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Effect of different starter cultures on chemical and microbial parameters of buckwheat honey fermentation

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ABSTRACT

The aim of this study was to analyze the microbiology of buckwheat honey fermentation inoculated with different starter cultures by culturing and PCR-DGGE, taking as a model for comparison a spontaneously fermented batch. The inoculants tested were (i) cider lees (from a cider factory), (ii) sourdough (from a bakery), and (iii) a commercial *Saccharomyces cerevisiae* strain. The results of the culturing and culture-independent techniques agreed well and detected the same dominant species along the fermentations. Our results suggest that *S. cerevisiae* strains, which constituted a majority population in all batches including the uninoculated one, carried out the fermentations. The highest microbial diversity was found at the beginning of the fermentation in the uninoculated batch; this contained in addition to *S. cerevisiae* bacteria (*Paracoccus* sp., *Staphylococcus* sp., and *Bacillus* sp.) and yeast (*Candida* sp.) species. *Candida* sp. was also common in batches inoculated with sourdough and cider lees cultures. *Lactobacillus* species were found throughout the fermentation of the sourdough-inoculated batch. Basic chemical analysis and testing trials demonstrated that the overall sensory acceptance of the four meads were highly similar. Yeast and bacteria isolated in this study could serve as a source of technologically relevant microorganisms for mead production.

1. Introduction

Although not a very popular drink at present, mead has seen its consumption steadily increase in Europe over the last few decades (Ramalhosa et al., 2011). Mead is a traditional alcoholic beverage made by fermenting honey diluted with water and having a final alcohol content ranging from 8% to 18% (Navrátil et al., 2001). The honey wort is sometimes boiled before the starter culture is added. This boiling step, a process called “saturation”, aims to killing pathogenic microorganisms in the wort. Currently, there is a limited amount of scientific literature regarding the fermentation of non-boiled honey musts (Czabaj et al., 2017; Kahoun et al., 2017; Wintersteen et al., 2005). To obtain products with different taste and aroma profiles, fruit juices can be added before fermentation. Mead can also be supplemented with herbs and spices after fermentation (McConnell and Schramm, 1995). In Poland, several meads are distinguished by the honey:water ratio of the initial wort, creating categories such as *półtorak* (1:0.5), *dwójniak* (1:1), *trójniak* (1:2) and *czwórniak* (1:3) (Czabaj et al., 2017), each requiring a different aging period. Polish meads are protected in the European Union under Traditional Specialty Guaranteed labels (European Union, 2008). In 2017, a new regulation added the term *staropolski tradycyjny* (Traditional Old Polish) to the categories, and these products are now listed as *półtorak staropolski tradycyjny*, and so on (European Union, 2017).

One of the main factors affecting the quality of mead is the honey used for fermentation. Industrial processes usually use multifloral honey instead of unifloral honey. As a normal practice, a small portion of buckwheat honey is usually also added to the wort to improve the fermentability of the must and to enhance the sensory attributes of the resulting mead. Buckwheat honey is a dark honey with well-recognized antioxidant properties (Gheldof et al., 2002). In sensory evaluation, buckwheat honey has been described, (depending on the panelists) as having buttery, vanilla, burnt sugar, floral, fruity/estery, and malty characteristics (Zhou et al., 2002); or as earthy, microbiological, and animal-like, with no berry-like, fruity, or floral attributes (Kortesniemi et al., 2018).

Spontaneous fermentation of honey is often used in traditional mead production although, on the industrial level, inoculation of the

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wort with pure cultures is a common practice. *Saccharomyces cerevisiae* strains from wine are commonly used as starter cultures (Mendes-Ferreira et al., 2010; Morales et al., 2013; Pereira et al., 2014; Sroka et al., 2017). Honey contains many nutrients, but lacks some others, as well as critical factors that may limit microbial growth. Therefore, the search for yeast strains with the appropriate growth and fermentation properties in honey is crucial (Pereira et al., 2009). Spontaneous fermentation and fermentation carried out with undefined microbial mixtures are considered good sources for the identification and selection of new and improved yeast starter strains (Teramoto et al., 2005; Pereira et al., 2009).

This article reports on the application of different inoculum regimens for the fermentation of buckwheat honey to obtain mead. This work aimed to investigate and compare the chemical and microbial parameters obtained during honey fermentation using different inocula, as well as those of the resulting meads. The honey must fermented in this study was not subjected to any thermal treatment. The composition and succession of the microbial populations during fermentation was assessed by culturing and by the culture-independent PCR-DGGE technique. The dominant microorganisms from the counting plates were then isolated and identified by molecular methods. Basic chemical parameters, such as pH, total extract, and ethanol content, were also determined. Finally, a multivariate analysis was performed to correlate microbial and chemical parameters under the different inoculation conditions. After fermentation, the final product was subjected to sensory analysis by the acceptance test.

2. Materials and methods

2.1. Materials

Unifloral honey from buckwheat (*Fagopyrum esculentum*) obtained from a local beekeeper from Pyzdry, Wielkopolska region, Poland, collected in 2016, was used in this work to prepare the mead. Honey was diluted with boiled tap water in a volumetric proportion of 1:2 (honey:water) to obtain a *trójniak* wort without additional ingredients, such as herbs or juice. Diammonium phosphate (DAP) was added as a nutrient (0.45 g/L) and, to limit spoilage and pathogenic microorganisms, the pH was adjusted to 3.6–3.7 using citric acid.

2.2. Fermentation of honey and sampling

The honey wort was divided into four batches. Three of the batches were independently inoculated with (i) cider lees (microbial residue from apple cider production), (ii) a sourdough starter from a local bakery, and (iii) the commercial yeast strain *Saccharomyces cerevisiae* (ex *bayanus*) BC S103 (Fermentis, Lesaffre, France); the fourth batch was left uninoculated, and underwent spontaneous fermentation. For the three inoculated batches the respective starter cultures were grown in sterilized Erlenmeyer flasks closed with a cotton plug. For this, 80 mL of honey must were inoculated with 800 µL of cider lees, 0.85 g of sourdough starter or 0.2 g of dry commercial yeast strain, respectively. Starters were then incubated on a shaker at 120 rpm for 48 h at 25 °C.The number of yeasts was determined immediately after inoculation in YGC medium. A concentration of about 10^7 CFU/mL was determined (Table 1). The fermentations were carried out in pre-sterilized bottles capped with fermentation locks (Biowin, Poland), in triplicate, at 25 °C, for 30 days. The final volume of each batch was 900 mL. Throughout the fermentation, samples were collected under sterile conditions on days 0, 3, 7, 15, and 30. Every time, 2 mL samples were collected in two separate Eppendorf tubes and a 50 mL sample was transferred to a Falcon tube. The samples were split into two aliquots; one was immediately processed for microbial analysis, and the other was kept frozen at −20 °C for biochemical tests.

2.3. Physicochemical analysis

Alcohol and sugar content were measured using an alcoholmeter (Alcolyzer Wine; Anton Paar, Austria) and a densitometer (DMA 5000 Density Meter, Anton Paar), respectively. The pH was measured with a pH meter (Crison, Barcelona, Spain).

2.4. Microbiological analysis

Microbiological counts of the viable cells were performed using the standard plate-counting method. Dilutions were prepared in sterile Eppendorf with a sterile saline solution (0.9%) with 200 µL of sample and 1800 µL of saline. Samples of mead and further decimal dilutions were plated in duplicate on different agar media, as indicated below. A 100 µL aliquot of the diluted samples was spread in each plate. All media were purchased from VWR International (Barcelona, Spain).

Total bacterial counts were determined on Plate Count Agar with Milk (PCAM) plates, which were incubated at 32 °C for 48–72 h. Yeast counts were determined on Yeast Extract Glucose Chloramphenicol agar (YGC) after incubation at 25 °C for 48–72 h. Enterobacteria and coliforms were counted on Violet Red Bile Dextrose agar (VRBD) and Violet Red Bile agar (VRB) plates, respectively. These media were both incubated for 24–48 h at 37 °C. Enterococci were assayed on Slanetz and Bartley medium at 42 °C for 48 h. Lactobacilli were determined on de Man, Rogosa, and Sharpe agar (MRS) at 32 °C for 48–72 h. To limit the growth of yeast, the MRS agar plates were supplemented with 100 µg/mL cycloheximide (Sigma-Aldrich, St. Louis, Mo., USA). Staphylococci were determined on Baird-Parker agar with added egg yolk after incubation at 37 °C for 48–72 h. Sporeformers were determined on PCAM after treatment of the sample at 80 °C for 10 min. Plates were then incubated at 32 °C for 48–72 h. Microorganisms were enumerated using two methods, the standard pour plate technique and the surface spread-plate technique. Enterobacteria, enterococci, and coliforms were determined using the pour-plate and overlay technique; dilutions were mixed with 15 mL of semisoft agar medium (0.75% agar) and poured onto Petri dishes. All other bacterial groups were enumerated with the spread plate technique.

After counting, colonies of all different morphologies were selected at random from the Petri dishes with PCAM, YGC, and MRS media and purified by subculturing onto Brain Heart Infusion agar (BHI) and Malt Extract agar (ME), for bacteria and yeast, respectively. 20% glycerol was added to the liquid cultures in the respective media, which were then held at −80 °C for storage and further identification.

2.5. Molecular identification of isolates

Isolates were identified by consecutive Amplified Ribosomal DNA Restriction Analysis (ARDRA), and by sequencing and sequence analysis of representative amplicons from all different ARDRA patterns. For this, cell-free extracts were obtained in line with the procedure described by Cherif-Antar et al. (2016), with minor modifications. For some isolates, DNA from overnight cultures was extracted and purified by using a GenElute Bacterial Genomic DNA kit (Sigma Aldrich, Germany). For bacteria, extracts and purified DNA were used as template sources to amplify the 16S rRNA gene using primers 27 F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), under the PCR conditions described by Cherif-Antar et al. (2016). To identify yeasts, amplifications were performed with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), under the PCR conditions described by White et al. (1990).

PCR products were purified using a PCR Clean-Up kit (Sigma-Aldrich). For the ARDRA analysis of bacteria and yeasts, the amplicons were digested with restriction enzymes HinfI, HhaI, and HaeIII (Takara, Japan) (Cherif-Antar et al., 2016), or with HinfI and HaeIII(Álvarez-Martín et al., 2007), respectively. After digestion, fragments were

Table 1
Microbial counts of total bacteria and yeasts during buckwheat honey fermentation of uninoculated wort (S) and of wort inoculated with a commercial *Saccharomyces cerevisiae* strain (Y), sourdough (B), and cider lees (C).

Day of fermentation	Total microbial counts in PCAM (log cfu mL^{-1})[a]				Yeast counts in YGC (log cfu mL^{-1})[a]			
	S	Y	B	C	S	Y	B	C
0	2.55 ± 0.20	6.08 ± 0.09	7.30 ± 0.08	7.31 ± 0.05	–	6.21 ± 0.18	7.02 ± 0.22	7.27 ± 0.01
3	4.56 ± 0.74	7.38 ± 0.03	8.39 ± 0.02	7.64 ± 0.23	4.03 ± 0.37	7.30 ± 0.14	7.97 ± 0.03	7.54 ± 0.11
7	8.48 ± 0.44	8.94 ± 0.34	9.72 ± 0.02	7.70 ± 0.38	8.33 ± 0.35	8.84 ± 0.33	8.85 ± 0.35	7.80 ± 0.35
15	7.80 ± 0.15	8.15 ± 0.54	8.13 ± 0.55	7.45 ± 0.58	7.76 ± 0.13	8.02 ± 0.47	7.86 ± 0.62	7.64 ± 0.51
30	6.80 ± 0.43	6.86 ± 0.39	4.75 ± 0.31	6.03 ± 0.27	6.38 ± 0.33	6.68 ± 0.26	4.77 ± 0.13	5.49 ± 0.24

—: not detected.
[a] Average of three independent fermentations.

separated by electrophoresis, visualized with ethidium bromide (0.5 μg/mL), and photographed. Representative amplicons of all ARDRA profiles were then sequenced and the sequences identified by comparison to those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and in the Ribosomal Database Project database using Classifier and Sequence Match tools (http://rdp.cme.msu.edu). Following Stackebrandt and Goebel (1994), sequences with an identity percentage of 97% or higher were considered to belong to the same species.

2.6. Extraction of total DNA from mead

To isolate the total microbial DNA during fermentation, samples were centrifuged and the supernatants were discarded. DNA was extracted and purified from the microbial pellets as described by Greco et al. (2014) with some modifications. Pellets were rinsed with a 0.9% NaCl solution, suspended in an extraction buffer (100 mM Tris-HCl, pH 9.5; 150 mM NaCl; 1.0% sarkosyl; 5 mM DTT), and incubated in a water bath at 37 °C for 1 h. After the incubation, the solution was transferred to a tube containing glass beads, and the cells were disrupted on a FastPrep FP120 Instrument (Thermo Savant-Bio101/Q-Biogen). Disruption of cells was performed at room temperature, and at maximum speed in three cycles of 1 min, with 1 min incubation on ice between cycles. Next, 20 μL proteinase K (10 mg/mL; Roche, Switzerland) was added and the solution was incubated at 56 °C for 30 min. Then 250 μL 10% SDS and 7 μL 1 M $CaCl_2$ were added, and the solution was incubated again at 56 °C for 15 min. After that, 200 μL molecular biology grade water (Sigma-Aldrich), 88 μL of 96% ethanol, and 200 μL 3 M potassium acetate were added and the solution was vortexed. One volume of phenol/chloroform was added, vortexed, and centrifuged at 16,100 × g for 5 min. The supernatant was moved to a new tube and the phenol/chloroform extraction was repeated until the interphase was clear, adding 100 μL of water between steps (to avoid decreasing the volume of the water phase). The supernatant was transferred to a new tube and one volume of chloroform was added to clear out phenol residues. Finally, the supernatant was transferred to a new tube and 1 mL of isopropanol was added and centrifuged at 4 °C for 30 min. The supernatant was discarded and the DNA pellet was washed with 100 μL of 70% ethanol. Pellets were left to dry in a SpeedVac concentrator 5301 (Eppendorf) for 10 min. The DNA was suspended in 100 μL of molecular biology grade water and frozen at −20 °C until analysis.

2.7. PCR-DGGE analysis

Total DNA from the mead fermentation was used as a template to amplify the V3 region of the bacterial 16S rRNA gene by PCR using the universal primers F357-GC, the universal primer F357 (5′-TACGGGAG GCAGCAG-3′) with GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGG GCGGGGGCACGGGGG-3′), and R518 (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993). The PCR conditions used for amplification were as reported by Muyzer et al. (1993). For eukaryotic organisms, the D1 domain of the 26S rRNA gene was amplified using primers NL1-GC (5′-GCCATATCAATAAGCGGAGGAAAAG-3′), in which the GC clamp was linked to primer NL1, and LS2 (5′-ATTCCCAAACAACTCGACTC-3′). Amplification was performed under the conditions described by Cocolin et al. (2002). The amplification reactions consisted of 3 μL of extracted DNA, Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark), 10 μM of the primers, and molecular biology grade water (Sigma-Aldrich), added to total volume of 50 μL. Amplification was tested by agarose (1%) gel electrophoresis in 1 × TAE buffer (Sigma-Aldrich), stained with ethidium bromide and visualized under UV light.

DGGE was carried out in a DCode apparatus (Bio-Rad, Richmond, CA, USA) at 60 °C on 8% polyacrylamide gels (acrylamide/N,N′-methylene bisacrylamide, 37/5/1, Bio-Rad, Richmond, CA, USA) with a denaturing urea formamide gradient of 40–60% at 75 V for 17 h in the case of the bacteria and 30–50% at 130 V for 4.5 h in the case of the yeasts (Flórez and Mayo, 2006) in 1 × TAE buffer (Sigma-Aldrich). Bands were stained with ethidium bromide and visualized as above.

After electrophoresis, the bands of interest were cut from the gel with a sterile blade and incubated overnight in 25 μL of molecular biology grade water at 4 °C for DNA diffusion. Three microliters of DNA solution were used as a template for reamplification with the same primers (but without the GC clamp) and PCR conditions. PCR products were purified using a PCR Clean-Up kit (Sigma-Aldrich) and sequenced. Sequences were identified by comparison to those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). Following Stackebrandt and Goebel (1994), sequences with an identity percentage of 97% or higher were considered to represent the same species.

2.8. Sensory acceptance test

Evaluation of the acceptability of the mead samples was tested with a randomized block design using a hybrid hedonic scale of 10 cm, following the method of Villanueva et al. (2005). As reported elsewhere (Ribeiro et al., 2018), three points were introduced to the scale: dislike extremely (0), neither like nor dislike (5) and like extremely (10). Attributes such as appearance, aroma, flavor, and overall acceptance were evaluated by a panel of consumers.

2.9. Statistical analysis

Tukey's Honestly Significant Difference (HSD) test, in conjunction with an ANOVA post-hoc analysis was carried out to search for significant differences between means. The nonparametric Kruskal–Wallis ANOVA and the post-hoc comparison of mean ranks of all pairs of groups was used for variables with nonnormal distribution. Principal component analysis (PCA) was used to show the relationship between variables and cases. The *K*-means algorithm with the *v*-fold cross-

validation algorithm was used to group samples and to show discriminant categorical variables. Statistical analysis was performed using Statistica software, version 12 (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Microbial diversity of fermentation by culturing

Table 1 presents the microbial counts enumerated on PCAM (total microbial counts) and YGC (total yeast counts) plates. In the spontaneous fermentation, rapid microbial growth was observed between day 0 and day 7. Notably, the yeasts, which were not detected at the beginning of fermentation, reached 8 logarithmic units on day 7. As expected, high yeast counts were already observed on day 0 in the fermentation inoculated with the commercial yeast, reaching the highest counts on day 7. In batches inoculated with the sourdough and cider lees, the total numbers of microorganisms at the beginning of the fermentation in both PCAM and YGC (thus corresponding to yeasts) were also high and comparable. A small decrease in both total and yeast counts was then observed in all fermentation from day 7 onwards, particularly in the batch inoculated with the sourdough. As for microorganisms of safety concern, low numbers of Enterobacteriaceae and coliforms were seen in the batches inoculated with sourdough and cider lees up to day 7 of fermentation. However, counts were always below the limit of detection ($< 10^2$ cfu/mL) in all four batches from day 15 onwards. Furthermore, counts of typical enterococci in Slanetz-Bartley and *Staphylococcus aureus* in Baird-Parker media were never scored.

In order to identify the dominant microorganisms in the fermentations, a number of colonies from each batch and sampling point representative of all morphotypes was selected. In total, 94 colonies were picked up from the PCAM (57), YGC (19), and MRS (18) plates, purified by subculturing in BHI or ME and identified by molecular methods. Digestion of rDNA amplicons with restriction enzymes grouped the isolates in 16 ARDRA profiles, of which eleven corresponded to bacteria (35 isolates) and five to yeasts (59 isolates) (Fig. 1). The bacteria profiles were then identified by amplicon sequencing and sequence comparison as *Lactobacillus plantarum/L. pentosus* (13 isolates), *Staphylococcus warneri/S. pasteuri* (7), *Staphylococcus epidermidis* (4), *Lactobacillus casei/L. paracasei* (3), *Paracoccus yeei* (2), *Paracoccus sanguinis* (1), *Paracoccus siganidrum* (1), *Kocuria rhizophila* (1), and *Bacillus licheniformis* (1). Two of the isolates were only identified to the genus level, one as a *Bacillus* sp. and the other one as a *Methylobacterium* sp. The five eukaryotic profiles were identified as belonging to *Saccharomyces cerevisiae* (24 isolates) or to species of *Candida*, such as *Candida magnoliae* (14), *Candida sorbosivorans* (14), and *Candida metapsilosis*/C. *parapsilosis* (6). Finally, a single isolate of *Sporobolomyces ruberrimus* was also identified.

The succession of the different species of bacteria and yeast identified through the sampling points along fermentation is summarized in Table 2. The microbial community of the uninoculated batch at the beginning of fermentation was found to remain diverse up to day 15. It consisted of bacteria (*Paracoccus* sp., *Staphylococcus* sp., and *Bacillus* sp.) and yeasts of *Candida* species. *Candida* spp. were also common up to day 7 in the batches inoculated with the sourdough and the cider lees. *Staphylococcus* sp. isolates were randomly identified from all batches between days 7 and 15, while *Lactobacillus* sp. were found in mead fermentations in the sourdough inoculated batch. As expected, the batch inoculated with the commercial yeast strain was dominated from the beginning by *S. cerevisiae*. By the end of all fermentations, *S. cerevisiae* was the only microorganism identified in the counting PCAM plates, except for the mead batch inoculated with the sourdough, in which *Lactobacillus* species were also seen at day 30.

3.2. Chemometric results

A matrix containing binary data (1: identified, 0: not detected) was performed to look for possible correlations between the inoculum regimen and the microorganisms detected through fermentation. Principal Components Analysis (PCA) of the data showed the two principal components (PCs) explained 57% of the variance (Fig. 2), which allowed establishing three clusters. From the comparison of loading and score plots (Fig. 2A and B), we conclude that the composition of the populations shifted from being very diverse in the earlier stages of fermentation to almost single species in batches inoculated with the sourdough (B) and cider lees (C). Samples from the mead obtained using the commercial strain (Y) are located in the first group, as *S. cerevisiae* dominated this batch from the start of the fermentation. As seen in the figure, the spontaneous fermentation (S0) was an outlier, and its position was determined by bacteria (*L. casei/L. paracasei*, *P. sanguinis*, *P. yeei*, *P. siganidrum*, *Bacillus* sp., *Methylobacterium* sp.). The profile of the uninoculated batch (S) after day 3 of was similar to that of the batch inoculated with cider lees (the third group). Further, the results also showed a strong negative correlation between *S. cerevisiae* and *Candida* sp. (Pearson's Phi correlation coefficients ranged from -0.58 to -0.76, $p < 0.05$), which indicates a strong competition between the two yeast species in mead.

The *K*-means algorithm was used to demonstrate which microorganisms governed the statistically significant differences between

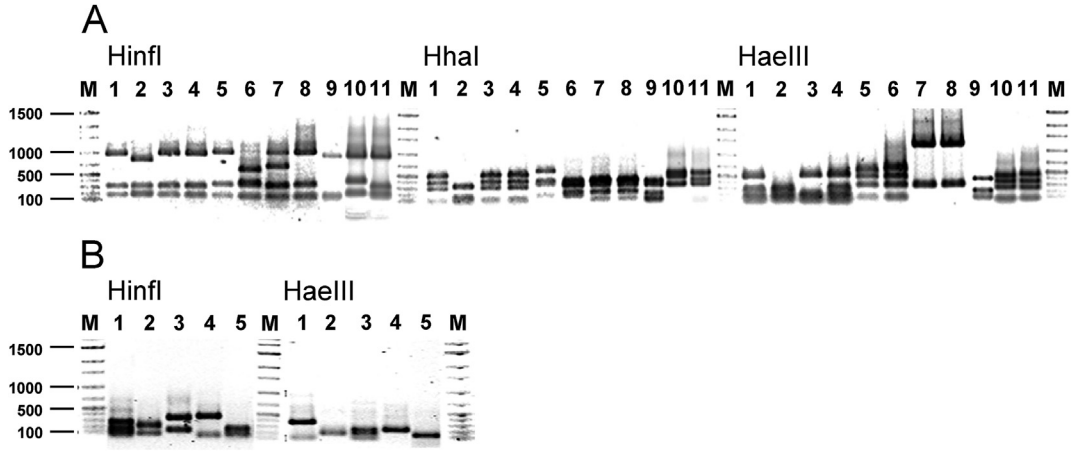

Fig. 1. ARDRA profiles of bacteria (A) and yeasts (B) after digestion with the indicated restriction enzymes. Order key in panel A: M, molecular weight markers; 1, *Paracoccus sanguinis*; 2, *Methylobacterium* sp.; 3, *Paracoccus siganidrum*; 4, *Paracoccus yeei*; 5, *Bacillus* sp.; 6, *Bacillus licheniformis*; 7, *Staphylococcus warneri/pasteuri*; 8, *Staphylococcus epidermidis*; 9, *Kocuria rhizophila*; 10, *Lactobacillus plantarum/L. pentosus*; 11, *Lactobacillus casei/L. paracasei.* Order key in panel B: M, molecular weight markers; 1, *Saccharomyces cerevisiae*; 2, *Candida magnoliae*; 3, *Sporobolomyces ruberrimus*; 4, *Candida metapsilosis/C. parapsilosis*; 5, *Candida sorbosivorans.*

Table 2
Identification of microorganisms along buckwheat honey fermentation of uninoculated wort (S) and of wort inoculated with a commercial *Saccharomyces cerevisiae* strain (Y), sourdough (B), and cider lees (C).

Day of sampling	S	Y	B	C
0	*Paracoccus sanguinis* (1)[a] *Paracoccus yeei* (2) *Methylobacterium* sp. (1) *Paracoccus siganidrum* (1) *Bacillus* sp. (1)	*Saccharomyces cerevisiae* (3)	*Lactobacillus plantarum/L. pentosus* (2) *Candida magnoliae* (2) *Candida sorbosivorans* (1) *Candida metapsilosis/C. parapsilosis* (4) *Sporobolomyces ruberrimus* (1)	*Candida magnoliae* (3) *Candida sorbosivorans* (6)
3	*Staphylococcus warneri/S. pasteuri* (1) *Staphylococcus epidermidis* (1) *Kocuria rhizophila* (1) *Candida metapsilosis/C. parapsilosis* (1) *Candida magnoliae* (1) *Candida sorbosivorans* (2)	*Staphylococcus warneri/S. pasteuri* (1) *Saccharomyces cerevisiae* (1)	*Lactobacillus plantarum/L. pentosus* (4) *Candida magnoliae* (1) *Candida sorbosivorans* (2) *Candida metapsilosis/C. parapsilosis* (1)	*Bacillus licheniformis* (1) *Candida magnoliae* (3) *Candida sorbosivorans* (1)
7	*Lactobacillus plantarum/L. pentosus* (2) *Staphylococcus epidermidis* (1) *Staphylococcus warneri/S. pasteuri* (2) *Candida magnoliae* (2) *Saccharomyces cerevisiae* (3)	*Staphylococcus epidermidis* (1) *Saccharomyces cerevisiae* (3)	*Lactobacillus plantarum/L. pentosus* (5) *Staphylococcus warneri/S. pasteuri* (2) *Candida magnoliae* (1) *Saccharomyces cerevisiae* (2)	*Staphylococcus warneri/S. pasteuri* (1) *Saccharomyces cerevisiae* (2) *Candida magnoliae* (1) *Candida sorbosivorans* (2)
15	*Saccharomyces cerevisiae* (1)	*Saccharomyces cerevisiae* (1)	*Lactobacillus casei/L. paracasei* (2) *Saccharomyces cerevisiae* (1)	*Staphylococcus epidermidis* (1) *Saccharomyces cerevisiae* (2)
30	*Saccharomyces cerevisiae* (2)	*Saccharomyces cerevisiae* (1)	*Lactobacillus casei/L. paracasei* (1) *Saccharomyces cerevisiae* (1)	*Saccharomyces cerevisiae* (1)

[a] In brackets, number of colonies identified of each species.

batches (data not shown). The same tree groups as with PCA were also obtained with this algorithm. The fermentation profiles were significantly ($p < 0.05$) differentiated by yeasts -namely *C. metapsilosis/C. parapsilosis*, *C. magnoliae*, *C. sorbosivorans*, and *S. cerevisiae*-, suggesting these organisms are the most competitive in mead.

3.3. Microbial diversity of fermentations as determined by PCR-DGGE

As regards the culture-independent analysis of mead fermentations, Fig. 3 shows the PCR-DGGE yeast profile of the four batches analyzed in this study. The results obtained with the PCR-DGGE technique agree well with those of the counting, and the eukaryotic microbiota of honey fermentation seems to be composed of *S. cerevisiae* alone, or in combination with species of *Candida*. A total of 26 bands from the gels were identified by reamplification, sequencing, and sequence comparison. As in the culture analysis, *S. cerevisiae* was detected in all DGGE gels (bands 1–10 in Fig. 3A, and bands 4, 5, 6, 12, 13 in Fig. 3B). *S. cerevisiae* was the only yeast detected in samples taken from the spontaneous fermentation and the commercial yeast fermentation. The sequence of the three bands (1, 2 and 3) in the upper part of the gel in Fig. 3B, which corresponded to the fermentation inoculated with the sourdough, were identified as having the same nucleotide identity to the closely related species *Candida orthopsilosis*, *C. parapsilosis*, or *C. metapsilosis*. In samples taken from the fermentation with cider lees as the starter, the bands appearing in the upper part of the gel (Fig. 3B, bands 10 and 11) were assigned to *C. magnoliae*. Further, bands appearing in the lower part of the gel in these two fermentations (Fig. 3B, bands 7, 8, 9, 14, 15, 16) were identified as *C. sorbosivorans*.

Attempts were also made to carry out a PCR-DGGE analysis of the majority bacterial populations during fermentation, but proved to be unsuccessful. The resulting DGGE bands were shown to consist of amplicons that could not be identified by reamplification or which belonged to yeast species. The large amount of yeast DNA in all samples interfered with amplification of bacterial-specific sequences. *In silico* analysis of the prokaryotic-specific primers used, which have been shown to work well in cheese (Flórez and Mayo, 2006; Alegría et al.,

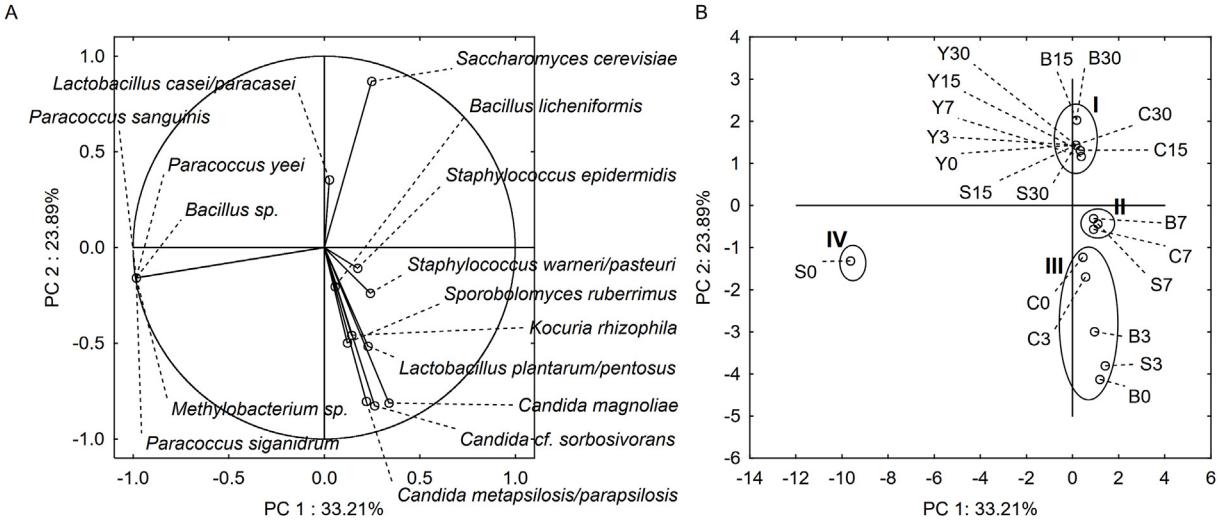

Fig. 2. Principal Component Analysis (PCA) of the loadings plot (A) and score plot (B) of the microorganisms identified in the buckwheat honey fermentations at days 0 (immediately after inoculation), 3, 7, 15, and 30 days of fermentation. S: spontaneous fermentation; Y: fermentation inoculated with the commercial yeast strain; B: fermentation inoculated with sourdough culture; C: fermentation inoculated with cider lees culture.

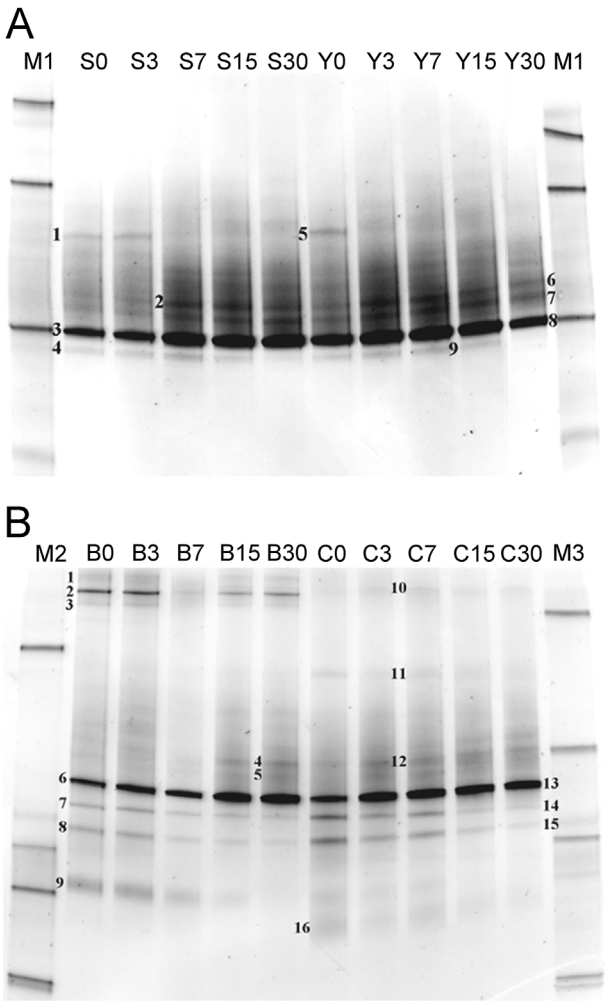

Fig. 3. Eukaryotic DGGE patterns of buckwheat honey wort at the beginning (immediately after inoculation) (0) and throughout fermentation (days 3, 7, 15, and 30). Panel A: S: spontaneous fermentation; Y: fermentation with a commercial yeast strain. Panel B: B: fermentation inoculated with a sourdough culture; C: fermentation inoculated with a cider lees culture. Numbered bands in the gels were isolated and identified as follows: Panel A, bands 1 through 10 were all identified as *Saccharomyces cerevisiae*; Panel B, bands 1, 2, and 3 as *Candida orthopsilosis/C. parapsilosis/C. metapsilosis* (same homology percentage); bands 4, 5, 6, 12, and 13 as *Saccharomyces cerevisiae*; bands 7, 8, 9, 14, 15, and 16 as *Candida sorbosivorans*; bands 10 and 11 as *Candida magnoliae*.

2009), showed that they matched ribosomal sequences from *S. cerevisiae* and other yeasts (data not shown).

3.4. Basic chemical parameters

The ethanol contents, total extracts (which describe the sugar content of the sample), and the pH values recorded during fermentation are shown in Table 3. The final ethanol concentration by the end of all fermentations was shown to be rather similar (ranging from 13.10% to 13.47%). However, the dynamics of ethanol production in the spontaneous fermentation was completely different to the others. This suggests that the *S. cerevisiae* strains present in the honey wort grew slowly until day 7, although they dominated the microbiota by the end of the fermentation. In contrast, ethanol production in the batch inoculated with the commercial strain started quickly (4.86% at day three). The batches started with sourdough and cider lees behaved similarly, having shorter delays in ethanol production than the commercial yeast, but reaching similar ethanol percentages by day 15. Despite the differences in the fermentation dynamics, meads from all batches met the

Table 3
Mean and standard deviation of alcohol content, total extract, and pH during buckwheat honey fermentation of uninoculated wort (S) and wort inoculated with a commercial *Saccharomyces cerevisiae* strain (Y), sourdough (B), and cider lees (C).

Day	Parameter															
	Alcohol content (%)				Total extract (°Brix)				pH							
	S	Y	B	C	S	Y	B	C	S	Y	B	C	S	Y	B	C
0	0.23^{aA} ± 0.01	0.59^{aB} ± 0.03	0.53^{aBC} ± 0.08	0.50^{aC} ± 0.03	23.05^{aC} ± 0.2	21.91^{aA} ± 0.1	22.28^{aB} ± 0.1	22.52^{aB} ± 0.2	3.71^{aA} ± 0.26	3.60^{aA} ± 0.09	3.58^{aA} ± 0.25	3.59^{aA} ± 0.09				
3	0.29^{aA} ± 0.04	4.86^{bC} ± 0.22	1.94^{bB} ± 0.20	1.89^{aB} ± 0.11	22.62^{aC} ± 0.2	15.20^{bA} ± 0.3	19.58^{bB} ± 0.4	19.89^{bB} ± 0.3	3.67^{aA} ± 0.12	3.31^{bB} ± 0.13	3.50^{abAB} ± 0.06	3.34^{abB} ± 0.06				
7	1.56^{aA} ± 0.03	9.66^{cC} ± 0.45	7.72^{cB} ± 0.47	6.80^{bB} ± 0.41	20.87^{bD} ± 0.3	8.56^{cA} ± 0.3	11.55^{cB} ± 0.2	13.07^{cC} ± 0.4	3.28^{a} ± 0.15	3.29^{b} ± 0.07	3.05^{c} ± 0.10	3.25^{b} ± 0.12				
15	11.70^{bA} ± 0.33	12.97^{dA} ± 0.12	12.66^{dA} ± 0.22	12.75^{cA} ± 0.44	6.74^{cC} ± 0.4	3.93^{dA} ± 0.4	4.68^{dB} ± 0.5	4.79^{dB} ± 0.2	3.34^{aA} ± 0.23	3.31^{bA} ± 0.07	3.12^{cA} ± 0.05	3.43^{abA} ± 0.16				
30	13.47^{bA} ± 0.19	13.39^{dA} ± 0.45	13.12^{dA} ± 0.27	13.10^{cA} ± 0.20	4.33^{dB} ± 0.1	3.35^{dA} ± 0.5	4.14^{dB} ± 0.4	4.31^{dB} ± 0.1	3.42^{aAB} ± 0.12	3.41^{abAB} ± 0.05	3.15^{bcA} ± 0.17	3.47^{abB} ± 0.08				

Lowercase letters: mean values at different fermentation days (columns); capital letters: mean values in fermentation with different inocula (rows). Superscript with different letters in columns or rows indicate statistical differences ($p < 0.05$).

ethanol content requirements of Polish mead (European Union, 2007). The initial total extract (TS) was 22–23 °Brix and dropped sharply in the batch inoculated with the commercial yeast. The TS dynamics followed a trend opposite to that of ethanol production. The correlation between alcohol production and sugar content was calculated as −0.9928. In any case, the sugars had almost disappeared in all fermentations by day 15. The pH at the beginning of fermentation ranged from 3.58 to 3.71, the highest being that of the spontaneously fermented batch. The pH value dropped rapidly between days 3 and 7 in the uninoculated batch and in the batch inoculated with the sourdough, while in the other two fermentations, the pH decreased constantly until day 30. It is worth noting that the lowest pH at the end of the fermentation was measured in the batch inoculated with the sourdough, which may be associated with the presence of viable lactobacilli at this time.

3.5. Sensory evaluation

To compare the meads produced under different inoculation regimes, a consumer sensory test was undertaken by 50 panelists. The experimental meads in this study (with final extracts below 3.5 °Bx and a minimal ethanol content of 13.1% v/v) were compared to a commercial semisweet multifloral mead sample, i.e. "*hidromiel*" from Spain (initial extract 22.8 °Bx and an ethanol content of 10.33% v/v) (Fig. 4). No differences were noted between samples in terms of appearance ($p = 0.727$); this factor was rated the highest. The buckwheat meads obtained the lowest marks for flavor, while the commercial mead obtained the highest scores. The aroma of mead from the spontaneous fermentation was intermediate between the commercial and all others. Overall acceptance of experimental meads looks to be a compromise between aroma and flavor evaluation.

4. Discussion

In this research, buckwheat honey-based wort was shown to have good fermentation properties, even in the absence of inoculation; only diammonium phosphate was supplemented for the fermentation. Buckwheat honey has been shown to be a good source of nutrients, and has high protein content and an abundance of minerals (Deng et al., 2018). Dark honeys are known for having higher mineral content than light ones (Da Silva et al., 2016), which is an additional benefit. It thus contains all the nutritive requirements to support rapid growth of *S. cerevisiae*, the microorganism that carries out the alcoholic fermentation

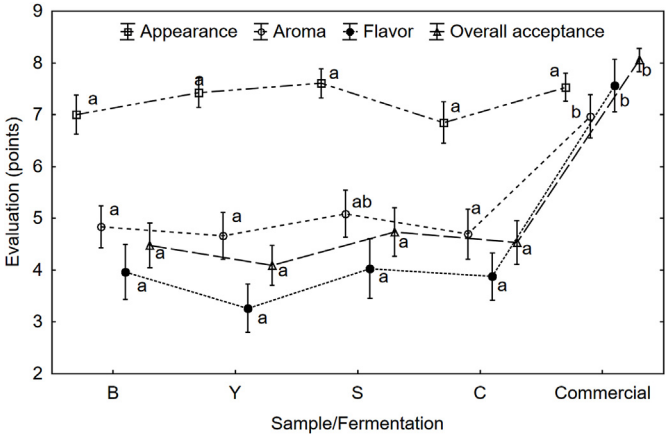

Fig. 4. Sensory acceptance profile of fermented buckwheat honey with different starter cultures and a commercial sample of mead. S: spontaneous fermentation; Y: fermentation with commercial yeast strain. B: fermentation inoculated with sourdough culture; C: fermentation inoculated with cider lees culture. Commercial: sample of multifloral mead ("*hidromiel*") purchased from a retail outlet. Different letters indicate statistically significant difference of mean values for organoleptic factors between samples ($p < 0.05$).

by converting sugars into alcohol. Further, the wort in this study was not heated prior to fermentation, which suggests that heating may be of interest for hygienic reasons (Kahoun et al., 2017; Wintersteen et al., 2005), but is not necessary to release nutritive substances needed by *S. cerevisiae* to grow. Finally, buckwheat honey is rich in volatile compounds, which can enhance the final sensory properties of the mead. In that sense, Pasini et al. (2013) have identified 118 volatile compounds in buckwheat honey, of which 3-methylbutanal and 2-methylbutanal seem to be the key compounds in its distinctive aroma (Plutowska et al., 2011).

Our results suggest that fermentations are carried out by *S. cerevisiae* strains. The commercial yeast batch began fermenting before all others, although a similar amount of ethanol was produced in all fermentations. *S. cerevisiae* is surely present in bread and cider cultures, as well as in the honey, in the case of the uninoculated batch. Although some authors have suggested that this species may appear from bees feeding on sugar pastes that contain this yeast (Kast and Roetschi, 2017), native honey strains of *S. cerevisiae* have been found in Portuguese (Carvalho et al., 2010, 2005) and Turkish (Senses-Ergul and Ozbas, 2006) meads. Further, *S. cerevisiae* has also been found as the most abundant yeast species throughout the whole process of fermentation of the traditional Sicilian "*Spiritu re' fascitrari*", a honey-based alcoholic beverage (Gaglio et al., 2017). Apart from the microorganisms involved, the type of honey used to produce mead has significant impact on its organoleptic evaluation. Gupta and Sharma (2009) reported that mead made from buckwheat honey had a very pungent, strong odor reminiscent of the strong flavor of raw buckwheat honey. Our results showed that spontaneous fermentation gave mead with intermediate acceptance for aroma between highly accepted "*hidromiel*" and others meads. The data also suggests that the strong aroma and flavor of buckwheat honey probably masks the positive and negative organoleptic effects produced by lactic acid bacteria, as no differences were observed between the sample inoculated with commercial yeast and the others.

The customary yeasts used in mead production are *S. cerevisiae* starter strains from wine and beer (Pereira et al., 2009). The microbiology of the traditional (uninoculated) processes is mostly unexplored, and studies of the fermentation ability of yeasts isolated from honey and mead are scarce (Pereira et al., 2009; Teramoto et al., 2005). Compared to wine must, undiluted honey contains three times more sugar content but 100 times less nitrogen. In addition, depending on the amount of water added to the honey, mead wort can be a more stressful medium than wine must due to the high osmotic pressure (Halawani and Shohayeb, 2011). Thus, wine yeast strains may not necessarily be suitable for mead production (Ramalhosa et al., 2011). In spite of this, it is worth noting that, in the mead obtained with the commercial yeast, no other microorganism appeared during the fermentation, which strongly indicates that this strain (isolated from Chardonnay grapes and used for the production of Champagne) is well adapted to growth in buckwheat honey wort. The same strain has also been used successfully for the fermentation of plums (Pielech-Przybylska et al., 2016). In the batch inoculated with the sourdough starter, live lactobacilli species were present alongside *S. cerevisiae* until the end of the fermentation. Species of *Lactobacillus* are common in bread dough (Lues et al., 1993). Indeed, positive interactions between yeast and LAB have been shown in many food fermentations (Álvarez-Martín et al., 2008; Ponomarova et al., 2017). Whether these microorganisms and the presence of *Candida* sp. at the beginning of some fermentation impart taste or aroma notes to the final mead deserves further research. The yeast and bacteria isolated from the batches inoculated with the cider lees and the sourdough, and those isolated from the uninoculated batch, might serve as a source of new technologically-relevant microorganisms for mead production.

In spite that PCR-DGGE suffers from the general limitations of all PCR-based techniques (primer specificity, preferential amplification, etc.) and also from specific drawbacks (formation of chimera, artifact amplicons, bands that do not re-amplify, etc.) (Wintzingerode et al.,

1997; Adil, 2015), it is still considered a useful technique for rapid microbial inspection of poorly characterized ecosystems, including food fermentations (Cocolin et al., 2013). In general, the results in this study of both culturing and culture-independent techniques agreed well with those from the cultures, as they showed *S. cerevisiae* as a dominant microorganism in all fermentations. Further, *Candida* species were also detected by this method in batches inoculated with sourdough and cider lees. The microbial species found in the batch inoculated with cider lees are the same that can be found on apples or in cider itself (mainly *Candida*) (Suárez Valles et al., 2007; Alonso et al., 2015). The PCR-based technique applied to the analysis of the cider apple microbiota showed that the fungal community there was quite homogenous as compared to the heterogeneous bacterial profile (Alonso et al., 2015). Further, *Saccharomyces cerevisiae* and *Saccharomyces bayanus* have already been reported to be present in Asturian cider by classical culturing (Suárez Valles et al., 2007). The presence of *Candida* sp. in honey is not surprising as this species is known to be osmotolerant (Pfannebecker et al., 2016); this is a key feature to develop in honey-derived environments.

Enterobacteriaceae, enterococci, and staphylococci are considered indicator populations of hygiene in food systems. Among all these populations, only enterobacteria counts were detected at the beginning of the fermentation of batches inoculated with the sourdough and cider lees. These bacterial types have been shown to be frequent contaminants in these foods (Alonso et al., 2015; Ercolini et al., 2013). The low pH of the wort (which continues to drop over the course of the fermentation process) may explain the inability of these populations to progress in the honey wort, which suggests that mead can be considered an unsuitable product for the development of food pathogens.

5. Conclusions

The microbial populations of buckwheat honey fermented under different inoculation regimes was investigated both by culturing and culture-independent techniques. The results of both methods agreed well and showed that *S. cerevisiae* dominated all fermentations, including the uninoculated one. The microbial community in the batch inoculated with the commercial yeast strain was the least diverse as it was dominated by the starter strain, showing that this microorganism can also be used for fermentation of non-thermal treated honey must. *S. cerevisiae* strains from the uninoculated batches, and from those inoculated with undefined sourdough and cider lees cultures, grew well and took over the alcoholic fermentation, converting all sugars into alcohol, as did the commercial strain. This suggests that starter-free fermentations might be a source for the identification and selection of new strains to complement or replace currently used commercial mead starters. Finally, the contribution of other microorganisms, such as lactobacilli and *Candida* species, to the final sensory profile of meads deserves further research.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.006.

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