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

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

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Editor in Chief of Journal of Food Science

Dr. Andrea Osimani, Ph.D.

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For Peer Review

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

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

2

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

4

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31 **Abstract**

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

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

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

46

47

48 **Pratical applications**

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

55

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

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61

62 **Introduction**

63 Beer is one of the world's most ancient and widely consumed fermented alcoholic beverages produced with water,
64 malted cereal grains (generally barley and wheat), hops, and yeast.
65 The popularity of niche beers has increased in recent years and the market is progressively shifting from mass-produced
66 lager brands to the richer flavors, quirky ingredients and striking aesthetics of craft beers which are characterized by a
67 unique aroma and taste (Canonico and others 2014; Aquilani and others 2015).
68 Beer is considered an unfavourable substrate of growth for many microorganisms due to several factors such as: i) the
69 ethanol concentration ranging from 0.5 to 10% (w/w), ii) the presence of hop compounds that have antimicrobial
70 activity, iii) the low pH (usually around 3.8-4.7), iv) the reduced oxygen content and nutrient availability, v) the high
71 CO_2 content (Sakamoto and Konings 2003; Manzano and others 2005; Rouse and Van Sinderen 2008; Menz and others
72 2010). Despite these hostile properties, there are a limited number of bacteria and yeasts which are capable of growth
73 and may spoil beer especially if it is not pasteurized or sterile-filtered as craft beer (Sakamoto and Konings 2003; Hill
74 2009; Menz and others 2010). In particular, lactic acid bacteria (LAB) are well-known as the main spoilage agents since
75 it has been assessed that they are responsible for 60-70% of all cases of beer spoilage (Sakamoto and Konings 2003;
76 Rouse and Van Sinderen 2008; Menz and others 2010). Among LAB, a few hetero- and homofermentative species
77 belonging to *Lactobacillus* and *Pediococcus* genera are recognized as the leading causes of beer spoilage events (Rouse
78 and Van Sinderen 2008). In particular, *Lactobacillus brevis* is the most frequent and difficult to eliminate, since it may
79 persist in the production environment also due to the ability of some *Lb. brevis* strains to form biofilms (Rouse and Van
80 Sinderen 2008; Leathers and others 2014). Other *Lactobacillus* species ascribed to *Lactobacillus lindneri*, *Lactobacillus*
81 *buchneri*, *Lactobacillus parabuchneri*, *Lactobacillus casei*, *Lactobacillus coryneformis*, *Lactobacillus malefermentans*
82 and *Lactobacillus curvatus*, have also been found as less common beer spoilers (Jespersen and Jakobsen 1996; Rouse
83 and Van Sinderen 2008). Among pediococci, several species have been reported in breweries, such as *Pediococcus*
84 *damnosus*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Pediococcus dextrinicus*, *Pediococcus inopinatus*, and
85 *Pediococcus parvulus* although *P. damnosus* is considered the main beer spoilage species together with, although to a
86 lesser extent, *Pediococcus dextrinicus* and *Pediococcus inopinatus* (Jespersen and Jakobsen 1996; Sakamoto and
87 Konings 2003; Rouse and Van Sinderen 2008). The growth of these bacteria during the brewing process implies a
88 competition for nutrients with yeast thus causing a reduction in the yeast fermentation and therefore decreased ethanol
89 yields (Rouse and Van Sinderen 2008). Furthermore, these bacteria are generally characterized by resistance to hop
90 compounds thus explaining their presence and growth in the final product (Rouse and Van Sinderen 2008; Haakensen

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91 and others 2009). The beer alteration caused by LAB spoilage consists in the production of off-flavours (mainly due to
92 diacetyl and lactic acid), changes in colour and an increase in turbidity (Rouse and Van Sinderen 2008; Menz and others
93 2010). Furthermore, this detrimental effect on beer quality is also the cause of huge economic losses in the brewery
94 sector (Sakamoto and Konings 2003). Sources of LAB contamination are widespread and they can mainly be traced to
95 the raw materials used for beer processing and to the brewery environment (indoor air, surfaces, equipment…).
96 Some papers have dealt with the detection of LAB spoilage in beers and breweries (Manzano and others 2005; Menz
97 and others 2010), another focused on methods to control microbial contamination by the use of specific cleaning
98 procedures (Manzano and others 2011), while others have been aimed at defining a rapid and sensitive method for the
99 detection of beer spoilage bacteria before the beer is bottled and sold (Takahashi and others 2000; Asano and others
100 2009).
101 However, contamination by beer spoilage bacteria in the brewing industry is still an unsolved problem especially in
102 craft beer production where the beer is often unpasteurized or sterile-filtered. Contamination by LAB is insidious and,
103 to our knowledge, there is currently no standardized and reliable method for the early detection of beer spoilage LAB.
104 The aim of this research study was to track and monitor beer spoilage LAB in the brewery and during the craft beer
105 production process by culture-dependent methods and PCR-DGGE. In detail, indoor air and work surface samples
106 collected in the brewery involved in the study, together with commercial active dry yeasts, exhausted yeasts, yeast
107 pellet (obtained after mature beer centrifugation), and spoiled beers were sampled and analyzed in order to identify the
108 contaminant LAB species and the source of LAB contamination.

109

110 **Materials and Methods**

111

112 Reference strains and culture conditions

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

117

118 Description of the brewery and beer production

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120 The brewery produces special craft beers made of malt, hops added with spelt or grass peas. The plant is divided into
121 five different environments (main room, milling station, cooking room, warehouse and bottled beer storage room) in
122 which all the different production activities are carried out (Figure 1). The main room is an open-space which houses
123 the fermenter, the centrifuge, the maturation tank and the bottler; the mill for the production of malt, spelt or grass pea
124 grist is housed in the milling station near the cooking room, the mashing tun is located in the latter and linked to the
125 fermenter through stainless steel tubes. After mashing and boiling (98 °C) the malt, hops, spelt/grass peas and water, the
126 wort is centrifuged, cooled at 12-20 °C and pumped to the fermenter; a commercial active dry yeast is added and the
127 fermentation is carried out at 20 °C for 6 days, after which the temperature is progressively lowered to 2 °C. At the end
128 of fermentation the beer is centrifuged at 8000 rpm and then transferred to the maturation tank where fructose syrup is
129 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
130 allow re-fermentation before final aging at 4 °C and storage at room temperature (Figure 2).
131

132 Microbiological analyses on brewery indoor air
133 Airborne bacterial contamination was studied using a calibrated impaction sampler (SAS Super 90, International-Pbi,
134 Milan) which was placed in the centre of each room at 1 m from the floor (Osimani and others 2013b). Using this active
135 air sampler, the microbial cells are impacted on agarized culture medium contact plates where they form colonies after
136 incubation. The density of microorganisms in a given air volume is therefore calculated by knowing both the sampled
137 air volume and the number of colonies grown on plates. The environments subjected to air sampling and monitored air
138 volumes are reported in Table 1.
139 Before sampling, the cover of the air sampler was cleaned with propanol, as advised by the manufacturer. To avoid
140 interference from outdoor air currents, all windows, if present, were kept closed during the sampling. For each area,
141 field blanks were obtained by loading and immediately unloading one set of sampling media. All the samples and
142 blanks were maintained under refrigerated conditions and taken for incubation to the laboratory on the day of collection.
143 Airborne LAB were counted on contact plates with MRS agar (Oxoid) containing 500 mg/L of cycloheximide to inhibit
144 the growth of eumycetes. Bacteria enumerations were carried out after 2 days incubation at 37 °C under anaerobiosis.

145

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

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150 disinfecting agent used for sanitization (Osimani and others 2011; Osimani and others 2013a; Osimani and others
151 2014). Samples were transferred to the laboratory under refrigerated conditions and immediately subjected to viable cell
152 counting; in more detail, samples were serially ten-fold diluted in a sterile peptone-saline solution (1 g/L peptone and
153 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
154 mg/L of cycloheximide incubated for 2 days at 37 °C under anaerobiosis.

155

156 pH measurements

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

160

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

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

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

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

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

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

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179 Bulk cell formation

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

184

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

186 The microbial DNA was extracted directly from the rehydrated yeasts, by-products (exhausted yeasts and pellet yeast)
187 and beer samples, using the PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA). In detail,
188 1 mL of each sample was centrifuged to produce a pellet that was processed according to the kit manufacturer's
189 instructions. The DNA quantity and purity were assessed by optical readings at 260, 280 and 234 nm, respectively,
190 using a UV-Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

191

192 DNA extraction from bulk cells and pure microbial cultures

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

196

197 PCR-DGGE analyses

198 To analyze both the DNA extracted directly from the samples and the DNA extracted from bulk cells, 100 ng of each
199 DNA were amplified through PCR in 50 µL reaction volume using the universal prokaryotic primers $338f_{GC}$ and 518r
200 that target the V3 region of the 16S rRNA gene (Osimani and others 2015).
201 Five microliters of each PCR product were checked by electrophoresis in 1.5% (w/v) agarose gels (Laboratorios
202 CONDA, Madrid, Spain) using a GeneRuler DNA Ladder mix (Thermo Scientific Fermentas, Pittsburgh, USA) as a
203 molecular weight standard (Osimani and others 2015). Gels were visualized under UV light and photographed with the
204 Complete Photo XT101 system (Explera, Jesi, Italy).
205 A vertical DCode electrophoresis system (Bio-Rad Laboratories, Hercules, USA) was used for the DGGE analysis.
206 PCR products (25 µL) obtained with primers $338f_{GC}$/518r were applied to 0.8 mm polyacrylamide gel [8% (w/v)
207 acrylamide/bisacrylamide gel 37.5:1], containing a 30-60% urea-formamide denaturing gradient increasing in the
208 direction of the electrophoresis (100% corresponds to 7 M urea and 40% (w/v) formamide), and run with 1X TAE

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209 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
210 °C. After electrophoresis, the gels were stained for 20 min in 1X TAE buffer containing SYBR Green I Stain 1X
211 (Lonza, USA), visualized under UV light and photographed with the Complete Photo XT101 system (Explera).
212 For the preliminary identification of DGGE bands, an identification ladder (Mix) was prepared by mixing suitable
213 amounts (5 µl) of the $338f_{GC}$/518r amplicons obtained from the pure cultures of the two bacteria reference strains.
214
215 Sequencing of the DGGE bands and sequence analysis
216 The DGGE bands were excised by the gels using sterile cutting tips and the DNA from each band was eluted in 50 µL
217 sterile deionized water overnight at +4 °C (Garofalo and others 2008). Five microliters of the eluted DNA were re-
218 amplified under the same conditions as described above, with the forward primer 338f without the GC clamp. These
219 PCR amplicons were purified using a GFX™ PCR DNA and Gel Band Purification Kit (Euroclone, Milan, Italy)
220 following the manufacturer's instructions and were then sent to Macrogen (Amsterdam, The Netherlands) for
221 sequencing. The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA
222 database using the basic BLAST search tools (Altschul and others 1990).
223
224 **Results and Discussion**
225 This study investigated three different kinds of spoiled craft beers produced with spelt and grass peas as raw materials.
226 The three beer samples were characterized by turbidity and the following pH values: 4.23 ± 0.1 (beer 1), 4.29 ± 0.1
227 (beer 2) and 4.03 (beer 3); beer 3 was also slimy. The microscopic analyses revealed coccal shaped bacterial cells in
228 beer 1, while beer 2 contained coccal shaped bacterial cells, both isolated and in tetrads, together with short bacilli, and
229 beer 3 contained yeast cells, coccal shaped bacterial cells and long and short bacilli. Viable counts on MRS agar ranged
230 from 7.0 x 10^4 to 4.8 x 10^6 (Table 2). From the PCR-DGGE analyses of the DNA recovered both directly from beers
231 and from bulk cells (from both high and low dilution agar plates) the closest relatives to *Lb. brevis* were found to be
232 ubiquitous (Table 2). This species identification was also confirmed by analyzing five other spoiled beers (4 beers
233 produced using spelt and 1 beer obtained using grass peas as raw materials) from different batches (data not shown). On
234 the contrary, no LAB viable counts and no bacterial cells were found on unspoiled beers.
235 LAB of the genera *Lactobacillus* and *Pediococcus* are considered the main cause of beer spoilage (Sakamoto and
236 Konings 2003; Rouse and Van Sinderen 2008; Menz and others 2010). PCR-DGGE was found to be a useful technique
237 for identifying the contaminant LAB species in the spoiled beers, which was ascribed to *Lb. brevis*. In detail, the species
238 *Lb. brevis* was characterized by four DGGE bands migrating at different positions in the acrylamide gel (data not

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239 shown). This is probably a result of the presence of multiple heterogeneous copies of the 16S rRNA gene within the
240 same species thus leading to a DGGE profile with multiple bands due to the different electrophoretic mobility of the
241 PCR amplicons in the gel. This PCR-DGGE bias has already been highlighted by other authors (Ercolini 2004; Cocolin
242 and others 2013; Garofalo and others 2015) who indicated the need to sequence all the visualized DGGE bands in order
243 to arrive at an exact determination of the sample biodiversity. The rigorous and reliable identification of the beer
244 spoilage species is fundamental in order to define an effective cleaning and sanitization plan specifically tailored to the
245 contaminant species (f.i. if the contaminant is a biofilm-producing species). In particular, the detection of *Lb. brevis*
246 confirmed previous studies that reported this obligate heterofermentative LAB species as the most common and
247 problematic beer spoilage species due to its optimal growth at 30 °C and at pH comprised between 4.0 and 6.0, its
248 physiological versatility, its resistance to hop compounds and the ability of some strains to develop biofilms (Sakamoto
249 and Konings 2003; Rouse and Van Sinderen 2008; Leathers and others 2014). Finally, it is worth noticing that
250 unspoiled beer did not show any LAB viable counts, thus indicating that LAB contamination and, in particular, that of
251 *Lb. brevis*, is directly linked to alterations in the beer.
252 In order to discover the source of *Lb. brevis* contamination a detailed monitoring plan was adopted which involved the
253 analysis not only of raw materials and by-products, such as active dry yeasts, exhausted yeasts, yeast pellet, but also of
254 the brewery environment (indoor air and some selected key work surfaces).
255 The results of the microbiological analyses carried out on three different commercial active dry yeasts (ADY), two
256 exhausted yeasts (EY), a yeast pellet (YP) and three different spoiled beers are reported in Table 2. Concerning the
257 three active dry yeasts, the LAB viable counts were very low or even absent in ADY 1. The closest relatives to *Pd.*
258 *acidilactici*/*Pediococcus stilesii* were found as a common species in the three active dry yeasts after enrichment through
259 PCR-DGGE analyses. Furthermore, the closest relatives to *Pd. acidilactici*/*Pd. stilesii* were found in ADY 1 after PCR-
260 DGGE analyses on DNA extracted directly from this matrix and in ADY 2 by analyzing the DNA from bulk cells. The
261 closest relatives to different *Lactobacillus* species were also identified among the three different active dry yeasts. In
262 detail, the closest relatives to *Lb. brevis* were found from ADY 1 after enrichment; the closest relatives to *Lb.*
263 *parabuchneri* were identified in ADY 2 by analyzing DNA obtained from bulk cells; the closest relatives to
264 *Lactobacillus xiangfangensis*/*Lactobacillus fabifermentans*/*Lactobacillus plantarum*/*Lactobacillus*
265 *paraplantarum*/*Lactobacillus pentosus* and *Lactobacillus fermentum* were revealed in ADY 3 by analyzing DNA from
266 bulk cells and DNA from cells after enrichment, respectively. Bacterial species were not detected by PCR-DGGE
267 analyses of DNA extracted directly from ADY 2 and 3, probably due to the low number of bacterial cells within these
268 samples. By contrast, the closest relatives to *Pd. acidilactici*/*Pd. stilesii* were found by analyzing DNA extracted

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269 directly from ADY 1 although no viable counts, and consequently bulk cells, were recovered. This result was probably
270 due to the presence of viable but non-cultivable (VBNC) cells within this active dry yeast sample.
271 The fact that of the three commercial active dry yeasts analyzed only one (ADY 1) showed the presence of *Lb. brevis*,
272 and immediately after the enrichment step, may indicate the low cellular number of *Lb. brevis* in this product. However,
273 the continuous addition of this active dry yeast during the beer production process may also represent a constant
274 inoculum of *Lb. brevis* that may multiply easily in the final product at the re-fermentation stage and during storage at
275 room temperature and may consequently be accumulated over time within the brewery environment. This latter
276 hypothesis seems to be confirmed by the fact that, in the absence of an efficient and frequent sanitization plan, after
277 about six months the beers were again spoiled by *Lb. brevis* (data not shown). Probably in this period *Lb. brevis*,
278 deriving from active dry yeasts, was able to accumulate and spread in the brewery environment and, hence, grow on
279 beer.
280 Other LAB species, ascribed to *Pd. acidilactici*/*Pd. stilesii*, *Lb. parabuchneri*, *Lb. xiangfangensis*/*Lb.*
281 *fabifermentans*/*Lb. plantarum*/*Lb. paraplantarum*/*Lb. pentosus* and *Lb. fermentum* were also found in the three
282 commercial active dry yeasts analyzed. Among these species only *Lb. parabuchneri*, has previously been identified as a
283 possible beer spoilage agent (Jespersen and Jacobsen 1996; Sakamoto and Konings 2003). In fact, although pediococci
284 are also known as beer spoilage agents, the species *Pd. acidilactici* has never been reported as producing defects in
285 mature beer (Sakamoto and Konings 2003). However, these findings were not considered to be a cause for concern
286 since they were not present within the spoiled beers.
287 The exhausted yeasts and the yeast pellet did not show the presence of bacterial DNA and of LAB colonies on MRS
288 agar plates. This result was probably due to the fact that, on the one hand, any bacterial cells present in the fermenter do
289 not precipitate to the bottom together with the exhausted yeasts, and on the other hand, the bacterial cells cannot be
290 collected in the yeast pellet after centrifugation due to the low speed applied which is necessary to partially keep the
291 yeasts in suspension for subsequent bottle re-fermentation.
292 In order to verify the possible presence of *Lb. brevis* in the brewery environment a monitoring plan that involved
293 microbiological analyses coupled with PCR-DGGE of the indoor air and selected work surfaces was applied.
294 The results of the microbiological analyses obtained using the calibrated impaction sampler in the brewery under study
295 are reported in Table 1. The LAB viable counts were generally low. The colonies were collected in bulk and the
296 extracted DNA was analyzed through PCR-DGGE using a universal primer for eubacteria. The closest relatives, the per
297 cent identities, and the accession numbers of the obtained sequences are given in Table 1. Several closest relatives to
298 species belonging to the genera *Staphylococcus* were found in the cooking room, milling station and in the bottler

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299 together with the closest relatives to *Leuconostoc pseudomesenteroides* in the latter case. The closest relatives to
300 *Acetobacter* sp. were also detected in the fermenter room.
301 The results of the microbiological analyses performed on the selected work surfaces are reported in Table 1. In detail,
302 viable counts were not found on MRS agar plates from the maturation tank either before or after cleaning nor on a
303 connection hose before cleaning. From 1 to 60 ufc/cm^2 were detected after the analysis of two different hoses before
304 cleaning and of the mill surface. The PCR-DGGE analyses of the DNA extracted from the bulk cells revealed the
305 presence of the closest relatives to *Enterobacteriacea* bacterium, *Salmonella enterica*, *Enterobacter* sp. and *Acetobacter*
306 sp. in the two hoses analyzed and the presence of the closest relatives to *Lactobacillus graminis*/*Lactobacillus curvatus*
307 on the surface of the mill.
308 *Lb. brevis* was never detected inside the brewery environment thus indicating that during the microbiological
309 monitoring period *Lb. brevis* was not widespread. However some other bacterial species which may be considered
310 dangerous for human health were found in the plant. In fact, an analysis of the indoor air showed that some species
311 belonging to the *Staphylococcus* genera were widespread, including *Staphylococcus saprophyticus* and *Staphylococcus*
312 *petrasii*/ *Staphylococcus jettensis*/*Staphylococcus hominis*/*Staphylococcus haemolyticus*. *St. petrasii*, *St. jettensis*, *St.*
313 *hominis*, and *St. haemolyticus* are coagulase-negative staphylococci which are phylogenetically related and
314 phenotypically similar (Pantůček and others 2013). These bacteria are ubiquitous and human common commensals
315 isolated mainly from the skin and mucous membranes of mammals. However, they have been recently recognized as
316 opportunistic pathogens in several human infections (Pantůček and others 2013) as also demonstrated for *St.*
317 *saprophyticus* which was found to be involved in urinary tract infection mainly in young women (Raz and others 2005).
318 Other taxa were also found, such as *Leuc. pseudomesenteroides* in the bottler indoor air and *Acetobacter* sp. in the
319 fermenter room. *Acetobacter* species belong to the group of acetic acid bacteria that are characterized by gram-negative,
320 rod-shaped cells, widespread on several cereals and fruits, with spoilage activity in some products such as wine, as a
321 result of their ability to oxidize sugars and alcohols to organic acids (Sengun and Karabiyikli 2011). *Acetobacter* sp.
322 were also found by analyzing a connection hose in the fermenter room before cleaning, thus showing the same
323 localization previously detected by analyzing the fermenter room indoor air. Members of the *Enterobacteriacea* family
324 were mainly found on the work surfaces analyzed. In particular, *S. enterica*, that was identified in a connection hose in
325 the fermenter room before cleaning, contains over 2,000 serovars. Some of these, such as *Salmonella enterica* serovar
326 Typhi, lead to systemic infections and typhoid fever, whereas others, such as *Salmonella enterica* serovar
327 Typhimurium, determine gastroenteritis (McClelland and others 2001). Although none of these pathogenic species are
328 able to grow in beer since they are inhibited by the low beer pH and therefore they do not represent a risk for the

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329 consumers, they may be harmful for the health of the people involved in the beer production process. Hence, an
330 implementation of the sanitization program and hygiene regimes in the brewery environment are necessary in order to
331 avoid any possible health risks for the brewery staff.
332 Considering the overall results the importance of a specific and efficient sanitization plan is confirmed, based on the
333 application of hygiene and good manufacturing practices which are often the most effective methods for managing
334 microbiological risk (Hill 2009). Therefore, the inalienable ingredients for an effective microbiological control must
335 include: i) a good plant design; ii) an efficient plant maintenance/renewal; iii) the use of cleaning-in-place; iv) effective
336 detergents and sanitizers and; v) a stringent microbiological monitoring (Hill 2009). As reported by Hill (2009), a
337 knowledge of spoilage microorganisms which may be present in the brewery environment and the control of microbial
338 fouling both play a pivotal role in the prevention of microbial beer spoilage. In addition, a proper microbiological
339 monitoring must be implemented since a low sample volume in relation to typical batch volumes (f.i. 250 mL samples
340 collected from more than 1000 hectoliters of beer) and the heterogeneity of the potential beer spoiling bacteria may
341 increase the difficulty in detecting trace contaminants (Hill 2009). Therefore, the enrichment step applied in this study
342 (5-7 days at 37 °C) and microbiological monitoring may be an efficient method for the early detection of LAB species
343 in the beer before bottling, thereby preventing huge economic losses. In fact the enrichment step may highlight the
344 presence of even a few cells of LAB contaminants deriving from commercial active dry yeasts, as found in this study.
345 In order to eliminate this contamination an appropriate sanitization plan has to be frequently applied within any brewery
346 that hosts beer spoilage LAB species deriving from commercial active dry yeasts which may accumulate after
347 continuous inoculum.
348
349 **Conclusions**
350 In conclusion, the PCR-DGGE technique coupled with a culture-dependent method that specifically envisages an
351 enrichment step was found to be a useful tool for identifying *Lb. brevis* as the beer spoilage species and the source of
352 beer contamination. The monitoring plan applied for the raw materials, by-products, final products and the brewery
353 environment was suitable for developing an efficient and thorough sanitization plan within the brewery, even if
354 currently there are no criticalities linked to the brewery environment in terms of beer spoilage agents.
355
356 **Author Contributions**
357 All the co-authors contributed to the planning of the study and the interpretation of the results. The sampling, culture-
358 dependant analyses and PCR-DGGE analyses were carried out by C. Garofalo, A. Osimani, V. Milanović and M.

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359 Taccari. The initial manuscript draft was written by C. Garofalo and A. Osimani; the draft was further critically read
360 and improved by all the co-authors.
361
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Table 1-Identification of lactic acid bacteria from environmental samples.

Air samples

Sample	Volume (L)	Counts (ufc/m^3)	Closest relatives	% Ident.[a]	Acc. no.[b]
Cooking room	100	1	*Staphylococcus* sp.	98%	GQ406605
Cooking room	1000	2	*Staphylococcus saprophyticus*	98%	AP008934[T]
Milling station	100	n.d.	n.d	n.d	n.d
Milling station	1000	6	*Staphylococcus* sp.	98%	JQ314011
Fermenter room	1000	6	*Acetobacter* sp.	98%	JQ314092
Bottler	100	1	*Staphylococcus petrasii/*	98%	JX139845
			Staphylococcus jettensis/	98%	JN092118
			Staphylococcus hominis/	98%	AB233326
			Staphylococcus haemolyticus	98%	D83367
Bottler	1000	2	*Leuconostoc pseudomesenteroides*	99%	HM443958

Surface samples

Sample	Area (cm^2)	Counts (ufc/cm^2)	Closest relatives	% Ident.[a]	Acc. no.[b]
Maturation tank (1) before cleaning	100	n.d.	n.d	n.d	n.d
Maturation tank (2) before cleaning	100	n.d.	n.d.	n.d.	n.d.
Maturation tank (1) after cleaning	100	n.d.	n.d.	n.d.	n.d.
Maturation tank (2) after cleaning	100	n.d.	n.d.	n.d.	n.d.
Hose (1) before cleaning	100	n.d.	n.d.	n.d.	n.d.
Hose (2) before cleaning	100	16	*Enterobacteriacea bacterium*	99%	HQ259700
			Acetobacter sp.	98%	JQ314092
Hose (3) before cleaning	100	60	*Enterobacteriacea bacterium*	99%	HQ259700
			Salmonella enterica	99%	CP007531
			Enterobacter sp.	99%	CP005991
Mill surface	100	1	*Lactobacillus graminis/*	99%	AB289145
			Lactobacillus curvatus	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.

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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	7.0×10^4	Beer	*Lactobacillus brevis*	99%	AB626062[T]
		Bulk cells	*Lactobacillus brevis*	99%	AB626062[T]
Beer 2	4.8×10^6	Beer	*Lactobacillus brevis*	99%	AB626062[T]
		Bulk cells	*Lactobacillus brevis*	99%	AB626062[T]
Beer 3	2.1×10^5	Beer	*Lactobacillus brevis*	99%	AB626062[T]
		Bulk cells	*Lactobacillus brevis*	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	*Pediococcus acidilactici/*	99%	FJ457014
			Pediococcus stilesii	99%	AJ973157[T]
		Bulk cells	n.d.		
		Enrichment	*Pediococcus acidilactici/*	99%	FJ457014
			Pediococcus stilesii	99%	AJ973157[T]
			Lactobacillus brevis	99%	AB626062[T]
ADY 2	2×10^0	ADY	n.d.		
		Bulk cells	*Pediococcus acidilactici/*	99%	FJ457014
			Pediococcus stilesii	99%	AJ973157[T]
			Lactobacillus parabuchneri	99%	AB370877[T]
		Enrichment	*Pediococcus acidilactici/*	99%	FJ457014
			Pediococcus stilesii	99%	AJ973157[T]
ADY 3	2×10^0	ADY	n.d.		
		Bulk cells	*Lactobacillus xiangfangensis/*	98%	AB907194[T]
			Lactobacillus fabifermentans/	98%	AB626075[T]
			Lactobacillus plantarum/	98%	FR775893[T]
			Lactobacillus paraplantarum/	98%	AB626065[T]
			Lactobacillus pentosus	98%	AB626060[T]
		Enrichment	*Pediococcus acidilactici/*	99%	FJ457014
			Pediococcus stilesii	99%	AJ973157[T]
			Lactobacillus fermentum	98%	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.

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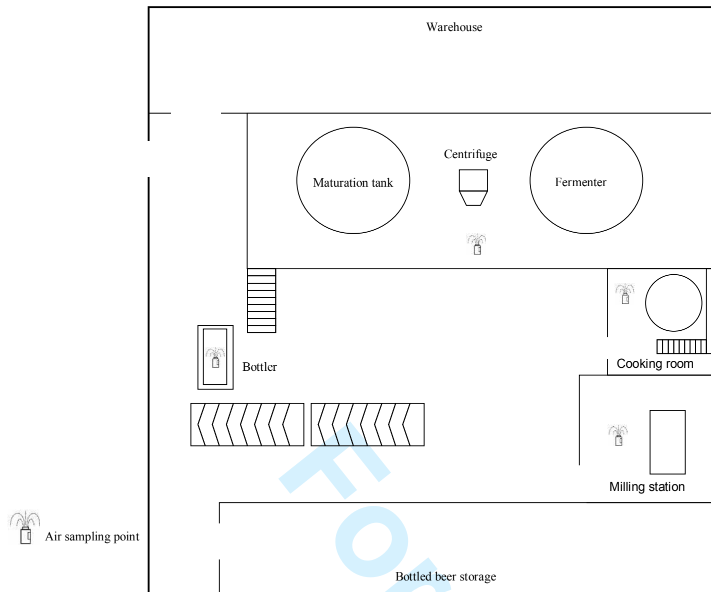

Figure 1-Brewery plant consisting of five different environments: main room, milling station, cooking room, warehouse and bottled beer storage room.

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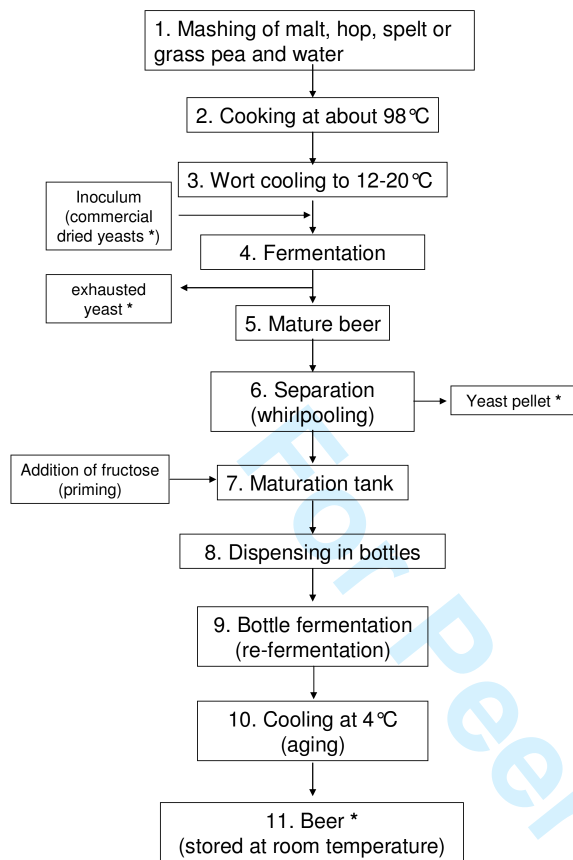

Figure 2-Beer production flow chart.

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