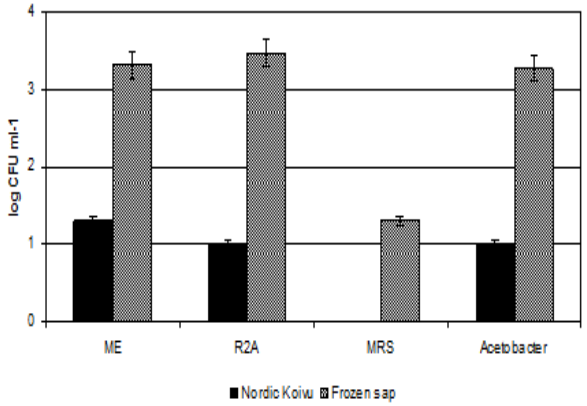

Figure 2. The number of microorganisms in bottled birch sap from Nordic Koivu and in frozen sap after defrosting: log CFU ml^{-1}. ME – medium for fungi; R2A – medium for aerobic bacterial plate count; MRS – medium for lactic acid bacteria; *Acetobacter* – medium for acetic acid bacteria

(*Brevundimonas vesicularis, Burkholderia cepacia, Chryseobacterium indologenes, Micrococcus luteus, Pseudomonas luteola, Sphingobacterium multivorum, Sphingomonas paucimobilis*) as well as yeast *Cryptococcus laurentii* synthesized yellow pigments. Colonies of P*antoea agglomerans* became blue during growth at 20°C which coincides with the observations of Fujikawa and Akimoto (2011). Yeast *Rhodotorula mucilaginosa* formed pink coloured colonies. Proliferation of non-pathogenic yellow pigment-producing microorganisms and pink-pigmented yeasts, which was richly represented in our sap, can change the colour of the sap. All three predominant yeast species belong to the basidiomycetous yeasts. Yeasts C*ryptococcus, Metschnikowia, Candida* and *Aureobasidium* species, *Nadsonia fulvescens, Guehomyces pullulans* and *Xanthophyllomyces dendrorhous* have been found in birch sap in other studies (Weber, 2006). We did not provide experiments with isolated pure cultures but it is ascertained that species of *Aeromonas hydrophila* (Rouf and Rigney, 1971), *Bacillus cereus* (Valero *et al*., 2003), *Kluyvera ascorbata, K. cryocrescens* (Farmer 3rd *et al.*, 1981), *Pantoea agglomerans* (Fujikawa and Akimoto, 2011), *Pseudomonas luteola* (homotypic synonym *Chryseomonas luteola*; Laurent *et al*., 2000) are psychrophilic or psychrotrophic or contain such strains. This explains the high concentration of microorganisms in the cold stored sap. All the above-mentioned species, with the exception of *Aeromonas hydrophila*, were isolated from birch sap after 38 days of storage. In order to ensure microbiological safety of birch sap, we recommend sterilization of the sap by filtration through membrane filters with pore diameter of 0.22

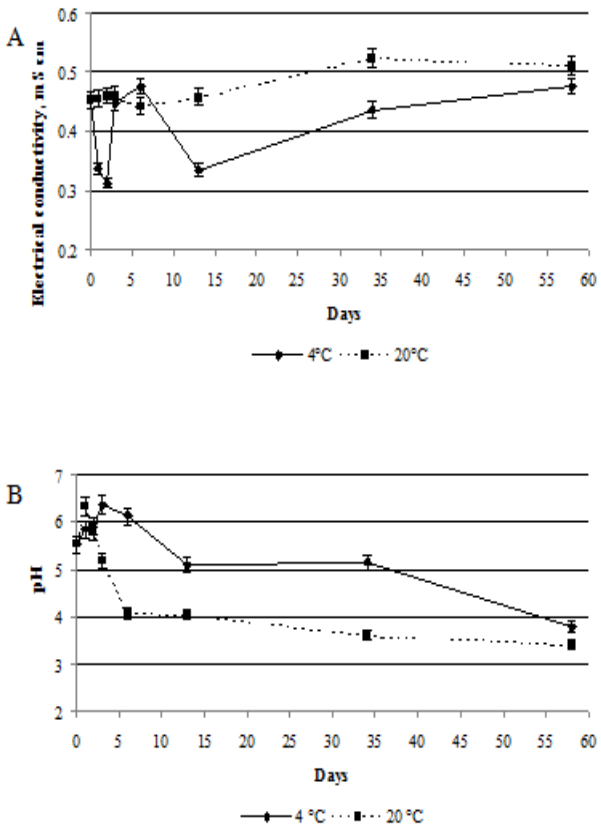

Figure 3. Changes of electrical conductivity (A) and pH (B) during storage of the sap at 4°C and 20°C

µm as one of the simplest methods.

Physicochemical properties and microbiological assessment of birch sap

Both investigated birch sap samples (from Latvia and Finland) had zero turbidity and browning index. The pH value, electrical conductivity and protein content was from 5.52, 0.453 mS cm^{-1} and 12.19 mg l^{-1} to 5.98, 0.487 mS cm^{-1} and 26.67 mg l^{-1}, respectively, lower in fresh frozen sap (Latvia) than in bottled sap (Finland). It is known that the initial content of proteins depends on the time of gathering. At the end of the flow period it may even be twice as big as at the beginning (Jiang *et al*., 2001).

Frozen sap after thawing contained considerably more live microorganisms from all of the investigated groups than bottled sap (Figure 2). There were 10 and 2900 bacterial CFU ml^{-1} and 20 and 2060 yeast CFU ml^{-1}, respectively, in bottled sap and in frozen sap. The smallest portion of the microorganisms, i.e. 20 CFU ml^{-1} from the frozen sap grew in the MRS. Microscopy showed no yeast cells, thus indicating the presence of lactic acid bacteria. 1856 CFU ml^{-1} from the frozen sap grew in the Acetobacter medium. Acetic acid bacteria represented 64% and 100% of total number of bacteria cultivated in the universal medium R2A in frozen sap and in bottled sap,

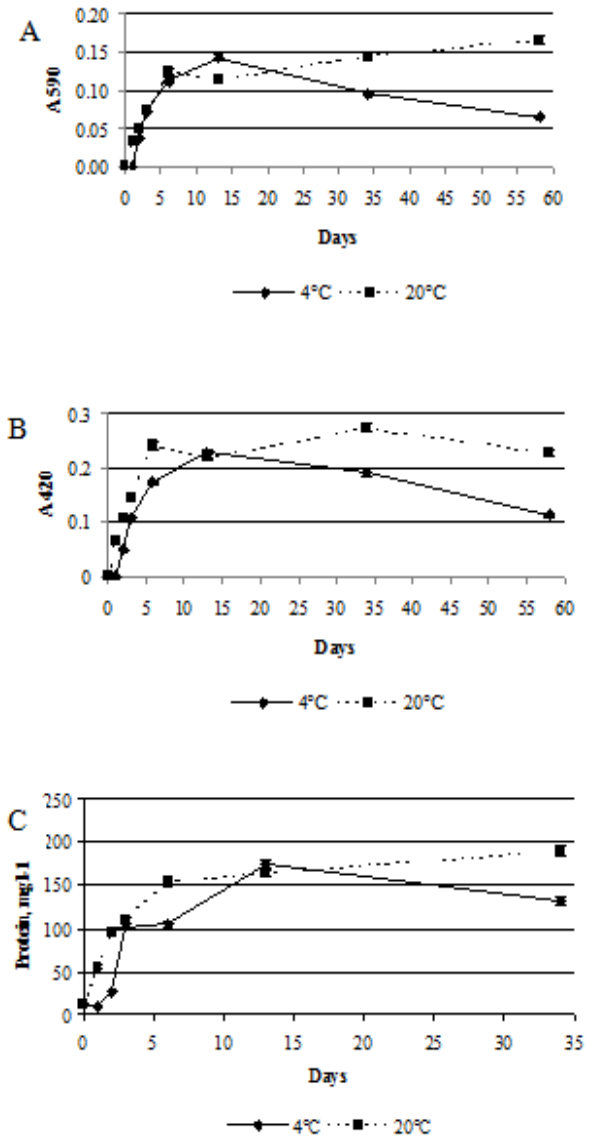

Figure 4. Changes of turbidity (A_{590} nm) (A), browning index (A_{420} nm) (B) and protein content (C) during storage of the sap at 4°C and 20°C

respectively.

Changes during storage of birch sap

Frozen sap after thawing was stored for 58 days at a temperature of 4°C or 20°C. Physical, chemical and microbiological analyses were performed during this period. Similar trends were found regarding the dynamics of electrical conductivity, turbidity, browning index and protein content, which increased faster and reached higher levels at 20°C than at 4°C. Electrical conductivity changed from initial 0.453 mS cm^{-1} to 0.524 mS cm^{-1} (Figure 3A). The increase had a uniform trend at 20°C and fluctuations during the first two weeks during storage of sap at 4°C. pH value decreased during two months to 3.79 and 3.41 at 4°C and 20°C, respectively, and this process was faster at 20°C (Figure 3B). Similar values (~3.4 and 3.95 after 40 days of storage at 25°C and 4°C) were detected in the sap of white birch Betula platyphylla (Jeong *et al*., 2013). Values of turbidity and browning showed a fast increase during the first two weeks (Figure 4). Turbidity and browning continued to slowly increase at 20°C whereas a gradual decrease of both values was detected at 4°C. Whereas Jeong *et al*. (2013) demonstrated a gradual increase of the browning index and turbidity during at least 40 days. A strong positive linear relationship was observed between these parameters with Pearson's correlation coefficient $r = 0.9698$ ($R^2 = 0.9405$ and $p < 0.00001$). Protein content in the sap increased rapidly from initial 12.19 mg l^{-1} to 173.82 mg l^{-1} after 13 days of storage at 4°C and a further increase was detected at 20°C (Figure 3C). A strong positive correlation was observed ($r = 0.9638$, $R^2 = 0.9289$, $p < 0.0001$) between protein content and turbidity of the sap. Turbidity refers to the concentration of microorganisms but correlation with protein content indicates microbial origin of the protein.

The number of yeasts (Figure 4A), aerobic bacterial plate count (Figure 1B) and number of acetic acid bacteria (Figure 1C) increased in the sap during the first week of storage at both temperature rates. Afterwards, the number of yeasts, aerobic plate count and acetic acid bacteria tended to decrease at 20°C which differs from data provided by Jeong *et al*. (2013) where no decrease was found during 40 days. A significant difference ($p < 0.05$) between concentrations of microbial CFU was detected. The refrigerator temperature (4°C) supported the growth of all groups of microorganisms better than room temperature (20°C) and the number of CFU ml^{-1} reached $7x10^9$ for yeasts, $2x10^{11}$ for total count of aerobic bacteria including $9x10^9$ acetic acid bacteria. The maximum of $4x10^9$ CFU ml^{-1} at room temperature was reached by aerobic bacterial plate count. The number of lactic acid bacteria did not exceed 170 CFU ml^{-1} at 20°C, which was detected after six days of storage or 80 CFU ml^{-1} at 4°C after three days of storage. From other parameters, only the browning index began to decrease after 35 days of storage at 20°C. In our experiments, the highest counts of microorganisms were detected after storage of the sap at 4°C. We think that this is due to the presence of psychrophilic microorganisms in the birch sap because indigenous microbial populations of temperate climate zone contain a lot of species with psychrophilic or psychrotrophic properties.

Conclusion

Defrosted birch sap underwent physicochemical and microbiological changes during two months' storage at temperatures of 4°C and 20°C. Similar trends were found regarding the dynamics of electrical conductivity, turbidity, browning index and protein content, which increased faster and reached higher levels at 20°C than at 4°C. PH value decreased faster at 20°C than at 4°C reaching 3.41 and 3.79 respectively after 58 days. Electrical conductivity increased during storage. Values of turbidity, browning and protein content showed a fast increase during the first two weeks. Afterwards, these parameters continued to slowly increase at 20°C whereas a gradual decrease was detected at 4°C. The number of CFU of microorganisms showed a rapid increase during the first week of storage without a significant difference regardless of the temperature. Afterwards, the number of microorganisms tended to decrease at 20°C. The highest counts were detected after storage of the sap at 4°C. Predominant culturable bacteria belonged to the *Alpha-, Beta-,* and *Gamma-Proteobacteria,* to the *Actinobacteria, Flavobacteria* and *Sphingobacteria*. Indicators of faecal pollution were not found. Three species of yeasts and 14 species of bacteria were identified. *Burkholderia cepacia* was recovered as predominant both before and after storage. The number of CFU of toxins-producing species *Bacillus cereus* as well as non-pathogenic *Bacilli* and *Gamma-Proteobacteria* increased during storage. Although legislative acts do not define the quality requirements for birch sap, it is important for it to be safe. Attention must be paid to the presence and growth of pathogenic bacteria and facultative pathogens.

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