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Original

The occurrence of beer spoilage lactic acid bacteria in craft beer production / Garofalo, Cristiana; Osimani, Andrea; Milanovic, Vesna; Taccari, Manuela; Aquilanti, Lucia; Clementi, Francesca. - In: JOURNAL OF FOOD SCIENCE. - ISSN 0022-1147. - ELETTRONICO. - 80:12(2015), pp. 2845-2852. [10.1111/1750-3841.13112]

Availability:

This version is available at: 11566/228807 since: 2022-06-01T17:24:24Z

Publisher:

Published

DOI:10.1111/1750-3841.13112

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(Article begins on next page)



The Monitoring of Beer Spoilage Lactic Acid Bacteria in Craft Beer Production

Journal:	<i>Journal of Food Science</i>
Manuscript ID:	Draft
Manuscript Type:	6 JFS: Food Microbiology and Safety
Date Submitted by the Author:	n/a
Complete List of Authors:	Garofalo, Cristiana; Università Politecnica delle Marche, Dipartimento di Scienze Agrarie, Alimentari ed Ambientali Osimani, Andrea; Università Politecnica delle Marche, Dipartimento di Scienze Agrarie, Alimentari ed Ambientali Milanović, Vesna; Università Politecnica delle Marche, Dipartimento di Scienze Agrarie, Alimentari ed Ambientali Taccari, Manuela; Università Politecnica delle Marche, Dipartimento di Scienze Agrarie, Alimentari ed Ambientali Aquilanti, Lucia; Università Politecnica delle Marche, Dipartimento di Scienze Agrarie, Alimentari ed Ambientali Clementi, Francesca; Università Politecnica delle Marche, Dipartimento di Scienze Agrarie, Alimentari ed Ambientali
Keywords:	craft beer, Lactobacillus brevis, air sampling, PCR-DGGE, brewery hygiene

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Manuscripts

Ancona, 21/04/2015

Editor in Chief of Journal of Food Science

Dr. Andrea Osimani, Ph.D.

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Dear Editor,

would you please consider the manuscript titled "The Monitoring of Beer-Spoilage Lactic Acid Bacteria in Craft Beer Production" for publication in Journal of Food Science. The aim of this research study was to track and monitor beer spoilage lactic acid bacteria inside a brewery and during the craft beer production process through culture-dependent methods and PCR-DGGE. The results obtained demonstrated the effectiveness of this combined approach. Furthermore, a deeper knowledge on beer spoilage agents and sources was achieved, which was fundamental for the implementation of a brewery sanitization plan and for preserving the quality of the final products.

The manuscript has been completely revised by an English mother tongue and has not been previously published or considered for publication elsewhere.

Thank you very much for your kind consideration.

Best regards

Andrea Osimani

The Monitoring of Beer Spoilage Lactic Acid Bacteria in Craft Beer Production

Short version of the title: (Beer spoilage lactic acid bacteria)

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Abstract

Beer is one of the world’s most ancient and widely consumed fermented alcoholic beverages produced with water, malted cereal grains (generally barley and wheat), hops, and yeast. Beer is considered an unfavourable substrate of growth for many microorganisms however, there are a limited number of bacteria and yeasts which are capable of growth and may spoil beer especially if it is not pasteurized or sterile-filtered as craft beer.

The aim of this research study was to track and monitor beer spoilage lactic acid bacteria (LAB) inside a brewery and during the craft beer production process. To that end, indoor air and work surface samples, collected in the brewery under study, together with commercial active dry yeasts, exhausted yeasts, yeast pellet (obtained after mature beer centrifugation), and spoiled beers were analyzed through culture-dependent methods and PCR-DGGE in order to identify the contaminant LAB species and the source of contamination. *Lb. brevis* was detected in a spoiled beer and in a commercial active dry yeast. Other LAB species and bacteria ascribed to *Staphylococcus* sp., *Enterobacteriaceae*, and *Acetobacter* sp. were found in the brewery.

In conclusion, the PCR-DGGE technique coupled with the culture-dependent method was found to be a useful tool for identifying the beer spoilage bacteria and the source of contamination. The monitoring of raw materials, by-products, final products and the brewery was useful for implementing a sanitization plan to be adopted in the production plant.

Pratical applications

In-depth studies of beer spoilage agents and sources are fundamental for the implementation of a brewery sanitization plan and for preserving the quality of the final products. The combination of a culture-dependent and -independent approach using PCR-DGGE and the monitoring plan applied along the craft beer production process were efficient in identifying the beer spoilage bacteria and the source of contamination. The importance of a specific and efficient sanitization plan is confirmed, based on the application of hygiene and good manufacturing practices which are often the most effective methods for managing microbiological risk.

Keywords: craft beer; *Lactobacillus brevis*; air sampling; PCR-DGGE; brewery hygiene

Introduction

Beer is one of the world's most ancient and widely consumed fermented alcoholic beverages produced with water, malted cereal grains (generally barley and wheat), hops, and yeast.

The popularity of niche beers has increased in recent years and the market is progressively shifting from mass-produced lager brands to the richer flavors, quirky ingredients and striking aesthetics of craft beers which are characterized by a unique aroma and taste (Canonico and others 2014; Aquilani and others 2015).

Beer is considered an unfavourable substrate of growth for many microorganisms due to several factors such as: i) the ethanol concentration ranging from 0.5 to 10% (w/w), ii) the presence of hop compounds that have antimicrobial activity, iii) the low pH (usually around 3.8-4.7), iv) the reduced oxygen content and nutrient availability, v) the high CO₂ content (Sakamoto and Konings 2003; Manzano and others 2005; Rouse and Van Sinderen 2008; Menz and others 2010). Despite these hostile properties, there are a limited number of bacteria and yeasts which are capable of growth and may spoil beer especially if it is not pasteurized or sterile-filtered as craft beer (Sakamoto and Konings 2003; Hill 2009; Menz and others 2010). In particular, lactic acid bacteria (LAB) are well-known as the main spoilage agents since it has been assessed that they are responsible for 60-70% of all cases of beer spoilage (Sakamoto and Konings 2003; Rouse and Van Sinderen 2008; Menz and others 2010). Among LAB, a few hetero- and homofermentative species belonging to *Lactobacillus* and *Pediococcus* genera are recognized as the leading causes of beer spoilage events (Rouse and Van Sinderen 2008). In particular, *Lactobacillus brevis* is the most frequent and difficult to eliminate, since it may persist in the production environment also due to the ability of some *Lb. brevis* strains to form biofilms (Rouse and Van Sinderen 2008; Leathers and others 2014). Other *Lactobacillus* species ascribed to *Lactobacillus lindneri*, *Lactobacillus buchneri*, *Lactobacillus parabuchneri*, *Lactobacillus casei*, *Lactobacillus coryneformis*, *Lactobacillus malefermentans* and *Lactobacillus curvatus*, have also been found as less common beer spoilers (Jespersen and Jakobsen 1996; Rouse and Van Sinderen 2008). Among pediococci, several species have been reported in breweries, such as *Pediococcus damnosus*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Pediococcus dextrinicus*, *Pediococcus inopinatus*, and *Pediococcus parvulus* although *P. damnosus* is considered the main beer spoilage species together with, although to a lesser extent, *Pediococcus dextrinicus* and *Pediococcus inopinatus* (Jespersen and Jakobsen 1996; Sakamoto and Konings 2003; Rouse and Van Sinderen 2008). The growth of these bacteria during the brewing process implies a competition for nutrients with yeast thus causing a reduction in the yeast fermentation and therefore decreased ethanol yields (Rouse and Van Sinderen 2008). Furthermore, these bacteria are generally characterized by resistance to hop compounds thus explaining their presence and growth in the final product (Rouse and Van Sinderen 2008; Haakensen

and others 2009). The beer alteration caused by LAB spoilage consists in the production of off-flavours (mainly due to diacetyl and lactic acid), changes in colour and an increase in turbidity (Rouse and Van Sinderen 2008; Menz and others 2010). Furthermore, this detrimental effect on beer quality is also the cause of huge economic losses in the brewery sector (Sakamoto and Konings 2003). Sources of LAB contamination are widespread and they can mainly be traced to the raw materials used for beer processing and to the brewery environment (indoor air, surfaces, equipment...). Some papers have dealt with the detection of LAB spoilage in beers and breweries (Manzano and others 2005; Menz and others 2010), another focused on methods to control microbial contamination by the use of specific cleaning procedures (Manzano and others 2011), while others have been aimed at defining a rapid and sensitive method for the detection of beer spoilage bacteria before the beer is bottled and sold (Takahashi and others 2000; Asano and others 2009). However, contamination by beer spoilage bacteria in the brewing industry is still an unsolved problem especially in craft beer production where the beer is often unpasteurized or sterile-filtered. Contamination by LAB is insidious and, to our knowledge, there is currently no standardized and reliable method for the early detection of beer spoilage LAB. The aim of this research study was to track and monitor beer spoilage LAB in the brewery and during the craft beer production process by culture-dependent methods and PCR-DGGE. In detail, indoor air and work surface samples collected in the brewery involved in the study, together with commercial active dry yeasts, exhausted yeasts, yeast pellet (obtained after mature beer centrifugation), and spoiled beers were sampled and analyzed in order to identify the contaminant LAB species and the source of LAB contamination.

Materials and Methods

Reference strains and culture conditions

Two bacterial reference strains, namely *Lb. brevis* DSMZ 20556^T and *Pd. pentosaceus* DSMZ 20336^T, were used as controls in the PCR-DGGE analyses. These cultures were purchased from the “Deutsche Sammlung von Mikroorganismen und Zellkulturen” (DSMZ Collection, Braunschweig, Germany, <http://www.dsmz.de/>). They were incubated at 30 °C for 48 h under anaerobiosis on MRS agar (Oxoid, Basingstoke, UK).

Description of the brewery and beer production

The brewery produces special craft beers made of malt, hops added with spelt or grass peas. The plant is divided into five different environments (main room, milling station, cooking room, warehouse and bottled beer storage room) in which all the different production activities are carried out (Figure 1). The main room is an open-space which houses the fermenter, the centrifuge, the maturation tank and the bottler; the mill for the production of malt, spelt or grass pea grist is housed in the milling station near the cooking room, the mashing tun is located in the latter and linked to the fermenter through stainless steel tubes. After mashing and boiling (98 °C) the malt, hops, spelt/grass peas and water, the wort is centrifuged, cooled at 12-20 °C and pumped to the fermenter; a commercial active dry yeast is added and the fermentation is carried out at 20 °C for 6 days, after which the temperature is progressively lowered to 2 °C. At the end of fermentation the beer is centrifuged at 8000 rpm and then transferred to the maturation tank where fructose syrup is added at 0.005% (v/v) for priming (48 h). After priming, the beer is bottled and left at 20 °C for 2 weeks in order to allow re-fermentation before final aging at 4 °C and storage at room temperature (Figure 2).

Microbiological analyses on brewery indoor air

Airborne bacterial contamination was studied using a calibrated impaction sampler (SAS Super 90, International-Pbi, Milan) which was placed in the centre of each room at 1 m from the floor (Osimani and others 2013b). Using this active air sampler, the microbial cells are impacted on agarized culture medium contact plates where they form colonies after incubation. The density of microorganisms in a given air volume is therefore calculated by knowing both the sampled air volume and the number of colonies grown on plates. The environments subjected to air sampling and monitored air volumes are reported in Table 1.

Before sampling, the cover of the air sampler was cleaned with propanol, as advised by the manufacturer. To avoid interference from outdoor air currents, all windows, if present, were kept closed during the sampling. For each area, field blanks were obtained by loading and immediately unloading one set of sampling media. All the samples and blanks were maintained under refrigerated conditions and taken for incubation to the laboratory on the day of collection. Airborne LAB were counted on contact plates with MRS agar (Oxoid) containing 500 mg/L of cycloheximide to inhibit the growth of eumycetes. Bacteria enumerations were carried out after 2 days incubation at 37 °C under anaerobiosis.

Microbiological analyses on work surfaces

Traditional hygiene swabbing was performed on work surfaces as detailed in Table 1. Microbiological samples were collected using sterile cotton swabs and tubes containing 10 mL of sterile 0.1% peptone solution (Oxoid, Basingstoke, UK) added with 30 g/L of Tween 80 (Liofilchem, Roseto, Italy) for the inactivation of any possible residues of the

disinfecting agent used for sanitization (Osimani and others 2011; Osimani and others 2013a; Osimani and others 2014). Samples were transferred to the laboratory under refrigerated conditions and immediately subjected to viable cell counting; in more detail, samples were serially ten-fold diluted in a sterile peptone-saline solution (1 g/L peptone and 8.5 g/L NaCl) and aliquots (0.1 mL) of each dilution were used for counting LAB on MRS agar (Oxoid) added with 500 mg/L of cycloheximide incubated for 2 days at 37 °C under anaerobiosis.

pH measurements

The pH potentiometric measurements on spoiled beer samples were carried out with a model 300 pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy). For each sample, three independent measurements were performed.

Microbiological analyses of the active dry yeasts, by-products and beers

In order to trace bacterial contamination the following samples were collected: i) three commercially active dry yeasts (A, B, C) in sterile vacuum packages routinely used by the brewery; ii) two samples of exhausted yeasts obtained from different batches; iii) a yeast pellet obtained after mature beer centrifugation; iv) two different batches of spoiled spelt beer (beer 1 and 2) and one batch of spoiled grass pea beer (beer 3). All of the samples were stored under refrigerated conditions until microbiological analyses.

Active dry yeasts were rehydrated following the manufacturer's instructions; 10 mL aliquots of rehydrated yeast, exhausted yeasts and yeast pellet were ten-fold diluted in a sterile peptone-saline solution and aliquots (0.1 mL) of each dilution were used for counting LAB on MRS agar (Oxoid) added with 500 mg/L of cycloheximide incubated for 2 days at 37 °C under anaerobiosis. In parallel, 10 mL aliquots of the same samples were subjected to enrichment in 90 mL of MRS broth (Oxoid) added with 500 mg/L of cycloheximide, incubated for 7 days at 37 °C and streaked on MRS agar (Oxoid) added with 500 mg/L of cycloheximide incubated for 2 days at 37 °C under anaerobiosis.

One mL aliquots of beer samples underwent LAB enumeration on MRS agar (Oxoid) added with 500 mg/L of cycloheximide incubated for 2 days at 37 °C under anaerobiosis.

For each beer sample, the morphology of the suspended microbial cells was examined using a light microscope under oil immersion (100x).

In parallel, the same analyses were conducted on unspoiled beers as controls.

Bulk cell formation

For all the samples, bulk cells were prepared after bacterial counting. Briefly, colonies were washed off the MRS agar media with 2 mL saline solution and glycerol (0.85% NaCl, 50% glycerol); colony washes were stored at -20 °C (Garofalo and others 2015). Low (confluent colonies) and high (colonies ranging from 30 to 300) sample dilution plates were considered for the beer samples.

Direct DNA extraction from active dry yeasts, by-products and beers

The microbial DNA was extracted directly from the rehydrated yeasts, by-products (exhausted yeasts and pellet yeast) and beer samples, using the PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA). In detail, 1 mL of each sample was centrifuged to produce a pellet that was processed according to the kit manufacturer's instructions. The DNA quantity and purity were assessed by optical readings at 260, 280 and 234 nm, respectively, using a UV-Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

DNA extraction from bulk cells and pure microbial cultures

Three hundred µL of each bacterial suspension, either from bulk cells, enrichments (where applied) or from the pure reference strains, underwent DNA extraction using the method proposed by Hynes and others (1992) with some modifications reported by Osimani and others (2015). The DNA quantity and purity were assessed as described above.

PCR-DGGE analyses

To analyze both the DNA extracted directly from the samples and the DNA extracted from bulk cells, 100 ng of each DNA were amplified through PCR in 50 µL reaction volume using the universal prokaryotic primers 338f_{GC} and 518r that target the V3 region of the 16S rRNA gene (Osimani and others 2015).

Five microliters of each PCR product were checked by electrophoresis in 1.5% (w/v) agarose gels (Laboratorios CONDA, Madrid, Spain) using a GeneRuler DNA Ladder mix (Thermo Scientific Fermentas, Pittsburgh, USA) as a molecular weight standard (Osimani and others 2015). Gels were visualized under UV light and photographed with the Complete Photo XT101 system (Explera, Jesi, Italy).

A vertical DCode electrophoresis system (Bio-Rad Laboratories, Hercules, USA) was used for the DGGE analysis. PCR products (25 µL) obtained with primers 338f_{GC}/518r were applied to 0.8 mm polyacrylamide gel [8% (w/v) acrylamide/bisacrylamide gel 37.5:1], containing a 30-60% urea-formamide denaturing gradient increasing in the direction of the electrophoresis (100% corresponds to 7 M urea and 40% (w/v) formamide), and run with 1X TAE

buffer (0.04 mol L^{-1} Tris-acetate, 0.001 mol L^{-1} EDTA). The gels were run at a constant voltage of 130 V for 4 h at 60 °C. After electrophoresis, the gels were stained for 20 min in 1X TAE buffer containing SYBR Green I Stain 1X (Lonza, USA), visualized under UV light and photographed with the Complete Photo XT101 system (Explera). For the preliminary identification of DGGE bands, an identification ladder (Mix) was prepared by mixing suitable amounts (5 µl) of the 338f_{GC}/518r amplicons obtained from the pure cultures of the two bacteria reference strains.

Sequencing of the DGGE bands and sequence analysis

The DGGE bands were excised by the gels using sterile cutting tips and the DNA from each band was eluted in 50 µL sterile deionized water overnight at +4 °C (Garofalo and others 2008). Five microliters of the eluted DNA were re-amplified under the same conditions as described above, with the forward primer 338f without the GC clamp. These PCR amplicons were purified using a GFXTM PCR DNA and Gel Band Purification Kit (Euroclone, Milan, Italy) following the manufacturer's instructions and were then sent to Macrogen (Amsterdam, The Netherlands) for sequencing. The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database using the basic BLAST search tools (Altschul and others 1990).

Results and Discussion

This study investigated three different kinds of spoiled craft beers produced with spelt and grass peas as raw materials. The three beer samples were characterized by turbidity and the following pH values: 4.23 ± 0.1 (beer 1), 4.29 ± 0.1 (beer 2) and 4.03 (beer 3); beer 3 was also slimy. The microscopic analyses revealed coccal shaped bacterial cells in beer 1, while beer 2 contained coccal shaped bacterial cells, both isolated and in tetrads, together with short bacilli, and beer 3 contained yeast cells, coccal shaped bacterial cells and long and short bacilli. Viable counts on MRS agar ranged from 7.0×10^4 to 4.8×10^6 (Table 2). From the PCR-DGGE analyses of the DNA recovered both directly from beers and from bulk cells (from both high and low dilution agar plates) the closest relatives to *Lb. brevis* were found to be ubiquitous (Table 2). This species identification was also confirmed by analyzing five other spoiled beers (4 beers produced using spelt and 1 beer obtained using grass peas as raw materials) from different batches (data not shown). On the contrary, no LAB viable counts and no bacterial cells were found on unspoiled beers.

LAB of the genera *Lactobacillus* and *Pediococcus* are considered the main cause of beer spoilage (Sakamoto and Konings 2003; Rouse and Van Sinderen 2008; Menz and others 2010). PCR-DGGE was found to be a useful technique for identifying the contaminant LAB species in the spoiled beers, which was ascribed to *Lb. brevis*. In detail, the species *Lb. brevis* was characterized by four DGGE bands migrating at different positions in the acrylamide gel (data not

shown). This is probably a result of the presence of multiple heterogeneous copies of the 16S rRNA gene within the same species thus leading to a DGGE profile with multiple bands due to the different electrophoretic mobility of the PCR amplicons in the gel. This PCR-DGGE bias has already been highlighted by other authors (Ercolini 2004; Coccolin and others 2013; Garofalo and others 2015) who indicated the need to sequence all the visualized DGGE bands in order to arrive at an exact determination of the sample biodiversity. The rigorous and reliable identification of the beer spoilage species is fundamental in order to define an effective cleaning and sanitization plan specifically tailored to the contaminant species (f.i. if the contaminant is a biofilm-producing species). In particular, the detection of *Lb. brevis* confirmed previous studies that reported this obligate heterofermentative LAB species as the most common and problematic beer spoilage species due to its optimal growth at 30 °C and at pH comprised between 4.0 and 6.0, its physiological versatility, its resistance to hop compounds and the ability of some strains to develop biofilms (Sakamoto and Konings 2003; Rouse and Van Sinderen 2008; Leathers and others 2014). Finally, it is worth noticing that unspoiled beer did not show any LAB viable counts, thus indicating that LAB contamination and, in particular, that of *Lb. brevis*, is directly linked to alterations in the beer.

In order to discover the source of *Lb. brevis* contamination a detailed monitoring plan was adopted which involved the analysis not only of raw materials and by-products, such as active dry yeasts, exhausted yeasts, yeast pellet, but also of the brewery environment (indoor air and some selected key work surfaces).

The results of the microbiological analyses carried out on three different commercial active dry yeasts (ADY), two exhausted yeasts (EY), a yeast pellet (YP) and three different spoiled beers are reported in Table 2. Concerning the three active dry yeasts, the LAB viable counts were very low or even absent in ADY 1. The closest relatives to *Pd. acidilactici*/*Pediococcus stilesii* were found as a common species in the three active dry yeasts after enrichment through PCR-DGGE analyses. Furthermore, the closest relatives to *Pd. acidilactici*/*Pd. stilesii* were found in ADY 1 after PCR-DGGE analyses on DNA extracted directly from this matrix and in ADY 2 by analyzing the DNA from bulk cells. The closest relatives to different *Lactobacillus* species were also identified among the three different active dry yeasts. In detail, the closest relatives to *Lb. brevis* were found from ADY 1 after enrichment; the closest relatives to *Lb. parabuchneri* were identified in ADY 2 by analyzing DNA obtained from bulk cells; the closest relatives to *Lactobacillus xiangfangensis*/*Lactobacillus fabifermentans*/*Lactobacillus plantarum*/*Lactobacillus paraplantarum*/*Lactobacillus pentosus* and *Lactobacillus fermentum* were revealed in ADY 3 by analyzing DNA from bulk cells and DNA from cells after enrichment, respectively. Bacterial species were not detected by PCR-DGGE analyses of DNA extracted directly from ADY 2 and 3, probably due to the low number of bacterial cells within these samples. By contrast, the closest relatives to *Pd. acidilactici*/*Pd. stilesii* were found by analyzing DNA extracted

directly from ADY 1 although no viable counts, and consequently bulk cells, were recovered. This result was probably due to the presence of viable but non-cultivable (VBNC) cells within this active dry yeast sample.

The fact that of the three commercial active dry yeasts analyzed only one (ADY 1) showed the presence of *Lb. brevis*, and immediately after the enrichment step, may indicate the low cellular number of *Lb. brevis* in this product. However, the continuous addition of this active dry yeast during the beer production process may also represent a constant inoculum of *Lb. brevis* that may multiply easily in the final product at the re-fermentation stage and during storage at room temperature and may consequently be accumulated over time within the brewery environment. This latter hypothesis seems to be confirmed by the fact that, in the absence of an efficient and frequent sanitization plan, after about six months the beers were again spoiled by *Lb. brevis* (data not shown). Probably in this period *Lb. brevis*, deriving from active dry yeasts, was able to accumulate and spread in the brewery environment and, hence, grow on beer.

Other LAB species, ascribed to *Pd. acidilactici*/*Pd. stilesii*, *Lb. parabuchneri*, *Lb. xiangfangensis*/*Lb. fabifermentans*/*Lb. plantarum*/*Lb. paraplantarum*/*Lb. pentosus* and *Lb. fermentum* were also found in the three commercial active dry yeasts analyzed. Among these species only *Lb. parabuchneri*, has previously been identified as a possible beer spoilage agent (Jespersen and Jacobsen 1996; Sakamoto and Konings 2003). In fact, although pediococci are also known as beer spoilage agents, the species *Pd. acidilactici* has never been reported as producing defects in mature beer (Sakamoto and Konings 2003). However, these findings were not considered to be a cause for concern since they were not present within the spoiled beers.

The exhausted yeasts and the yeast pellet did not show the presence of bacterial DNA and of LAB colonies on MRS agar plates. This result was probably due to the fact that, on the one hand, any bacterial cells present in the fermenter do not precipitate to the bottom together with the exhausted yeasts, and on the other hand, the bacterial cells cannot be collected in the yeast pellet after centrifugation due to the low speed applied which is necessary to partially keep the yeasts in suspension for subsequent bottle re-fermentation.

In order to verify the possible presence of *Lb. brevis* in the brewery environment a monitoring plan that involved microbiological analyses coupled with PCR-DGGE of the indoor air and selected work surfaces was applied.

The results of the microbiological analyses obtained using the calibrated impaction sampler in the brewery under study are reported in Table 1. The LAB viable counts were generally low. The colonies were collected in bulk and the extracted DNA was analyzed through PCR-DGGE using a universal primer for eubacteria. The closest relatives, the percent identities, and the accession numbers of the obtained sequences are given in Table 1. Several closest relatives to species belonging to the genera *Staphylococcus* were found in the cooking room, milling station and in the bottler

together with the closest relatives to *Leuconostoc pseudomesenteroides* in the latter case. The closest relatives to *Acetobacter* sp. were also detected in the fermenter room.

The results of the microbiological analyses performed on the selected work surfaces are reported in Table 1. In detail, viable counts were not found on MRS agar plates from the maturation tank either before or after cleaning nor on a connection hose before cleaning. From 1 to 60 ufc/cm² were detected after the analysis of two different hoses before cleaning and of the mill surface. The PCR-DGGE analyses of the DNA extracted from the bulk cells revealed the presence of the closest relatives to *Enterobacteriaceae* bacterium, *Salmonella enterica*, *Enterobacter* sp. and *Acetobacter* sp. in the two hoses analyzed and the presence of the closest relatives to *Lactobacillus graminis*/*Lactobacillus curvatus* on the surface of the mill.

Lb. brevis was never detected inside the brewery environment thus indicating that during the microbiological monitoring period *Lb. brevis* was not widespread. However some other bacterial species which may be considered dangerous for human health were found in the plant. In fact, an analysis of the indoor air showed that some species belonging to the *Staphylococcus* genera were widespread, including *Staphylococcus saprophyticus* and *Staphylococcus petrasii*/*Staphylococcus jettensis*/*Staphylococcus hominis*/*Staphylococcus haemolyticus*. *St. petrasii*, *St. jettensis*, *St. hominis*, and *St. haemolyticus* are coagulase-negative staphylococci which are phylogenetically related and phenotypically similar (Pantůček and others 2013). These bacteria are ubiquitous and human common commensals isolated mainly from the skin and mucous membranes of mammals. However, they have been recently recognized as opportunistic pathogens in several human infections (Pantůček and others 2013) as also demonstrated for *St. saprophyticus* which was found to be involved in urinary tract infection mainly in young women (Raz and others 2005). Other taxa were also found, such as *Leuc. pseudomesenteroides* in the bottler indoor air and *Acetobacter* sp. in the fermenter room. *Acetobacter* species belong to the group of acetic acid bacteria that are characterized by gram-negative, rod-shaped cells, widespread on several cereals and fruits, with spoilage activity in some products such as wine, as a result of their ability to oxidize sugars and alcohols to organic acids (Sengun and Karabiyikli 2011). *Acetobacter* sp. were also found by analyzing a connection hose in the fermenter room before cleaning, thus showing the same localization previously detected by analyzing the fermenter room indoor air. Members of the *Enterobacteriaceae* family were mainly found on the work surfaces analyzed. In particular, *S. enterica*, that was identified in a connection hose in the fermenter room before cleaning, contains over 2,000 serovars. Some of these, such as *Salmonella enterica* serovar Typhi, lead to systemic infections and typhoid fever, whereas others, such as *Salmonella enterica* serovar Typhimurium, determine gastroenteritis (McClelland and others 2001). Although none of these pathogenic species are able to grow in beer since they are inhibited by the low beer pH and therefore they do not represent a risk for the

consumers, they may be harmful for the health of the people involved in the beer production process. Hence, an implementation of the sanitization program and hygiene regimes in the brewery environment are necessary in order to avoid any possible health risks for the brewery staff.

Considering the overall results the importance of a specific and efficient sanitization plan is confirmed, based on the application of hygiene and good manufacturing practices which are often the most effective methods for managing microbiological risk (Hill 2009). Therefore, the inalienable ingredients for an effective microbiological control must include: i) a good plant design; ii) an efficient plant maintenance/renewal; iii) the use of cleaning-in-place; iv) effective detergents and sanitizers and; v) a stringent microbiological monitoring (Hill 2009). As reported by Hill (2009), a knowledge of spoilage microorganisms which may be present in the brewery environment and the control of microbial fouling both play a pivotal role in the prevention of microbial beer spoilage. In addition, a proper microbiological monitoring must be implemented since a low sample volume in relation to typical batch volumes (f.i. 250 mL samples collected from more than 1000 hectoliters of beer) and the heterogeneity of the potential beer spoiling bacteria may increase the difficulty in detecting trace contaminants (Hill 2009). Therefore, the enrichment step applied in this study (5-7 days at 37 °C) and microbiological monitoring may be an efficient method for the early detection of LAB species in the beer before bottling, thereby preventing huge economic losses. In fact the enrichment step may highlight the presence of even a few cells of LAB contaminants deriving from commercial active dry yeasts, as found in this study. In order to eliminate this contamination an appropriate sanitization plan has to be frequently applied within any brewery that hosts beer spoilage LAB species deriving from commercial active dry yeasts which may accumulate after continuous inoculum.

Conclusions

In conclusion, the PCR-DGGE technique coupled with a culture-dependent method that specifically envisages an enrichment step was found to be a useful tool for identifying *Lb. brevis* as the beer spoilage species and the source of beer contamination. The monitoring plan applied for the raw materials, by-products, final products and the brewery environment was suitable for developing an efficient and thorough sanitization plan within the brewery, even if currently there are no criticalities linked to the brewery environment in terms of beer spoilage agents.

Author Contributions

All the co-authors contributed to the planning of the study and the interpretation of the results. The sampling, culture-dependant analyses and PCR-DGGE analyses were carried out by C. Garofalo, A. Osimani, V. Milanović and M.

Taccari. The initial manuscript draft was written by C. Garofalo and A. Osimani; the draft was further critically read and improved by all the co-authors.

References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215(3):403-10.
- Ampe F, Ben Omar N, Moizan C, Wachter C, Guyot JP. 1999. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Appl Environ Microbiol* 65(12):5464-73.
- Aquilani B, Laureti T, Poponi S, Secondi L. 2015. Beer choice and consumption determinants when craft beers are tasted: An exploratory study of consumer preferences. *Food Qual Prefer* 41:214-24.
- Asano S, Iijima K, Suzuki K, Motoyama Y, Ogata T, Kitagawa Y. 2009. Rapid detection and identification of beer-spoilage lactic acid bacteria by microcolony method. *J Biosci Bioeng* 108(2):124-9.
- Canonico L, Comitini F, Ciani M. 2014. Dominance and influence of selected *Saccharomyces cerevisiae* strains on the analytical profile of craft beer refermentation. *J Inst Brew* 120:262-67.
- Ercolini D. 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J Microbiol Methods* 56:297-314.
- Cocolin L, Alessandria V, Dolci P, Gorra R, Rantsiou K. 2013. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *Int J Food Microbiol* 167:29-43.
- Garofalo C, Osimani A, Milanović V, Aquilanti L, De Filippis F, Stellato G, Di Mauro S, Turchetti B, Buzzini P, Ercolini D, Clementi F. 2015. Bacteria and yeast microbiota in milk kefir grains from different Italian regions, *Food Microbiol* 49:123-33.
- Garofalo C, Silvestri G, Aquilanti L, Clementi F. 2008. PCR-DGGE analysis of lactic acid bacteria and yeast dynamics during the production processes of three varieties of Panettone. *J Appl Microbiol* 105(1):243-54.
- Haakensen M, Schubert A, Ziola B. 2009. Broth and agar hop-gradient plates used to evaluate the beer-spoilage potential of *Lactobacillus* and *Pediococcus* isolates. *Int J Food Microbiol* 130:56-60.
- Hill AE. 2009. Microbiological stability of beer. *Beer* 163-83.
- Hynes WL, Ferretti JJ, Gilmore MS, Segarra RA. 1992. PCR amplification of streptococcal DNA using crude cell lysates. *FEMS Microbiol Lett* 73(1-2):139-42.

- 389 Jespersen L, Jakobsen M. 1996. Specific spoilage organisms in breweries and laboratory media for their detection.
390 Int J Food Microbiol 33:139-55.
- 391 Leathers TD, Bischoff KM, Rich JO, Price NPJ, Manitchotpitit P, Nunnally MS, Anderson AM. 2014. Inhibitors of
392 biofilm formation by biofuel fermentation contaminants. Bioresource Technol 169:45-51.
- 393 Manzano M, Giusto C, Bartolomeoli I, Buiatti S, Comi G. 2005. Microbiological analyses of dry and slurry yeasts
394 for brewing. J Inst Brew 111(2):203-8.
- 395 McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F,
396 Hou S, Layman D, Leonard S, Nguyen C, Scott K, Holmes A, Grewal N, Mulvaney E, Ryan E, Sun H, Florea L,
397 Miller W, Stoneking T, Nhan M, Waterston R, Wilson RK. 2001. Complete genome sequence of *Salmonella*
398 *enterica* serovar Typhimurium LT2. Nature 413:852-6.
- 399 Menz G, Andrighetto C, Lombardi A, Corich V, Aldred P, Vriesekoop F. 2010. Isolation, identification, and
400 characterisation of beer-spoilage lactic acid bacteria from microbrewed beer from Victoria, Australia. J Inst Brew
401 116(1):14-22.
- 402 Osimani A, Babini V, Aquilanti L, Tavoletti S, Clementi F. 2011. An eight-year report on the implementation of
403 HACCP in a university canteen: impact on the microbiological quality of meals. Int J Env Health Res 21:120-32.
- 404 Osimani A, Aquilanti L, Tavoletti S, Clementi F. 2013a. Evaluation of the HACCP system in a University Canteen:
405 microbiological monitoring and internal auditing as verification tools. Int J Env Res Public Health 10: 1572-85.
- 406 Osimani A, Aquilanti L, Tavoletti S, Clementi F. 2013b. Microbiological monitoring of air quality in a university
407 canteen: an 11-year report. Environ Monit Assess 185:4765-74.
- 408 Osimani A, Garofalo C, Clementi F, Tavoletti S, Aquilanti L. 2014. Bioluminescence ATP monitoring for the
409 routine assessment of food contact surface cleanliness in a University canteen. Int J Env Res Public Health 11:
410 10824-37.
- 411 Osimani A, Garofalo C, Aquilanti L, Milanović V, Clementi F. 2015. Unpasteurised commercial boza as a source
412 of microbial diversity. Int J Food Microbiol 194:62-70.
- 413 Pantůček R, Švec P, Dajcs JJ, Machová I, Černohlávková J, Šedo O, Gelbíčová T, Mašlaňová I, Doškař J, Zdráhal
414 Z, Růžicková V, Sedláček I. 2013. *Staphylococcus petrasii* sp. nov. including *S. petrasii* subsp. *petrasii* subsp. nov.
415 and *S. petrasii* subsp. *croceilyticus* subsp. nov., isolated from human clinical specimens and human ear infections.
416 Syst Appl Microbiol 36:90-95.
- 417 Raz R, Colodner R, Kunin CM. 2005. Who are you-*Staphylococcus saprophyticus*? Clin Infect Dis 40:896-8.

- 418 Rouse S, Van Sinderen D. 2008. Bioprotective potential of lactic acid bacteria in malting and brewing. *J Food*
419 *Protect* 71:1724-33.
- 420 Sakamoto K, Konings WN. 2003. Beer spoilage bacteria and hop resistance. *Int J Food Microbiol* 89:105-24.
- 421 Sengun IY, Karabiyikli S. 2011. Importance of acetic acid bacteria in food industry. *Food control* 22:647-56.
- 422 Takahashi T, Nakakita Y, Watari J, Shinotsuka K. 2000. Application of bioluminescence method for the beer
423 industry: sensitivity of MicroStar™-RMDS for detecting beer spoilage bacteria. *Biosci Biotechnol Biochem*
424 65:1032-7.
- 425

For Peer Review

Table 1-Identification of lactic acid bacteria from environmental samples.

Air samples						Surface samples					
Sample	Volume (L)	Counts (ufc/m ³)	Closest relatives	% Ident. ^a	Acc. no. ^b	Sample	Area (cm ²)	Counts (ufc/cm ²)	Closest relatives	% Ident. ^a	Acc. no. ^b
Cooking room	100	1	<i>Staphylococcus</i> sp.	98%	GQ406605	Maturation tank (1) before cleaning	100	n.d.	n.d	n.d	n.d
Cooking room	1000	2	<i>Staphylococcus saprophyticus</i>	98%	AP008934 ^T	Maturation tank (2) before cleaning	100	n.d.	n.d.	n.d.	n.d.
Milling station	100	n.d.	n.d	n.d	n.d	Maturation tank (1) after cleaning	100	n.d.	n.d.	n.d.	n.d.
Milling station	1000	6	<i>Staphylococcus</i> sp.	98%	JQ314011	Maturation tank (2) after cleaning	100	n.d.	n.d.	n.d.	n.d.
Fermenter room	1000	6	<i>Acetobacter</i> sp.	98%	JQ314092	Hose (1) before cleaning	100	n.d.	n.d.	n.d.	n.d.
Bottler	100	1	<i>Staphylococcus petrasii</i> /	98%	JX139845	Hose (2) before cleaning	100	16	<i>Enterobacteriaceae bacterium</i>	99%	HQ259700
			<i>Staphylococcus jettensis</i> /	98%	JN092118				<i>Acetobacter</i> sp.	98%	JQ314092
			<i>Staphylococcus hominis</i> /	98%	AB233326						
			<i>Staphylococcus haemolyticus</i>	98%	D83367						
Bottler	1000	2	<i>Leuconostoc pseudomesenteroides</i>	99%	HM443958	Hose (3) before cleaning	100	60	<i>Enterobacteriaceae bacterium</i>	99%	HQ259700
									<i>Salmonella enterica</i>	99%	CP007531
									<i>Enterobacter</i> sp.	99%	CP005991
									<i>Lactobacillus</i>	99%	AB289145
						Mill surface	100	1	<i>Lactobacillus curvatus</i>	99%	AB289077

Percentage of identical nucleotides in the sequence obtained from the isolates and the sequence of the closest relative found in the GenBank database; ^b Accession number of the sequence of the closest relative found by BLAST search; ^T Type strain; n.d. not detected.

Table 2-Identification of lactic acid bacteria from beers, exhausted yeasts, yeast pellet and active dry yeasts.

Sample	Counts (cfu/mL)	DNA source	Closest relatives	% Ident. ^a	Acc. no. ^b
Beer 1	7.0 x 10 ⁴	Beer	<i>Lactobacillus brevis</i>	99%	AB626062 ^T
		Bulk cells	<i>Lactobacillus brevis</i>	99%	AB626062 ^T
Beer 2	4.8 x 10 ⁶	Beer	<i>Lactobacillus brevis</i>	99%	AB626062 ^T
		Bulk cells	<i>Lactobacillus brevis</i>	99%	AB626062 ^T
Beer 3	2.1 x 10 ⁵	Beer	<i>Lactobacillus brevis</i>	99%	AB626062 ^T
		Bulk cells	<i>Lactobacillus brevis</i>	99%	AB626062 ^T
EY 1	n.d.	EY	n.d.		
		Bulk cells	n.d.		
		Enrichment	n.d.		
EY 2	n.d.	EY	n.d.		
		Bulk cells	n.d.		
		Enrichment	n.d.		
YP	n.d.	YP	n.d.		
		Bulk cells	n.d.		
		Enrichment	n.d.		
Sample	Counts (cfu/g)	DNA source	Closest relatives	% Ident. ^a	Acc. no. ^b
ADY 1	n.d.	ADY	<i>Pediococcus acidilactici</i> / <i>Pediococcus stilesii</i>	99% 99%	FJ457014 AJ973157 ^T
		Bulk cells	n.d.		
		Enrichment	<i>Pediococcus acidilactici</i> / <i>Pediococcus stilesii</i>	99% 99%	FJ457014 AJ973157 ^T
			<i>Lactobacillus brevis</i>	99%	AB626062 ^T
ADY 2	2 x 10 ⁰	ADY	n.d.		
		Bulk cells	<i>Pediococcus acidilactici</i> / <i>Pediococcus stilesii</i>	99% 99%	FJ457014 AJ973157 ^T
			<i>Lactobacillus parabuchneri</i>	99%	AB370877 ^T
		Enrichment	<i>Pediococcus acidilactici</i> / <i>Pediococcus stilesii</i>	99% 99%	FJ457014 AJ973157 ^T
ADY 3	2 x 10 ⁰	ADY	n.d.		
		Bulk cells	<i>Lactobacillus xiangfangensis</i> / <i>Lactobacillus fabifermentans</i> / <i>Lactobacillus plantarum</i> / <i>Lactobacillus paraplantarum</i> / <i>Lactobacillus pentosus</i>	98% 98% 98% 98% 98%	AB907194 ^T AB626075 ^T FR775893 ^T AB626065 ^T AB626060 ^T
		Enrichment	<i>Pediococcus acidilactici</i> / <i>Pediococcus stilesii</i> <i>Lactobacillus fermentum</i>	99% 99% 98%	FJ457014 AJ973157 ^T AB289105 ^T

EY exhausted yeast; YP yeast pellet; ADY active dry yeast; cfu colony forming units; n.d. not detected; ^a Percentage of identical nucleotides in the sequence obtained from the isolates and the sequence of the closest relative found in the GenBank database; ^b Accession number of the sequence of the closest relative found by BLAST search; ^T Type strain.

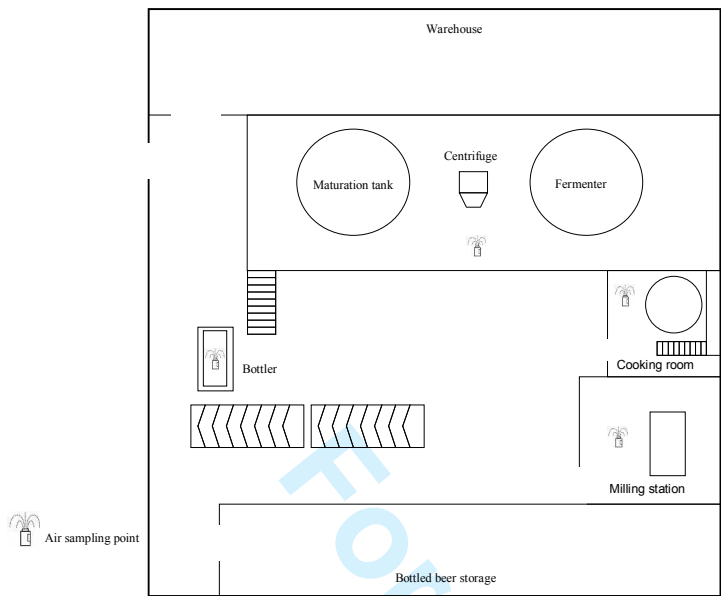
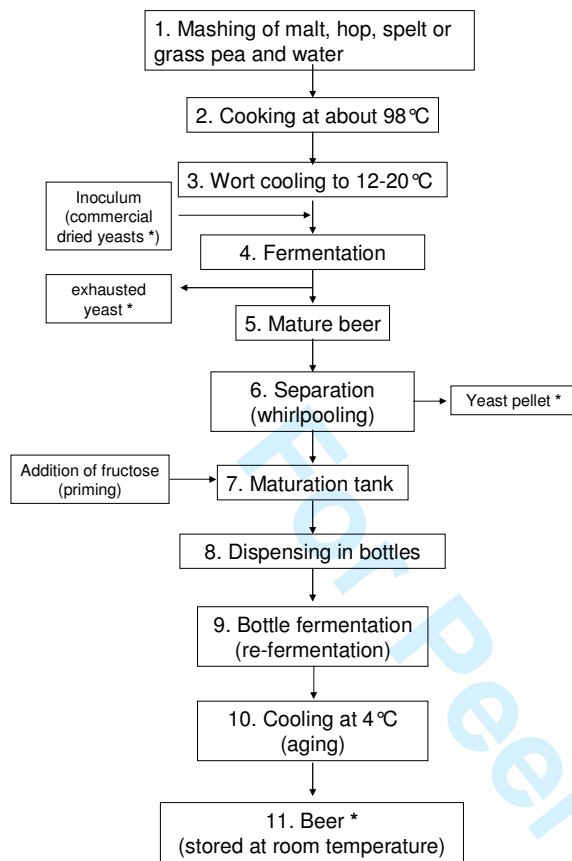


Figure 1-Brewery plant consisting of five different environments: main room, milling station, cooking room, warehouse and bottled beer storage room.



* ingredients, by-products and end-products sampled

Figure 2-Beer production flow chart.