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ORIGINAL PAPER

Reduction of malic acid in bilberry juice by *Lactiplantibacillus plantarum*-mediated malolactic fermentation

Martina K. Bergentall[1] · Loredana Malafronte[1,2] · Dorine As[1,3] · Emeline Calmet[1,4] · Petter Melin[5]

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Abstract

Bilberries (*Vaccinium myrtillus*) are the most common wild berries in Northern Europe. A substantial amount of the berries are picked with the objective to extract highly valued products such as anthocyanins. A smaller amount of the bilberries is used to make jams and drinks, and these are generally restricted to the domestic market. One reason is the sour taste, partly as a result of the high content of malic acid. By using certain strains of lactic acid bacteria with the ability to convert malic acid to lactic acid, the taste is predicted to be more pleasant. This process is called malolactic fermentation, and historically it has mostly been used in winemaking. After testing five different starter cultures, we identified that the strain, *Lactiplantibacillus plantarum* LP58, can rapidly convert malic acid to lactic acid without any loss of sugar or citric acid, which strongly indicates a successful malolactic acid fermentation. As it has been reported that other strains of *L. plantarum* can be used as biopreservative agents, the resulting product was also tested in terms of microbial safety after prolonged storage, and by means of metagenome sequencing. The obtained product was quite tolerant to microbial growth, but this observation was rather due to an initial heat treatment than the addition of lactobacilli. Potentially, starter cultures with documented biopreservative activity can be combined with *L. plantarum* LP58 to obtain a more stable product. Until then, the fermented bilberry juice must be processed and preserved like non-fermented bilberry products.

Keywords Bilberries · Lactic acid bacteria · Malolactic fermentation · Shelf life · Metagenome sequencing

Introduction

In Sweden, bilberries, or European blueberry (*Vaccinium myrtillus*), are the most naturally abundant wild berry species. It covers 11% of the Swedish forests with a natural production of more than 400,000 tons per year. Most of the bilberries harvested in Sweden are usually cleaned and frozen after picking. Subsequently, they are exported and used for extraction of high valued bio-products like anthocyanins from the skin and omega-3 oil from the seeds for health supplements and cosmetic products [1]. A smaller volume, limited to the domestic market, is used for berry-based food and drink products, e.g. jams, juice concentrates, soups, and dried berries [2]. To promote the use of Swedish bilberries as a food product globally, there is a need to design more sustainable processes and products that take advantage of the bilberry nutritional profile and limit waste. In this work, the focus is on non-alcoholic bilberry beverages that are naturally rich in sugars, but the sour taste can be one explanation why they are not widely used as beverages. Controlling the acidity of bilberry juice is a key for promoting consumption. The main substance that is responsible for the sour taste is malic acid [3]. Therefore, malolactic fermentation (MLF) can be a strategy for deacidification of the juice and improving its sensory acceptance. During this fermentation, L-malic acid is converted into equimolar amounts of L-lactic acid, which gives rise to a more pleasant taste since it is perceived as less acidic at invariant pH. Historically, MLF has been mostly studied in wine as secondary fermentation to modify the flavour properties and reduce sourness [4–6].

✉ Petter Melin
petter.melin@ri.se

1 Department of Agriculture and Food, RISE Research Institutes of Sweden, Göteborg, Sweden

2 Present Address: Pharmaceutical Technology and Development, Operations, AstraZeneca, Gothenburg, Sweden

3 Present Address: Hedelab, Mons, Belgium

4 Present Address: Fleury Michon, Pouzauges, France

5 Department of Agriculture and Food, RISE Research Institutes of Sweden, Uppsala, Sweden

Recently, this process has been getting more attention for the fermentation of berry juices including bilberries [3, 7, 8].

MLF is dependent on lactic acid bacteria (LAB) and there are several strains with reported activity, e.g. in the species of *Oenococcus oeni*, *Lactoplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Lactobacillus acidophilis*, *Pediococcus pentosaceus* and *Pediococcus acidilactici* [9–12]. *L. plantarum* is of particular interest because of its large diversity of enzymes, shorter incubation time, better conversion of malic acid into lactic acid and better aromatic profile given in wine and mulberry juice [7, 13, 14]. Furthermore, *L. plantarum* can improve the shelf life of fermented foods [15, 16] and thereby shows an interesting potential for fermenting berry juice. The mechanisms are not fully elucidated, but several *L. plantarum* strains have the capacity to produce antimicrobial substances, e.g. cyclic dipeptides and phenyllactic acid that have antifungal activities and thereby the potential to improve product shelf-life [17, 18].

The main goal of this work is to identify LAB strains that are able to perform malolactic fermentation in bilberry juices for exclusive and maximum malic acid reduction. In addition, we investigate the microbial community and the potential of improved product stability as a result of fermentation.

Materials and methods

Microorganisms

Frozen organic bilberries *(Vaccinium myrtillus)* were locally purchased and stored at −20 °C. The following bacterial starter cultures were obtained from SACCO S.R.L. (Italy): *Lactiplantibacillus plantarum* (LP58, LP01), *Pediococcus pentosaceus* (PP02), *Lactobacillus acidophilus* (LA03), *Pediococcus acidilactici* (PA09). All strains were supplied as freeze-dried products and stored at −20 °C until use.

Juice making

Berries were thawed overnight at 5 °C before pressing. Bilberry juice was prepared with a hydraulic press (Hafico, Germany) applying a pressure of 50 kg/cm^2 for 1 min, followed by a pressure of 100 kg/cm^2 for 1 min. Every pressing was performed with a load of 700 g of berries. The juice was filled in sterile bottles and kept at −20 °C for at least 24 h before fermentation.

Heat treatment and inoculation with starter cultures

Heat treatments of bilberry juices prior to fermentation were performed in 500 mL aliquots using a water bath (Julabo SW23) set at 65 °C and 50 rpm shaking speed. The temperature in the juice was monitored using a thermocouple (Armatherm GTH 1160 digital thermometer, Germany) and when the temperature of the juice reached 64.5 °C, the treatment continued for another 5 min, and the juice was cooled down quickly to room temperature for subsequent addition of starter cultures.

The starter cultures were rehydrated by resuspending approximately 1 g in 10 mL of sterile 0.5% NaCl water for 1 h before inoculation. Each sample consisting of 200 mL of bilberry juice was inoculated with approximately 7*10^7 CFU/mL LAB and incubated during 14 days at 25 °C, and for some samples, with an additional inoculation, also with 7*10^7 CFU/mL LAB, after 7 days. In addition, two different controls without inoculation were prepared, with and without heat treatment.

Determination of microbial counts and shelf life

During fermentation, viable LAB were estimated using De Man, Rogosa and Sharpe (MRS) agar supplemented with 1 mg/L Amphotericin B. Colonies on the MRS plates were enumerated after incubation under anaerobic conditions at 30 °C for 2–3 days. Yeast and mould growth was monitored using the selective dichloran rose bengal chloramphenicol (DRBC) media and counted after 5 days at 25 °C. Total aerobic count was monitored using Tryptic soy agar (TSA) plates and counted after 2–3 days at 30 °C. Using the same methodology, the effect of the different treatments on the shelf life of the juices after storage at 4 and 25 °C was examined. Samples were taken after 1, 2 and 3 months.

Nutritional quantities

During the malolactic fermentation process, L-malic acid and L-lactic acid concentrations were continuously measured using an absorbance detection method (L-Malic Acid Assay Kit K-LMAL-116A and L-Lactic Acid Assay Kit, K-LATE, Megazyme) according to the manufacturer's instructions. The juice samples were diluted ten times with deionized water before analysis. The absorbances were measured at 340 nm by a spectrophotometer (BECKMAN DU530 UV–Vis).

To monitor the sugar level throughout the MLF, a refractometer was used to monitor °Brix, where 1 degree Brix (°Bx) represents 1 g of sucrose in 100 g of solution [19]. The pH was monitored during the fermentation using a pH-meter (HI-8418 HANNA instruments). For a subset of samples, the quantities of organic acids and of individual sugars were analyzed externally at Eurofins Food & Feed Testing. The organic acids were analysed using HPLC (Eurofins, Cuneo, Italy), whereas the total sugar content was analysed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) according

to AOAC official method 982.14 (Eurofins, Lidköping, Sweden).

Metagenome sequencing

The following samples of bilberry juices were analysed with metagenomic sequencing: non-heat-treated juice before and after 14 days of incubation, and heat-treated juice inoculated with LP58 after 14 days (with and without reinoculation of LP58 at day 7). DNA was extracted from the remaining pellet after centrifugation of the juice samples at approximately 10 000*g* for 10 min. The pellet was washed in physiological saline solution and DNA extraction was performed using Invitrogen PureLink™ Microbiome DNA Purification Kit (ThermoFisher Scientific). The sequencing was performed externally by Eurofins Genomics including taxonomic profiling using Krona plots [20].

Results

Screening of bacterial strains

Bilberry juice was obtained by pressing frozen bilberries. Applied press conditions were based on previous experiences (unpublished data) and each batch containing 5.5 kg bilberries resulted in 3 L of bilberry juice. Five different starter-culture strains were added to heat-treated bilberry juice. During the fermentation, the pH, the concentrations of L-malic acid and L-lactic acid, and total sugar contents were frequently measured for up to 16 days. From these initial experiments, two *Lactiplantibacillus plantarum* strains (LP01 and LP58) were efficient in transforming L-malic to L-lactic acid without sugar consumption. The two *Pediococcus* strains, PA09 and PP02, and *Lactobacillus acidophilus* (LA03) were not able to perform MLF efficiently under the tested conditions. Examples of individual and valid fermentations with the different starter cultures are shown in Fig. 1a–e. The fermentations shown in Fig. 1 had stable °Brix and pH values during the fermentation and were between 12–13°Brix with a pH ranging from 2.9 to 3.1 (a typical pattern is shown in Fig. 1f). When comparing the results from the two *L. plantarum* strains, it was evident that LP58 was the most efficient starter culture with respect to successful acid conversion. Therefore, additional fermentations for subsequent experiments in this study were performed using the *L. plantarum* LP58 starter culture. To verify the necessity of heat treatment prior to addition, the *L. plantarum* LP58 was added to bilberry juice without an initial heat treatment. In this fermentation, the total level of sugar was reduced, similar to the non-heated control, and thereby considered unsuccessful (data not shown).

Nutritional analysis and conversion of juice fermented with *L. plantarum* LP58

To verify the MLF and to further elaborate on the change of nutrient composition obtained by fermentation with LP58, samples were externally analysed using chromatography-based methods rather than the enzymatic assays used previously. The results obtained were similar and are shown in Fig. 2. Besides conversion of malic and lactic acids, and sugars, the concentration of citric acid was intact during fermentation. Other measured sugars and organic acids were under the limit of detection and are thus not presented.

Additional fermentations to confirm the MLF potential of *L. plantarum* LP58 and to evaluate the conversion efficacy in different juice preparations

New fermentations, using juices from different batches were carried out using the best performing starter culture, *L plantarum* LP58. There were some differences compared to the initial screening experiments: The fermentations were always terminated after 14 days, and for some fermentations the starter culture was added again after 7 days. The reason for this reinoculation was to ensure that all malic acid was converted to lactic acid. Samples for pH and nutritional analyses were taken at fixed time points. Also, from the same fermentations, samples were taken for microbial counting during and after the fermentation, and for some samples, and time points, microbial DNA was extracted. In essence, results from the initial screening were confirmed, i.e. showing reduced concentration of malic acid and increased concentration of lactic acid, with a slight increase in conversion observed for the reinoculated samples (Fig. 3). The initial °Brix values varied between 7 and 13 between the samples. but remained constant during the fermentations, except for the non-heat-treated samples (sample 1 decreased with one °Brix during fermentation and sample 2 decreased with two °Brix). Notably, the four samples with lower starting L-malic acid (see Fig. 3a) concentration also had lower °Brix values (data not shown). The pH values differed between 2.6 and 3.1 for the samples and were stable during the 2-week fermentation for all samples except the non-heat-treated samples which decreased by 0.4 and 0.2 in samples 1 and 2, respectively (data not shown).

The efficacy of the L-malic conversion into L-lactic was calculated by dividing the L-lactic production (L-lactic acid content at after 13/14 days—L-lactic acid content from start) with the amount of L-malic acid degraded (L-malic acid from the beginning—L-malic acid content at 13/14 days). This was done with values from a total of eight individual fermentations with *L. plantarum* LP58, where two of them were derived from reinoculated samples (Table 1).

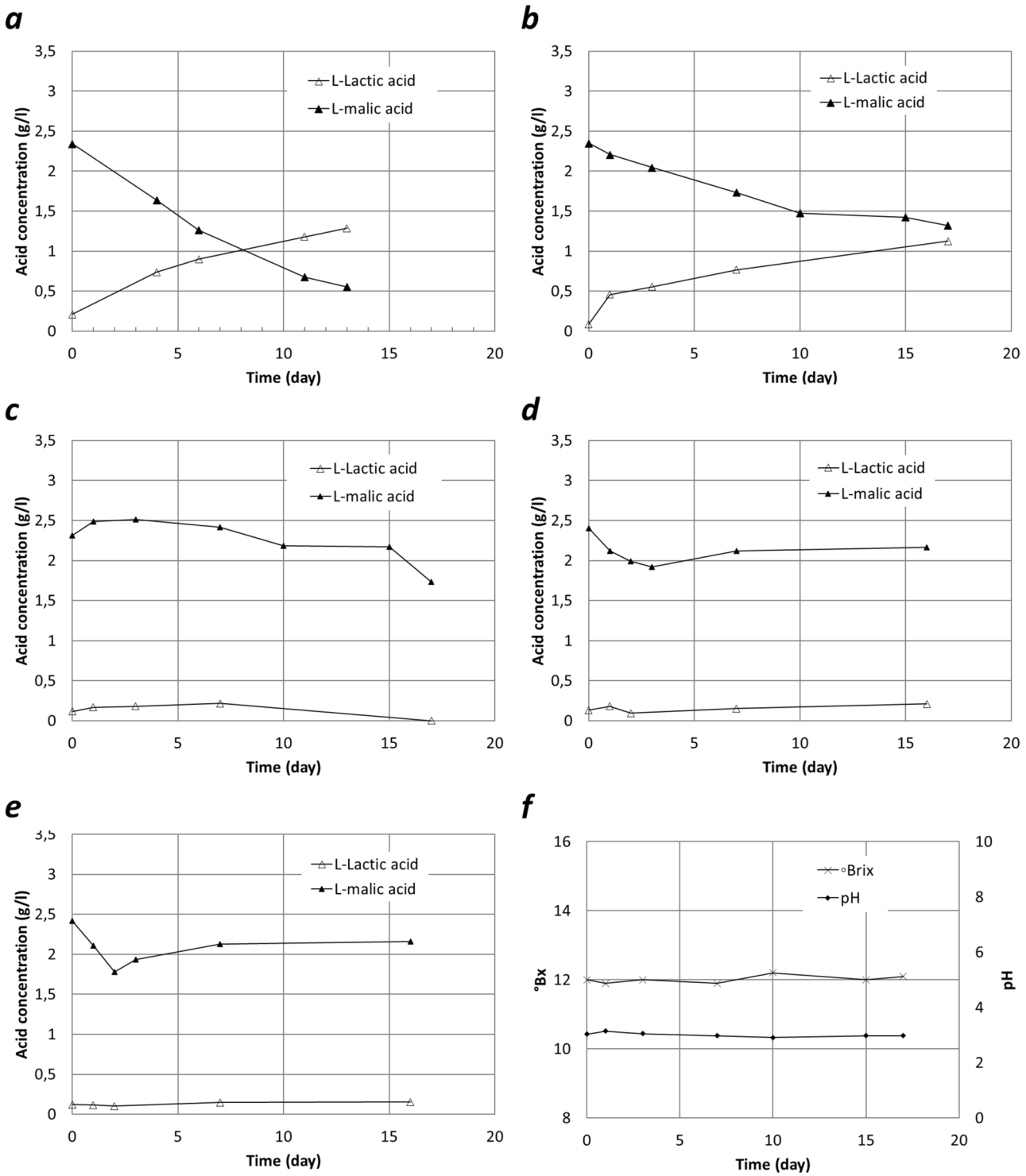

Fig. 1 Representative evolution of L-lactic acid and L-malic acid concentration for the different strains: *L. plantarum* LP58 (**a**), *L. plantarum* LP01 (**b**), *P. pentoaceus* PP02 (**c**), *L. acidophilus* LA03 (**d**) and *P. acidilactici* PA09 (**e**), and evolution of °Brix and pH for *L. plantarum* LP01 to demonstrate a standard °Brix and pH evolution in the fermentations (**f**)

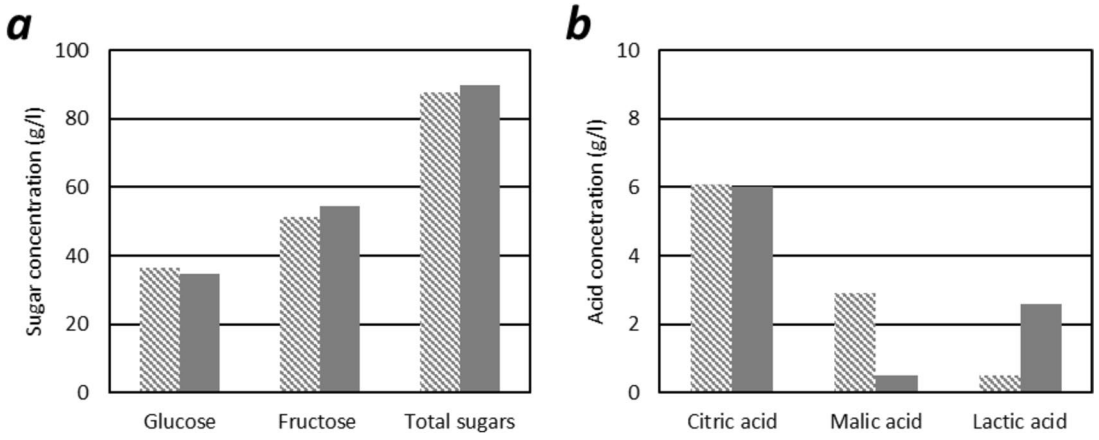

Fig. 2 Glucose, fructose and total sugar concentration before and after fermentation (**a**). Concentrations of citric, malic and lactic acid concentrations before and after fermentation analysed with HPLC (**b**). Striped bars represent heat-treated bilberry juice prior to fermentation, whereas dark grey bars represent bilberry juice after 13 days of inoculation with *L. plantarum* LP58

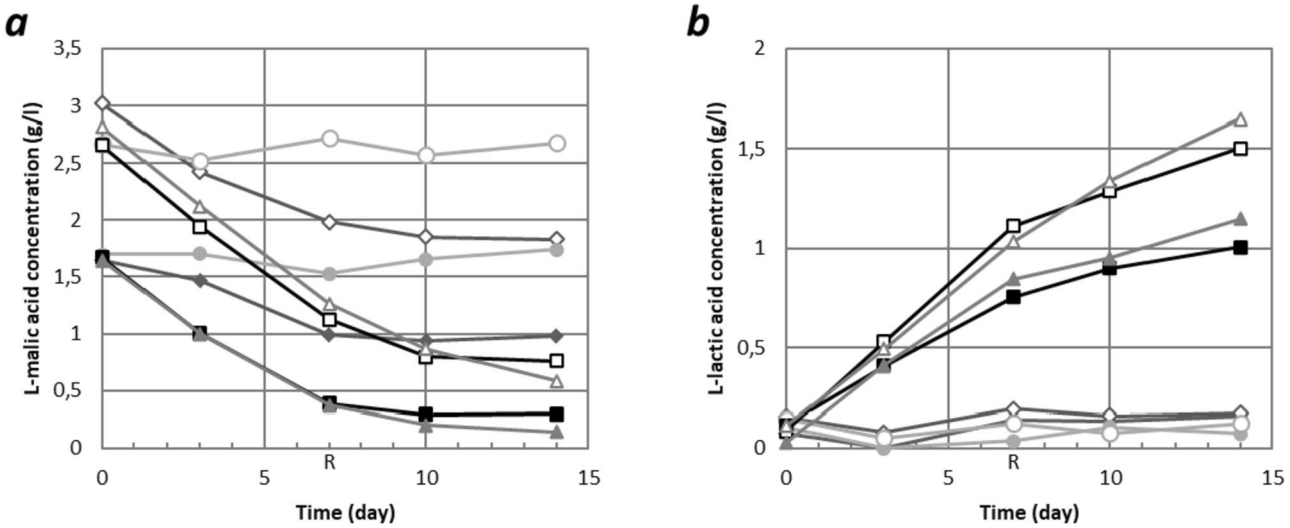

Fig. 3 Evolution of L-malic acid concentration (**a**) and L-lactic acid concentration (**b**) as a function of the fermentation time. Non-heat-treated controls (filled and non-filled rhomb), heat-treated controls (filled and non-filled circle), inoculated with *L. plantarum* LP58 (filled and non-filled square), and inoculated with *L. plantarum* LP58 and reinoculated after day 7 with the same strain (filled and non-filled triangle). Filled symbols indicate odd number samples and non-filled symbols indicate even number samples. *R* indicates the time of reinoculation for samples 7 and 8

Microbial flora during fermentation and effects of treatment on shelf life

To test the population dynamics of the starter culture and the potential of prolonged storage of the fermented juices, microbial contents were estimated during fermentation and after storage for up to 3 months. Traditional plating techniques were used on the same eight samples that previously were used to confirm the MLF. Estimated numbers of the starter culture are depicted in in Fig. 4. In the samples without addition of starter cultures, never more than one colony

Table 1 Conversion efficiency of *L. plantarum* LP58

Parameter	Conversion efficiency based on weight	Molar conversion efficiency[a]
Mean ($\bar{x}$) conversion efficacy	0.67	0.99
Standard deviation (s)	0.076	0.11
Standard error (s_x)	0.027	0.04

[a]Based on the molecular weights of L-lactic acid (90,08 g/mol) and L-malic acid (134.1 g/mol), respectively

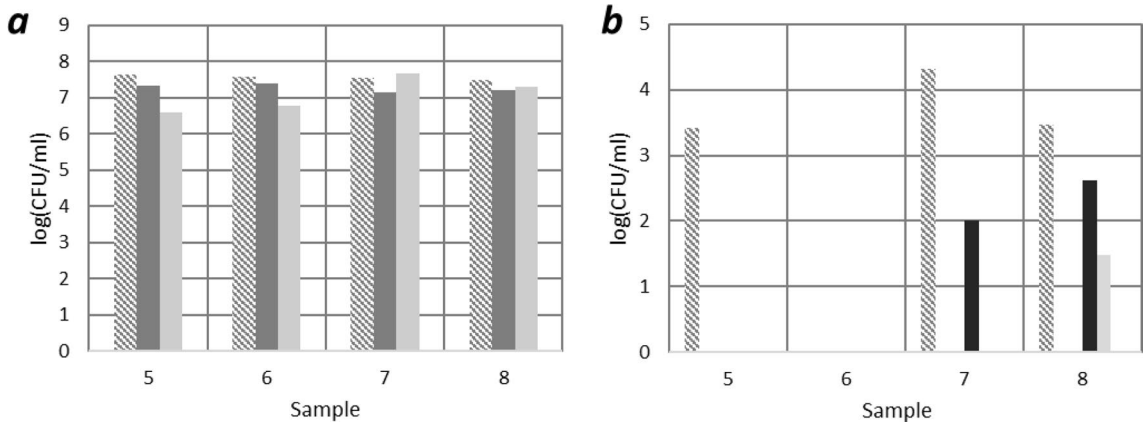

Fig. 4 Representation of the LAB evolution during fermentation (**a**). Striped bar on the day of inoculation, dark grey bar after 1 week (before reinoculation for sample 7–8) and light grey bar at the end of fermentation. Number of lactic acid bacteria after 1 month, 2 months and 3 months are shown at two different temperatures, 4 and 25 °C (**b**). The striped bar represents the number of LAB after 1 month at 25 °C, black bar represents LAB after 1 month at 4 °C and the light grey bar represents LAB after 2 months at 4 °C. As in Fig. 3, the sample numbers 5–6 refer to samples only inoculated at the start of the fermentation, while samples 7–8 also were reinoculated at day 7. Samples without bars are below limit of detection that were 1 CFU/mL (**a**) and 10 CFU/mL (**b**)

could be observed on MRS media in non-diluted samples (data not shown).

Besides population dynamics of the starter culture, the total growth of bacteria and fungi was estimated during fermentation and after storage (Fig. 5). When counting fungi (Fig. 5b, e, f), a noticeable observation was that yeasts were dominant in samples 1 and 2 (non-heat-treated controls), while moulds were dominant in all the other samples where fungal growth was observed (data not shown).

Fermentation effects on the microbial community

To obtain more insight into how fermentation affects the overall microbial community with focus on potential spoilage microorganisms, the bilberry juices were subjected to metagenome sequencing before and after fermentation. As a result of the DNA extraction protocol, it was not possible to perform any analyses on the thermal-treated control. Hence, results are restricted to the non-heated control before and after 14 days of incubation at 25 °C, and with samples with LP58 as starter culture after 14 days (with and without reinoculation of LP58 at day 7). Before heat treatment, the dominant microbe in the bilberry juice was *Frateuria aurantia*. After 14 days, it was still common but partly outcompeted by other bacteria. In the samples inoculated with starter culture, *L. plantarum* was totally dominant. To identify potential spoilage organisms, the species distributions were also analysed using Krona plots. For none of the four samples, no common spoilage bacterium or yeast was detected. For filamentous fungi, approximately 50 different species could be identified and among those were up to 35 Aspergilli, with little variation between samples. The two dominant fungi in all samples were *Aspergillus lentelus* and *Botrytis cinerea*.

A short summary of sequencing results is given in Table 2 and the sequencing reports can be found as supplementary data (technical report and Krona plots as separate HTML files).

Discussion

In the present work, several LAB starter cultures were evaluated in terms of MLF efficacy. Based on experimental observations, it is evident that the two *L. plantarum* strains were the most efficient of the tested starter cultures to perform MLF in bilberry juice. However, no firm conclusions can be drawn regarding why the other strains were not as efficient, at least not in the tested time frame of 2 weeks. It could be due to the inability to perform MLF under the tested conditions, or due to the bilberry juice not being a preferred substrate for these particular strains.

The superior strain with respect to the ability to perform MLF effectively was *L. plantarum* LP58, in which we could observe a molar conversion rate of malic acid to lactic acid close to 1.0 with no sugar consumption. Unless there is a degradation of more complex carbohydrates, not detected in the analysis, it can be concluded that all lactic acid molecules were derived from malic acid. This observation is further supported by the fact that citric acid is not consumed, as this is also a known metabolic pathway to synthesize lactic acid through the intermediate oxaloacetate [21]. This in in contrast to the reports by Viljakainen and Laakso where citric acid was consumed using *O. oeni* as starter culture, with

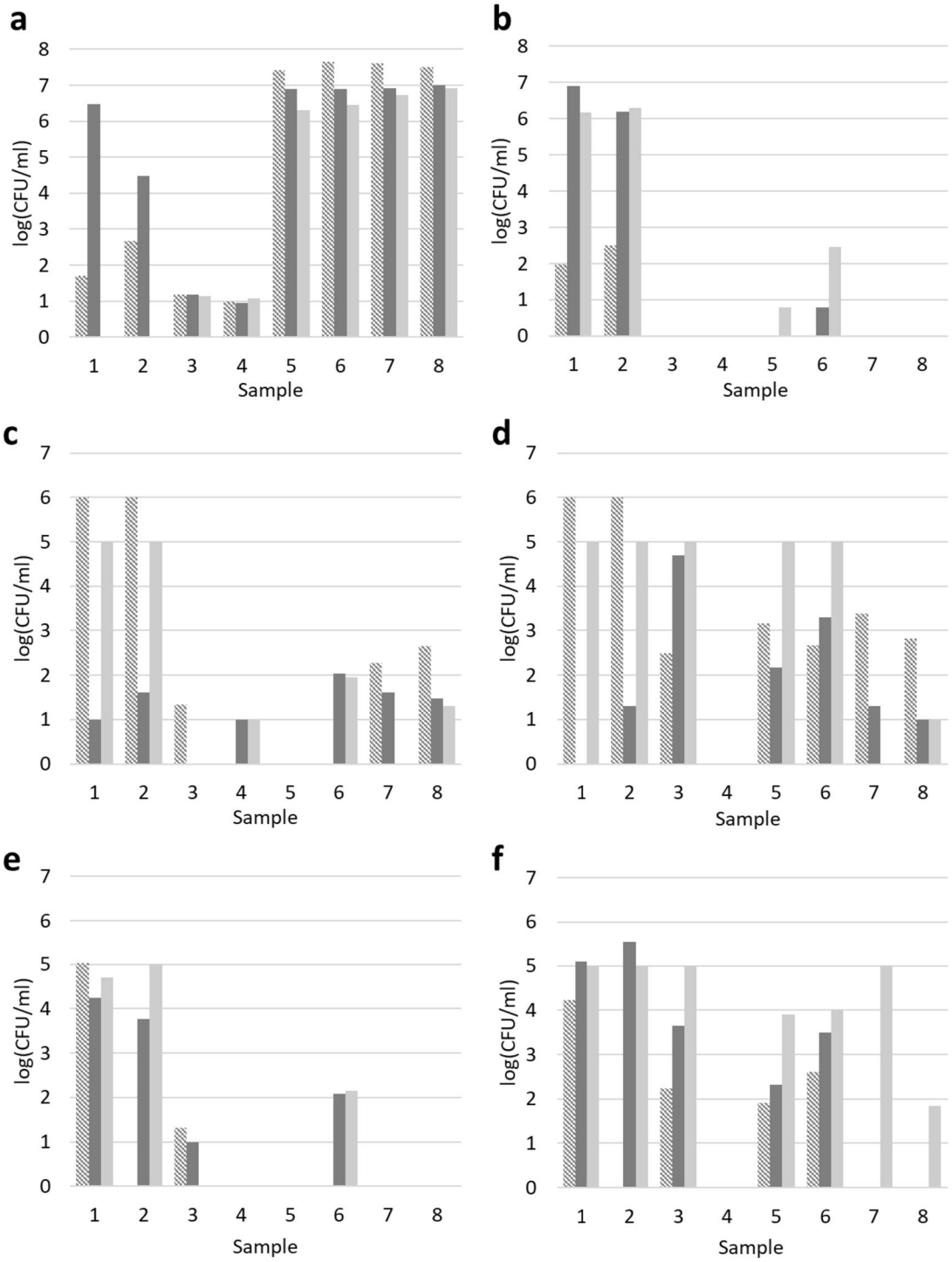

Fig. 5 Observed colony forming units of bacteria (TSA plates) and fungi (DRBC) plates. Total bacterial growth during fermentation (**a**), total fungal growth during fermentation (**b**), bacterial growth after storage of 1, 2 and 3 months at 4 °C (**c**), bacterial growth at 25 °C (**d**), fungal growth at 4 °C (**e**) and fungal growth at 25 °C (**f**). Striped bars represent start of fermentation in (**a**, **b**) and month 1 in (**c**–**f**), dark grey bars represent day 7 in (**a**, **b**) and month 2 in (**c**–**f**), whereas a light grey bar represents day 14 in (**a**, **b**) and month 3 in (**c**–**f**). The individual samples used are the same as used in previous experiments (see Fig. 3), i.e. non-heat-treated controls (1–2), heat-treated controls (3–4), inoculated with *L. plantarum* LP58 (5–6) and inoculated with *L. plantarum* LP58 and reinoculated after day 7 with the same strain (7–8). Note, for some samples, in figures **c**–**f**, with high numbers (> 4 log CFU/mL), exacts numbers are rather unprecise due to very high number of colonies on some of the plates. On day 0, the number of bacteria after inoculation is shown, and after 7 days the number of bacteria before reinoculation for sample 7–8 is presented (**a**, **b**). Samples without bars are below limit of detection that were 1 CFU/mL (**a**, **b**) and 10 CFU/mL (**c**–**f**)

Table 2 Percentages of organism distributions based on metagenome analyses

Sample[a]	Bacteria	fungi	Dominant bacterial species[b]	Second dominant bacterial species[b]	Third dominant bacterial species[b]	Fourth dominant bacterial species[b]	Dominant yeast species[c]	Dominant mould species[c]
2, day 0	83.72	12.52	*F. aurantia*, 53	*Glucanobacter oxydans,* 15	*Tanticharoenia sakaeratensis*, 5	*Rouxiella chamberiensis*, 4	*Pichia membramifaciens*, 8	*Aspergillus lentelus/Botrytis cinerea*, 17
2, day 14	96.76	2.08	*Glucanobacter oxydans,* 38	*Komagataeibacter xylinus*, 13	*Komagataeibacter saccharivorans*, 13	*F. aurantia*, 11	*Pichia membramifaciens*, 4	*Aspergillus lentelus*, 28
6, day 14	99.37	0.08	*L. plantarum*, 97	*Lactobacillus herbarum*, 0.8	*Lactiplantibacillus fabifermentans*, 0.3	*Lactiplantibacillus xiangfangensis*, 0.2	*Pichia membranifaciens*, 2	*Aspergillus lentelus*, 19
8, day 14	99.42	0.01	*L. plantarum*, 97	*Lactobacillus herbarum*, 0.9	*Lactiplantibacillus fabifermentans*, 0.3	*Lactiplantibacillus xiangfangensis*, 0.2	*Pichia membranifaciens*, 6	*Botrytis cinerea*, 17

[a]Sample numbers refer to the same batches of fermentation as used in Figs. 3, 4, 5

[b]Percentage is in relation to all bacterial species

[c]Percentage is in relation to all fungal species

less efficient conversion of malic acid, and some indication of sugar reduction. [3] As observed, initial concentration of malic and citric acids and the sugar concentration in the bilberries juices in the two studies are mostly identical, and the observed difference in MLF efficacy can probably be explained by the use of different starter cultures.

The metagenome sequencing of some of the samples of bilberry juices did not provide any results of great interest in relation to MLF. However, it did support that the starter culture LP58 was correctly classified and that even after heat treatment and successful fermentation, there were several potential spoilage microorganisms that could grow, a fact also confirmed in the shelf-life studies. Moreover, to our knowledge, this is the first observation of *F. aurantia* in bilberries; originally this bacterium was identified in raspberries and in the lily flower *Lilium auratum* [22]. The presence of *B. cinerea* was not surprising and justifies previous use of this fungus as model in shelf-life studies of bilberries [23, 24].

The microbial count during the fermentation and the subsequent shelf-life study can be interpreted, as addition of *L. plantarum* LP58 did not have a huge impact on microbial growth compared to the unfermented control. Another observation is that the viability of *L. plantarum* LP58 is rather low, as the number of countable LAB starts to decline during fermentation. After longer storage, other bacteria and fungi start to grow but in less number. This indicates that the bilberry juice is not a suitable habitat for the starter culture and that the strain is outcompeted by other microorganisms, an observation also noted by Viljakainen and Laakso [3]. When examining total bacterial counts, there are some variations even between identical samples, although most pronounced in the controls without starter culture. Despite this variation, the total numbers of microorganisms in bilberry juice are not in contrast to what has been reported previously [25]. For inoculated samples, the variation is smaller, and a plausible explanation is that most of the countable total bacteria refers to the starter culture. Although, less fungal growth observed in fermented samples, based on the high variation in fungal growth after storage, it is not possible to draw any firm conclusion related to fungal growth and thereby not supporting that the starter culture has any antifungal activity. The sample variation and inaccuracy in colony counting do not change the overall conclusion related to food safety, as stored samples cannot be considered as safe after storage under the tested conditions. When counting yeasts and moulds, an interesting observation is that in the non-heat-treated controls, only yeast growth was observed, while in the other samples only moulds. As vegetative mould spores, in general, have lower inactivation temperatures than yeasts [26], it is likely that observed mould growth has been originating from germinated ascospores that survived the heat treatment, but are not dependent on a thermal activation, e.g. *Aspergillus nidulans* [27], which was identified in the metagenome sequencing. This fact could explain why mould growth was not observed in all samples stored at 4 °C and with delayed growth at 25 °C (see Fig. 5e, f). However, any comparison between colony counting and metagenome sequencing must be considered with caution. For example, all extracted DNA might not derive from viable microorganisms, and the used culture media might not enable growth of all the present microorganisms.

To conclude, the *L. plantarum* strain LP58 can efficiently be used as a starter strain when fermenting bilberry juice. The rapid conversion of malic acid to lactic acid without reducing the sugar concentration, make it a potential strain that can be used to improve the taste of bilberry juice. However, as there is no significant evidence of microbial

inhibition in inoculated samples compared to the control, it cannot efficiently be used to improve the rather short shelf life of the product at ambient temperatures. It could be possible to isolate strains with high MLF ability that also has strong antimicrobial properties, or *L. plantarum* LP58 can be combined with one or more additional starter cultures providing a product with additional antimicrobial properties [28]. Alternatively, if the intention is to produce alcoholic beverages, the MLF can be combined with wine yeast and produced ethanol can serve as a microbial inhibitor [29]. Evidently, at this stage, to produce a safe bilberry juice that has underwent MLF, it is necessary to, like for other juices, include an inactivation step, probably using higher temperatures than the 65 °C used here, combined with safe production processes and packaging, and possibly combined with addition of weak acid preservatives [30].

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-023-04435-2.

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Declarations

Conflict of interest The authors have no competing interests or fundings that are directly or indirectly related to the submitted work.

Compliance with ethics requirement In the study, the work was restricted to harvested bilberries and microorganisms. Hence, this study does not contain any studies with human participants or animals performed by any of the authors.

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