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Detection and Identification of Lactic Acid Bacteria in Semi-Finished Beer Products Using Molecular Techniques †

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Abstract: Beer has been generally recognized as a microbiologically stable beverage. However, deviations in the brewing process may occur due to the activity of lactic acid bacteria (LAB). The growth of LAB during the brewing process implies a competition for nutrients with yeasts, causing decreased ethanol yields. Moreover, quality degradation caused by LAB spoilage can be observed due to the production of off-flavors (high indications of diacetyl and lactic acid), changes in color and excessive turbidity. This study aims at the microbiological investigation of non-pasteurized beer products, before and after filtration, with main emphasis on the detection and molecular characterization of the biodiversity of LAB. Sampling was performed at selected points in a beer production line on industrial scale to determine the population of Total Viable Counts (TVC), yeasts and LAB. The samples are classified in the "Lager" category, fermented using strains of *Saccharomyces pastorianus*. The sampling points included the pre- and post- filtration step, the buffer line, the filling tank, the packaged but non-pasteurized product and finally the packaged pasteurized product to confirm the effectiveness of heat treatment. Samples were collected in two different batch productions. The results showed that the population of LAB was relatively low. Specifically, before filtration levels were 1.52 log CFU/mL and 3.44 log CFU/mL in the first and second batch, respectively. This microbial group was not enumerated (<1.0 log CFU/mL) afterwards in all sampling points. A total of 80 LAB species were initially analyzed by rep-PCR, using the (GTG)5 primer to discriminate the isolates. Representative isolates (20) were selected for further identification using the conserved 16S rRNA region to be sequenced. Three different species were present in both batch productions namely *Lactobacillus brevis*, *Lactobacillus backii* and *Lactobacillus harbinensis*.

Keywords: lager beer; lactic acid bacteria; yeasts; Lactobacillus brevis; Lactobacillus backii; Lactobacillus harbinensis

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1. Introduction

Beer is an undistilled alcoholic beverage derived from a source of starch. Brewing beer differs from wine fermentation in that for brewing, a source of starch must first be converted into fermentable sugars. There are four main ingredients in beer: water, malt, hops and yeast. Even though there is a great variety of products around the world, beer products are divided in two main categories based on the yeast strain involved in processing. More specifically, *Saccharomyces pastorianus* is used to produce "Lager" beer and *Saccharomyces cerevisiae* is used for "Ale" [1]. The distinctions between the yeasts used in ale and lager brewing are mainly focused on their optimal growth temperature and sugar utilization. Ale yeast operates at room temperature (*ca.* 18–22 °C), ferments quickly and produces the characteristic "fruitiness" of most ales while lager yeast prefers lower temperatures (*ca.* 8–15 °C), ferments slowly and utilizes more wort sugars, resulting in a

cleaner, crisp taste. When it comes to utilization, lager yeast strains can utilize maltotriose more rapidly than ale strains and mixtures of galactose and maltose simultaneously, whereas ale strains prefer to utilize maltose [2]. Traditionally, ale yeasts are regarded as top fermenters that form a frothy yeast head on the surface of the fermenting tanks, which is skimmed off to be used for subsequent brews, while lager ones are bottom fermenters that form little surface head and are recovered from the bottom of the fermenter. Today, this is a less useful distinction as many types of ale yeast now have the capacity to fall out of solution and settle at the bottom of the tank [3]. The aim of this study was the microbiological investigation of non-pasteurized lager beer on industrial scale before and after filtration, as well as to determine the population of Total Viable Counts, yeasts and LAB. Additionally, the biodiversity of LAB was elucidated at species level using molecular techniques.

2. Materials and Methods

2.1. Beer Samples, Transportation and Fermentation Procedures

Samples of lager beer from two different batch productions were obtained from an industry located in Central Greece. The sampling points (Figure 1) included the pre- (a) and post- (b) filtration step, the buffer line (c), the filling tank (d), the packaged non-pasteurized product (e) and finally the packaged pasteurized product (f) to confirm the effectiveness of heat treatment. Beer was subjected to fermentation and maturation according to processing procedures employed by the industry. Specifically, wort and yeast strains of *Saccharomyces pastorianus* were processed in industrial fermentation vessels, in which fermentation was undertaken at relatively cold temperatures (*ca.* 10–14 °C) for approximately 6 days. After the end of fermentation, the yeast settled at the bottom of the tank was removed and the maturation phase was initiated. Maturation consisted of two stages, a warm rest (Ruh) to achieve the breakdown of unwanted volatile components followed by cold storage at ca. 2–3 °C so that the yeast in suspension could finalize the flavor profile of the beer [4]. All samples were collected aseptically and transported to the laboratory under refrigeration (ca. 6 °C). Upon arrival, the samples were maintained at 2 °C until analysis.

2.2. Microbiological Analyses

Beer samples were subjected to microbiological analysis for the determination of the population of total viable counts (TVCs), yeasts and lactic acid bacteria (LAB) using the following media: (a) Plate count agar (PCA) for the enumeration of total viable counts (TVCs) after incubation at 25 °C for 3 days; (b) Rose Bengal Chloramphenicol agar (RBC) incubated at 25 °C for 3–5 days for the enumeration of yeasts; and (c) de Man–Rogosa–Sharpe agar (MRS) supplemented with cycloheximide 0.5% and incubated at 30 °C for 5 days for the enumeration of LAB. After visible growth, 20% of the colonies from the proper dilution of the MRS agar plates were randomly selected and purified on the same MRS (Cycloheximide-free) medium, followed by a second incubation phase at the same conditions. Isolates were further characterized based on their colony characteristics, cell morphology and Gram staining.

2.3. Molecular Analyses

Rep-PCR was employed to characterize the biodiversity of 80 LAB isolates using the oligonucleotide (**GTG)5** (5-GTGGTGGTGGTGGTG-3) primer and its optimal PCR program was followed [5]. Banding profiles of the rep-PCR products were undertaken by the electrophoresis, visualized after staining with ethidium bromide under ultraviolet light and were analyzed by the Bionumerics software. Dendrogram generated by Dice/UPGMA analysis and the similarities were calculated using Pearson correlation and Unweighted Pair Group Method with Arithmetic mean (Supplementary Figure S1). Finally,

20 representative isolates were selected for partial sequencing analysis of 16S rRNA region [5].

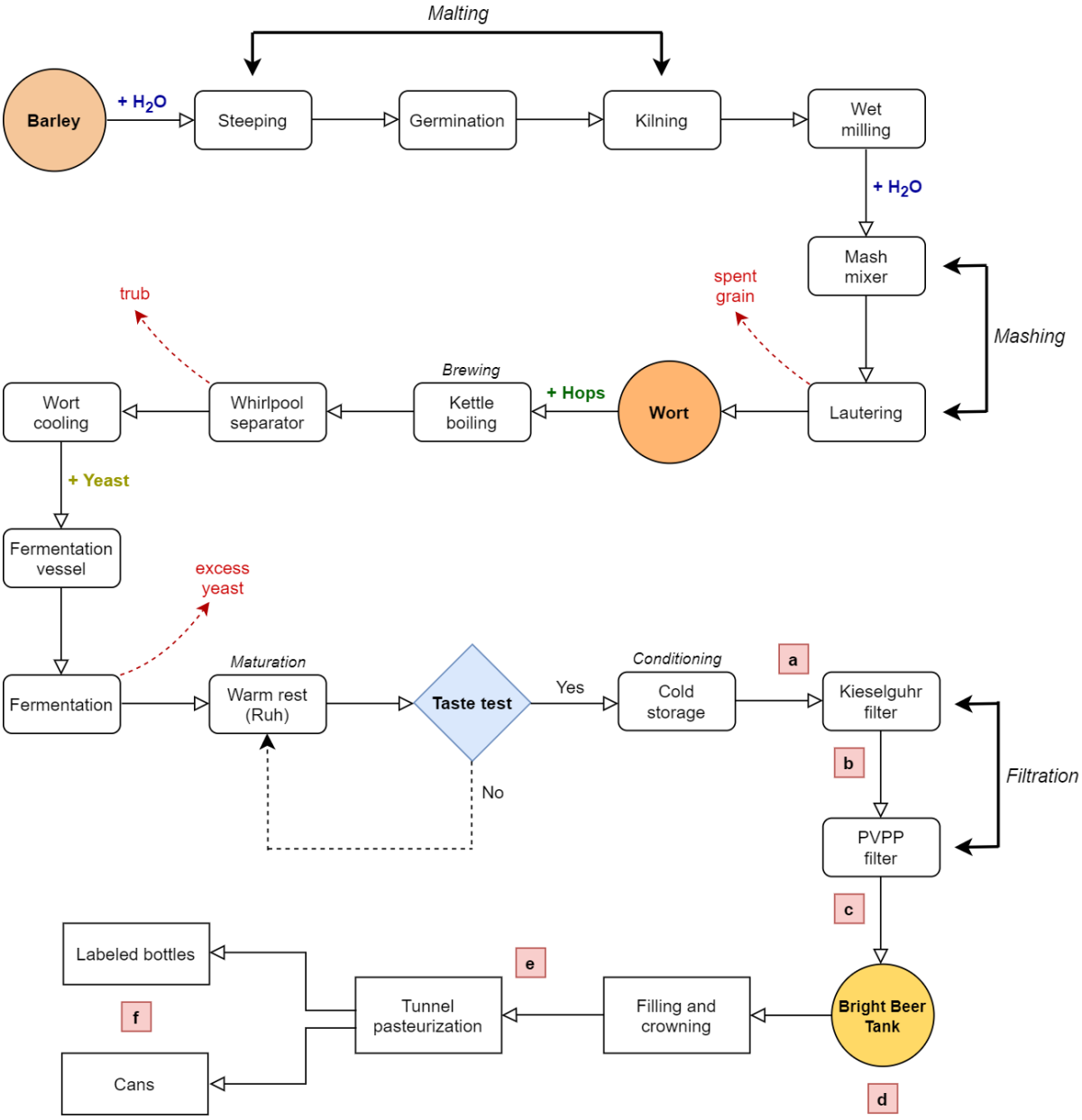

Figure 1. Flowchart of Lager-type beer production. Sampling points are highlighted as letters in red squares.

3. Results and Discussion

3.1. Microbiological Results

Despite the fact that there were 6 sampling points, yeast presence was enumerated only before and after filtration. More specifically, before Kieselguhr filtration yeast levels were 5.40 log CFU/mL and 4.98 log CFU/mL in the first and second batch, respectively. However, it needs to be noted that post-filtration yeast absence (<1.0 log CFU/mL) was observed only in the first batch production, while low population levels of 1.36 log CFU/mL were counted in the second batch. Taking into consideration that another poly-vinylpyrrolidone (PVPP) filter interfered between sampling points (b) and (c) (Figure 1) and knowing that yeast absence was also observed in the second batch after sampling point (c), it is safe to attribute these results to effective filtration conditions. As a fact, this work is in good agreement with previous researchers who studied the determination of

optimal Kieselguhr doses and filtration time to improve beer filtration [6]. The results also showed that the population of LAB was relatively low. Before Kieselguhr filtration, levels were 1.52 log CFU/mL and 3.44 log CFU/mL in the first and second batch respectively. No LAB population could be enumerated (<1.0 log CFU/mL) afterwards in all sampling points.

3.2. Molecular Identification of LAB

The results of the total of 80 LAB isolates which were subjected to molecular analysis to characterize the biodiversity of this microbial group are presented in Figure 2. The LAB species *Lactobacillus brevis, Lactobacillus backii and Lactobacillus harbinensis* were the only 3 identified species among the samples. In general, *L. brevis* was the most dominant in both batches but there was an opposing trend concerning the two other LAB species on each batch production. Specifically, *L. harbinensis* was abundant (46.9%) with *L. backii* presenting a low isolation frequency (6.3%) in the first batch, while *L. backii* was abundant (39.6%) with *L. harbinensis* presenting a low isolation frequency (10.4%) in the second batch. The composition of the microbial community reported in this work is in line with previous publications investigating bacterial biofilms as a possible source of contamination in a brewery environment or elucidating metabolic pathways of LAB as spoilage agents in beer products [7–9].

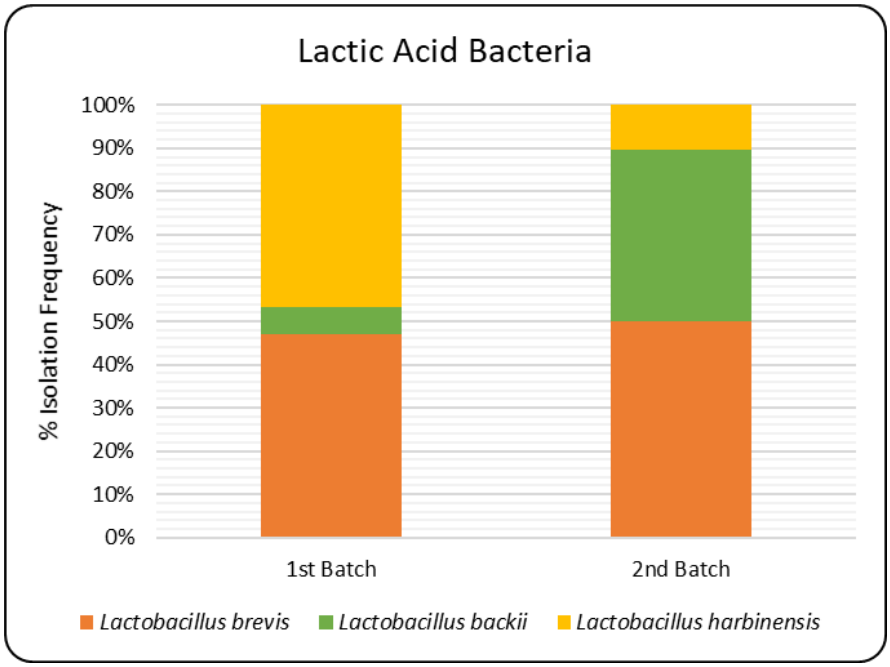

Figure 2. Isolation frequency (%) of LAB in the 1st and 2nd batch productions.

4. Conclusions

The present research elucidates the diversity of LAB isolated from industrially fermented and non-pasteurized Lager-type beer, one of the most economically important beer products in Greece. The relatively low population before the first sampling point (Kieselguhr filtration) indicates that hygienic conditions are partially successful. No enumerated population of LAB, after Kieselguhr filtration, advocates the production of a safe and high-quality final product. As a potential subject for further investigation, it would be interesting to examine the mechanisms that make it difficult for LAB cells to get over the Kieselguhr filter even though its pore diameter is multiple times wider than the dimensions of bacterial cells.

Supplementary Materials:

Author Contributions: Conceptualization, G.T., P.T. and E.Z.P.; methodology, G.T. and P.T.; investigation, G.T. and P.T.; resources, E.Z.P.; writing—original draft preparation, G.T.; writing—review and editing, G.T., P.T. and E.Z.P.; visualization, G.T. and E.Z.P.; supervision, E.Z.P. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

LAB	Lactic Acid Bacteria
TVC	Total Viable Count
PCA	Plate Count Agar
RBC	Rose Bengal Chloramphenicol
MRS	Man Rogosa Sharpe
PCR	Polymerase Chain Reaction
CFU	Colony Forming Unit

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