Molecular identification and physiological characterization of yeasts, lactic acid bacteria and acetic acid bacteria isolated from heap and box cocoa bean fermentations in West Africa

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Running title: Technological microbiota in West African cocoa bean fermentations

Abstract: Yeast, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) populations, isolated from cocoa bean heap and box fermentations in West Africa, have been investigated. The fermentation dynamics were determined by viable counts, and 106 yeasts, 105 LAB and 82 AAB isolates were identified by means of rep-PCR grouping and sequencing of the rRNA genes. During the box fermentations, the most abundant species were *Saccharomyces cerevisiae, Candida ethanolica, Lactobacillus fermentum, Lb. plantarum, Acetobacter pasteurianus* and *A. syzygii*; while *S. cerevisiae, Schizosaccharomyces pombe, Hanseniaspora guilliermondii, Pichia manshurica, C. ethanolica*, *H. uvarum*, *Lb. fermentum*, *Lb. plantarum, A. pasteurianus* and *A. lovaniensis* were identified in the heap fermentations. Furthermore, the most abundant species were molecularly characterized by analyzing the rep-PCR profiles. Strains grouped according to the type of fermentations and their progression during the transformation process was also highlighted. The yeast, LAB and AAB isolates were physiologically characterized to determine their ability to grow at different temperatures, as well as at different pH, and ethanol concentrations, tolerance to osmotic stress, and lactic acid and acetic acid inhibition. Temperatures of 45°C, a pH of 2.5 to 3.5, 12% (v/v) ethanol and high concentrations of lactic and acetic acid have a significant influence on the growth of yeasts, LAB and AAB. Finally, the yeasts were screened for enzymatic activity, and the *S. cerevisiae, H. guilliermondi, H. uvarum* and *C. ethanolica* species were shown to possess several enzymes that may impact the quality of the final product.

Keywords: cocoa beans, heap fermentation, box fermentation, microbial dynamics, rep-PCR, physiological characterization

40 **1. Introduction**

41 Different aspects can affect cocoa beans fermentations and therefore the quality of the final
42 products. Fruit pods of the *Theobroma cacao* tree are opened manually and the beans, with the
43 surrounding pulp, are fermented using traditional procedures, mainly heap and box fermentations,
44 for four to seven days and thereafter dried.

45 Local practices, related to the management of cocoa bean fermentation, such as good harvesting
46 practices, careful selection and correct handling of the beans, can influence cocoa quality (Lima et
47 al. 2011; Papalexandratou et al. 2011; Trognitz et al. 2013). Another important aspect to consider is
48 the fermentation process and, more specifically, its microbial ecology. Most of the microorganisms
49 that naturally contaminate the beans come from outer surface of the pods, the hands of the workers,
50 from the machetes used to open the pods, from the baskets used to transport the beans, plants
51 materials and, above all, from boxes with residues of previous fermentations and plantain leaves
52 (Schwan and Wheals 2004).

53 Fermentation process involved complex microbial activities and biochemical changes that have
54 been recently deeply reviewed by Schwan and Fleet (2014). The microorganisms responsible for the
55 fermentation are yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB), moreover
56 species of *Bacillus* sp, other bacteria and filamentous fungi could also grow with consequent
57 influence on quality of the process. First main event regards microbial activities on the
58 mucilaginous pulp that results in the production of alcohol and acids, and an increasing of
59 temperature. Secondly, fermentation is essential for the death of the embryo of the seed, which
60 takes place through the production of heat and acetic acid. This is also important to eliminate
61 bitterness and astringency and, lastly, for the formation of aroma precursors. High sugar
62 concentration, high temperatures, pH changes, ethanol production, metabolism of organic acids and
63 as a result their concentrations, turn cocoa beans fermentation into a stressful environment for
64 microorganism growth. They exert a selection and inhibition on the natural occurring microbiota,
65 while promoting those populations, which are better adaptable to these conditions. Another aspect

66 that influence the quality of the process involves enzymatic activities. Particularly, yeast
67 pectinolytic enzymes are considered to have an important role in degradation and solubilisation of
68 the pulp, that allows penetration of oxygen into the fermenting mass enabling aerobic acetic acid
69 bacteria to grow as reported by Schwan and Fleet (2014).
70 In this study, the dynamics of yeasts, LAB and AAB, obtained from two different types of cocoa
71 bean fermentation, that is, heap and box fermentations, have been investigated using a culture-
72 dependent microbiological method and molecular techniques. Furthermore, the ability of isolates to
73 tolerate different stress conditions has been tested in order to provide useful information on their
74 ability to initiate and carry out the fermentation. Moreover, a panel of yeast's enzymatic activities
75 potentially impacting the course of the fermentation and the quality of the final product were
76 determined.
77
78 **2. Materials and Methods**
79 **2.1 Cocoa bean fermentations**
80 Cocoa pods, of Forastero hybrid, were harvested in Ivory Coast (West Africa) during main crop
81 (October to December) by means of traditional procedures, and opened manually with a machete.
82 Two different spontaneous cocoa beans fermentation were carried out, that is, heap (H) and box (B)
83 fermentations. Both were performed in triplicate using three different batches of fresh beans and
84 located in the plantation area, under a roof. The heap fermentations involved 50 kg of fresh cocoa
85 pulp-bean mass being piled into a heap on top of plantain leaves placed on the ground, and the heap
86 being covered with other leaves. The box fermentations instead involved 1600 kg of fresh cocoa
87 pulp-bean mass being placed in a wooden box, resulting in 100 cm of depth, arranged in tiers, with
88 one slightly raised above the other, in order to facilitate the turning phase. In both cases, the mass
89 was turned after 48 and again after 96 h using a shovel, until the beans were visually homogenous.
90 After the fermentation processes, the cocoa beans were dried under the sun for five to ten days,
91 depending on the weather. The beans were turned until the humidity level was lower than 8% and

92 this was performed weighing 10 g of grounded beans and by using a moisture analyzer (Mettler
93 Toledo, Milan, Italy).

94 **2.2 Sampling procedure**

95 Samples were taken at the beginning of the fermentation (time zero) and after 1, 2, 4 and 6 days,
96 and microbiological analysis was performed immediately after sampling. Cocoa beans coming from
97 different points of the box/heap were mixed and collected in sterile bags. Twenty-five grams of
98 cocoa beans and the adhering pulp were added to 100 ml of Ringer's solution (Oxoid, Milan, Italy)
99 and homogenized manually. Serial dilutions in Ringer were prepared and analyzed on several
100 microbiological media. The yeasts were enumerated by spreading on WL Nutrient agar (WLN,
101 Oxoid) containing 1 µg/mL tetracycline (Sigma-Aldrich, Milan, Italy); the LAB were counted by
102 means of pour plate inoculation on de Man Rogosa Sharp agar (MRS, Oxoid) supplemented with 2
103 µg/mL of natamycin (DSM Food Specialities, The Netherlands); the AAB were determined by
104 spreading on Acid Acetic Medium agar (AAM, 1% glucose, 0.8% yeast extract, 1.5%
105 bacteriological peptone, 15 g/L agar, Oxoid) containing 2 µg/mL of natamycin (DSM Food
106 Specialities). Plates were incubated at 37°C for 2 days for MRS and at 30°C for 3 to 5 days for both
107 AAM and WLN media. After the incubation period, the colonies were counted (the mean and
108 standard deviation were calculated). Five colonies randomly selected for each microbial group
109 investigated were streaked for purification. The purified isolates were stored at -20°C in YEPD
110 broth (2% glucose, 1% yeast extract, 1% bacteriological peptone, Oxoid) for yeasts, and in MRS
111 broth and AAM broth for LAB and AAB, respectively. The broths all contained 25% of glycerol
112 (Sigma). The temperatures and pH values were measured directly on the fermentation mass
113 immediately before taking the samples, using a digital pH meter provided with a temperature probe
114 (Mettler Toledo).

115 **2.3 Statistical analysis**

116 The data obtained from the pH, temperatures and yeasts, as well as the LAB and AAB counts of the
117 H and B fermentations were analyzed using one-way Analysis of Variance (ANOVA). ANOVA

118 analysis was performed using the Statistica software package (version 7.1, StatSoft Inc., Tulsa, OK,
119 USA).

120 **2.4 DNA extraction from pure cultures**

121 DNA was extracted from 1 ml of an overnight culture of yeasts, LAB and AAB, as previously
122 described in Cocolin et al. 2000, 2004). The DNA was quantified using a Nanodrop Instrument
123 (Spectrophotometer ND-1000, Thermo Fisher Scientific, Milan, Italy) and then standardized at 100
124 ng/l.

125 **2.5 Identification and characterization of the isolates**

126 The isolates were identified by means of rep-PCR grouping and rRNA gene sequencing. Rep-PCR
127 was performed using a single oligonucleotide primer $(GTG)_5$ (Versalovic et al., 1994), as previously
128 described by Dal Bello et al. (2010). Generated dendrograms were analyzed in order to identify
129 clusters with a coefficient of similarity higher than 85%. Then, representatives of each group and
130 isolates with unique rep-PCR profiles were identified by 16S and 26S rRNA gene sequencing for
131 bacteria and yeasts, respectively. RNA genes were amplified with the P1V1-P4V3 and NL1-NL4
132 primers for bacteria and yeasts, respectively (Klijn et al., 1991; Kurtzman and Robnett, 1997). The
133 PCR products were purified by means of a PCR Extract Mini Kit (5PRIME, Milan, Italy) and sent
134 to a commercial sequencing facility (MWG Biotech, Ebersberg, Germany). The obtained sequences
135 were compared with those present in GenBank, using the Blast search program (Altschul et al.,
136 1997).

137 The rep-PCR profiles of the most abundant species, namely *S. cerevisiae* (35 isolates), *Lb.*
138 *fermentum* (51 isolates) and *A. pasteurianus* (51 isolates) were further analyzed to study the
139 intraspecific biodiversity. Arbitrary selected coefficients of similarity of 80% for the yeast and the
140 LAB, and of 75% for the AAB were used.

141 **2.6 Screening for tolerance to stress conditions**

142 An analysis of tolerance to stress conditions was carried out by monitoring the growth of all the
143 isolates at different temperatures, pH values and concentrations of ethanol, glucose and fructose, as

144 already suggested (Daniel et al. 2009; Lefeber et al. 2010; Pereira et al. 2012). Briefly, after
145 centrifugation of the overnight cultures, the pellets were washed in Ringer's solution and then
146 suspended in 200 µl of the same solution, and this was followed by dilution in order to normalize
147 the inoculum concentration at the same absorbance value of 0.02 OD. The cultures were inoculated
148 directly in 96-well plates in Yeast Nitrogen Base (YNB, Oxoid), MRS and Mannitol Yeast Extract
149 Peptone (2.5% D-mannitol, 0.5% yeast extract, 0.3% bacteriological peptone, Oxoid) media for
150 yeasts, LAB and AAB, respectively. Eight, 10, or 12% (vol/vol) of ethanol; 5, 15, or 30% (wt/vol)
151 of glucose and 5, 15 or 30% (wt/vol) of fructose were added to the media. Growth was measured at
152 30, 37, and 45°C, for temperature tolerance, and the media were adjusted to pH 2.5, 3.5 and 4.5 for
153 the pH tolerance. Furthermore, the LAB and AAB cultures were screened for lactic acid and acetic
154 acid resistance: 1, 2, 4 or 5% of lactic acid and acetic acid were added to the media separately
155 (Pereira et al. 2012). The yeast, LAB and AAB cultures inoculated in the media without any
156 modification were used as the reference. Growth was evaluated by measuring the absorbance value
157 at 630 nm (Biotek ELx808, Milan, Italy) at 0, 24 and 48 hours for the yeasts, at 0, 12 and 24 for the
158 LAB and at 0, 24 and 36 hours for the AAB. All the experiments were performed in triplicate; the
159 mean OD value of the three biological replicates for each stress condition was compared with the
160 OD value of the reference condition in order to define a growth percentage.

161 **2.7 LAB acidification properties**

162 The acidification activity of the LAB isolates was evaluated on the basis of the pH values. Fifty
163 millilitres of reconstituted 10% (wt/vol) UHT skimmed milk (LABM, Heywood, UK) was
164 inoculated with 2% (vol/vol) of an overnight LAB culture (Dandoy et al., 2011). The cultures were
165 then incubated at 37°C and the pH was measured immediately after the inoculation at 4, 8, 12, 24
166 and 48 hours, using a Basic 20 pH meter (Crison, Modena, Italy). The acidification analysis was
167 performed in triplicate and the average value of each time point was used to construct the
168 acidification curve for each LAB.

169 **2.8 Screening of yeasts for enzymatic activities**

The yeast strains were analyzed for enzymatic activities, and in particular for the esterase, β-glucosidase, glycosidase, protease and pectinase activities, according to the methods reported in Englezos et al. (2015). Screening of enzymatic activity was performed in triplicate on agar plates using 2 µl of YEPD overnight yeast cultures. The positive yeast controls, used as the reference in order to verify the reliability of the tests, were part of the collection of the Department of Forestry, Agriculture and Food Sciences, University of Torino, Italy.

3. Results

3.1 Microbiological analysis of the box and heap fermentations

The temperature, pH and counts of yeasts, LAB and AAB of the B and H fermentations are reported in Table 1.

The temperatures values were significantly different between B and H fermentations at all the sampling times; the greatest difference was recorded at 24 hours ($P<0.0001$). Significant differences were observed between the B and H fermentations at 48, 96 and 144 hours in the yeast and LAB counts (Tab. 1) and at 96 hours in the case of the AAB counts. No significant differences were recorded for the pH values ($P>0.05$).

3.2 Identification of isolates and species dynamics during fermentations.

A total of 450 colonies were isolated throughout the fermentation processes from the WLN, MRS and AAM plates. The colonies were identified as yeasts (106 isolates), LAB (105 isolates) and AAB (82 isolates). The rest of the colonies did not belong to these categories and were discarded, as referring mainly to *Enterococcus* genera. The results of the identification and of the species dynamics are shown in Figures 1 and 2. *S. cerevisiae* was the most abundant species, in the case of the box fermentations (Fig. 1A), and these were followed by *Candida ethanolica*. *S. cerevisiae* was isolated in almost all the sampling points (except for day 4), while *C. ethanolica* was detected in the last two days of fermentation (Fig. 2A). *Torulaspora delbrueckii* was found in the first 48 hours (Fig. 2A). *S. cerevisiae* was once again one of the most abundant species in heap fermentations,

196 together with *Schizosaccharomyces pombe,* (Fig. 1A), that was recorded from the first until the last
197 day of fermentation (Fig. 2A). *H. uvarum* was the most isolated species at time zero. *Lb. fermentum*
198 and *Lb. plantarum* were the most prevalent species among the 105 LAB strains (Fig. 1B), and they
199 dominated in both the box and heap fermentations (Fig. 2B). *Acetobacter pasteurinaus* was the
200 most important AAB species in box and heap fermentations as shown in Fig. 1C. *A. sygyzii* and *A.*
201 *lovaniensis* dominated box and heap fermentations, respectively, for the first 48 hours, both
202 followed by *A. pasteurianus* (Fig. 2C).

203 **3.3 Intraspecies biodiveristy**

204 *S. cerevisiae* (35 isolates), *Lb. fermentum* (51 isolates) and *A. pasteurianus* (51 isolates) as the most
205 abundant species were further investigated. Seven clusters were identified for *S. cerevisiae* (Fig.
206 3A), 13 for *Lb. fermentum* (Fig. 3B) and 13 for *A. pasteurianus* (Fig. 3C). Isolates generally
207 grouped according to the type of fermentation (B or H), except for clusters II and VI in case of *S.*
208 *cerevisiae* (Fig. 3A), clusters I, IV, VI and VIII for *Lb. fermentum* (Fig. 3B) and clusters II, IV, V,
209 VI and VII for *A. pasteurianus* (Fig. 3C).

210 For *S. cerevisiae*, cluster I was mainly composed of isolates at 1, 2 and 6 days of fermentation,
211 while most of the isolates in cluster III were obtained at day 0 (Fig. 3A). Clusters IV, V, VI and VII
212 contained strains isolated in the first stages of the fermentation processes. As can be seen in Figure
213 3B, *Lb. fermentum* isolates in clusters III, V, VI, IX and XIII came from the first 2 days of
214 fermentation, unlike those in clusters II, IV and VIII, in which isolates of days 4 and 6 were also
215 present. Clusters VII, X, XI and XII were composed of strains isolated only at days 4 and 6 of
216 sampling. Regarding *A. pasteurianus* (Fig. 3C), clusters I, III, IX, XI, XII and XIII contained
217 isolates from the last stage of the heap fermentation. None of the identified clusters grouped strains
218 isolated form the first to the last day of fermentation.

219 **3.4 Growth in stressful conditions**

220 The complete set of results pertaining to the physiological characterizations is presented in
221 Supplementary Tables 1, 2 and 3 for the yeasts, LAB and AAB, respectively. The growth

222 percentage was determined by comparing each isolate grown in the media with and without any
223 modification, where 100% means that the isolate grew in the same way as in the reference
224 condition. Isolates that did not exceed a percentage of growth ratio >10% were considered as not
225 grown. In some cases, the data showed an increased growth value compared to the reference
226 conditions, which means values higher than 100% (Supp. Tab. 1, 2 and 3).
227 Only a few strains of *C. ethanolica*, *P. kudriavzevii*, *P. manshurica*, *H. opuntiae*, *S. cerevisiae* and
228 *Sch. pombe* were able to grow at 45°C (Suppl. Tab. 1). The yeasts were able to grow at pH 3.5 and
229 4.5, while the growth at pH 2.5 did not on average, exceed 66%, with respect to the reference
230 condition. *S. cerevisiae*, *C. ethanolica*, *H. uvarum* and *P. manshurica* were able to cope with the pH
231 stress at pH 2.5. Fifty-four and 38% of the total isolates were able to grow at 8, 10 and 12% of
232 ethanol, respectively. *S. cerevisiae* showed the highest growth at 12% (Suppl. Tab. 1). All the yeast
233 species grew well at 5, 15 and 30 % of glucose and fructose. Among the strains that were able to
234 grow with 30% of sugars, *C. ethanolica, H. uvarum*, *P. manshurica, S. cerevisiae, Sch. pombe* and
235 *T. delbrueckii* showed the highest growths.
236 LAB growth was, on average, higher at 37°C than at 30° or 45°C (Suppl. Tab. 2). Only a few
237 strains of *Lb. fermentum* (55%), *Lb. plantarum* (41%) and *Lb. rhamnosus* (4%) exceeded 50% of
238 the growth ratio at pH 3.5 (Suppl. Tab. 2), and the average growth reached a maximum of 36% at
239 pH 2.5. In the presence of 8% and 10% ethanol the average growth of yeast isolates was close to
240 40%; the *Lb. plantarum, Lb. fermentum* and *Leuc. pseudomesenteroides* species were able to grow
241 with 12% of ethanol. At this latter concentration, 36% of the *Lb. plantarum* strains reached more
242 than 90% of growth, compared to the reference condition (Suppl. Tab. 2). Almost all of the isolates
243 were able to grow with 30% of glucose and fructose (Suppl. Tab. 2). Eighty-five percent of the
244 LAB strains were able to grow at 1% of lactic acid, but the average growth did not reach more than
245 38%, except for a few isolates of *Lb. plantarum* and *Lb. fermentum,* which were able to grow with a
246 maximum value of 4% of lactic acid (Suppl. Tab. 2). As far as acetic acid tolerance is concerned,
247 the mean growth was around 20%, with the only exception being a few strains that were able to

grow until 5% of acetic acid. Among these, 45% belonged to *Lb. fermentum*, 53% to *Lb. plantarum* and 2% to *Leuc. pseudomesenteroides* (Suppl. Tab. 2).

All the AAB isolates grew well under both the 30 and 37°C conditions, only a few strains were able to grow at 45°C above 40%, with respect to the reference condition: 73% belonged to *A. pasterurianus*, 17% to *A. lovaniensis*, 5% to *A. syzygii* and 5% to *G. saccharovirans* (Suppl. Tab. 3). All the strains were able to grow at pH 2.5, but only a few strains of *A. pasteurianus* grew to 50%, with respect to the reference condition. The AAB showed an average value of 63 and 56% for 8 and 10% of ethanol, respectively.Forty percent of the *A. pasteurianus* and 15% of the *A. lovaniensis* strains grew more than 50% in 12% of ethanol. Within the isolates that grew with 30% of glucose, 70% belonged to *A. pasteurianus*, 25% to *A. lovaniensis* and 5% to *A. sygyzii* (Suppl. Tab. 3). With 30% of fructose, *A. pasteurianus* (65%), *A. lovaniensis* (20%), A. *sygyzii* (12%) and *G. saccharovirans* (3%) were able to grow more than 50%, with respect to the reference condition, as can be observed in Supplementary Table 3. The average growth values of the AAB strains at 1% of lactic acid was 37% and 25% for 2 and 4% lactic acid concentrations, respectively. Among the isolates that were able to grow with 5% of lactic acid, 70% were *A. pasteurianus*, 15% were *A. lovaniensis* and 12 and 3% were *A. sygyzii* and *G. Saccharovirans*, respectively (Suppl. Tab. 3). The average growth for the AAB isolates at the 1% of acetic acid condition was 33%, compared to the reference condition, and around 25% for 2, 4 and 5% of acetic acid. Those isolates that were able to reach more than 25% with 5% of acetic acid were composed of 79% of *A. pasteurianus*, 13% of *A. lovaniensis,* 5% of *A. sygyzii* and 3% of *G. saccharovirans* (Suppl. Tab. 3).

3.5 Acidification activity of the LAB

Considering an initial pH value of 6.48, the *Lb. plantarum* and *Lb. fermentum* isolates reached a pH of 6.10 in 12 hours and the pH values were 5.31 and 5.54, respectively, after 48 hours. In the case of *Lb. rhamnosus* and *Leuc. pseudomesenteroides*, the pH was lower than 6.00 after 12 hours, and at the end of the acidification process, the *Lb. rhamnosus* pH value was 4.33 and the *Leuc. pseudomesenteroides* value was 4.56.

3.6 Enzymatic activities

Among the studied yeast isolates, 30% of the yeasts were positive to ß-glucosidase activity, and were mainly composed of *S. cerevisiae* (53%), *H. uvarum* (20%) and *H. guillermondii* (16%) species (Suppl. Tab. 1). Forty-six percent showed glycosidase activity, which mostly belonged to *S. cerevisiae* (48%), and this was followed by *H. guilliermondi*, *H. uvarum* and *C. ethanolica* (Suppl. Tab. 1). Only 10% of the total yeast isolates were positive to protease activity, 66% of which belonged to *S. cerevisiae*, as reported in Supplementary Table 1. Nineteen percent of the strains resulted positive to pectinase activities, primarily *S. cerevisiae* (55%), which was followed by *C. ethanolica* (35%), *H. guillermondii* (5%) and *Sch. pombe* (5%, Suppl. Tab. 1). Only 4% of the strains showed esterase activity: the *H. opuntiae*, *P. manshurica, T. delbrueckii* and *Sch. pombe* species.

4. Discussion

The ecology of heap and box fermentation methods has already been investigated (Camu et al. 2007; Daniel et al. 2009; De Melo Pereira et al. 2013; Jespersen et al. 2005; Lagunes Gálvez et al. 2007; Meersman et al. 2013; Nielsen et al. 2006, 2007; Papalexandratou et al. 2011, 2013;), however, only a few studies have made a comparison of the microbial diversity of these two traditional methods.

Cocoa bean fermentations were characterized by quite high initial counts of yeasts, LAB and AAB and by a lower maximum temperatures compare to the existing literature. Differences in initial microbial counts may depend from pod ripeness and postharvest pod age. Here, significant differences between the box and heap fermentations were mainly observed in the yeast and LAB counts, as reported in Table 1. Regarding the temperature, most studies reports values increasing up to 50°C during cocoa beans fermentation (Ardhana and Fleet, 2003; De Melo Pereira et al., 2013; Lagunes Gálvez et al., 2007; Papalexandratou et al., 2011b), while in this study the recorded temperatures were never above 42°C. The same trend was observed in all replicates, thereby it was

very consistent. Heap fermentation was characterized by a slower increase in temperature than the box one, which may be correlated to the lower amount of volume that characterized this method. Interesting to notice the lack of growth ability of almost all AAB observed at 45°C (Suppl. Tab. 3) suggesting a specific adaptation of those isolates to the fermentation processes investigated.

The molecular identification of the isolates pointed out a major complexity of the yeast population with respect to the LAB and AAB groups. In box fermentation, the predominance of *S. cerevisiae* in the first phase of the process was more evident with respect to the heap fermentation. This species has already been noted to be involved in box (Ardhana and Fleet 2003; De Melo Pereira et al. 2013; Meersman et al. 2013; Nielsen et al. 2007; Papalexandratou et al. 2011, 2013) and heap fermentations (Daniel et al. 2009; Jespersen et al. 2003; Meersman et al. 2013; Nielsen et al. 2005, 2007; Papalexandratou et al. 2011). The presence of *P. kudriavzevii, T. delbrueckii* and *H. opuntiae* has also been recorded during box fermentations (Daniel et al. 2009; Hamdouche et al. 2015; Ho et al. 2014; Meersman et al. 2013; Papalexandratou et al. 2013), but not during heap one. Heap fermentation was dominated by different species: first *H. uvarum* that was seen as the most abundant species at starting point, then, *S. cerevisie* until 48 h followed by *H. guilliermondii* together with *C. ethanolica.* Here, *C. ethanolica* was dominant also in the last phase of box fermentation while, in other studies, it was only found during heap fermentations (De Melo Pereira et al. 2013; Nielsen et al. 2007).

The LAB populations were similar in both fermentations. *Lb. fermentum* was the most dominant species during both the heap and box cocoa bean fermentations, as already observed in other studies (Camu et al. 2007, 2008; Kostinek et al. 2008; Lefeber et al. 2011; Nielsen et al. 2007; Papalexandratou et al. 2011). *Lb. plantarum* was also dominant during both fermentations, although it was isolated more frequently in the box one.

A. pasteurianus was the most important species in the AAB group and its presence has already been reported in other studies (Camu et al. 2007; Cleenwerck et al. 2008; Lefeber et al. 2012; Nielsen 2006; Papalexandratou et al. 2011). Another two species of AAB have been isolated in this study:

326 *A. syzygii,* in the box fermentations and *A. lovaniensis* in the heap fermentations, their presence was
327 in both cases significant since took a predominant role in the first 24 h.
328 The results of the molecular characterization highlighted that the fermentation method had an
329 important impact on the biodiversity of *S. cerevisiae, Lb. fermentum* and *A. pasteurianus*. Strains
330 that dominated the box and heap fermentations did not grouped together and this can be explained
331 taking into consideration the microbial contamination from previous fermentations, in case of
332 boxes, and from plantain leaves for heap fermentations. Furthermore, intraspecific characterization
333 suggests a progression of different strains during the fermentation, probably correlated to the
334 capability of strains to better adapt to the stress conditions, which characterize the process.
335 The response of isolates to stress conditions, in terms of growth capability, has been investigated
336 using different parameters, in order to provide useful information on their ability to initiate and
337 carry out the fermentation. Furthermore, conditions were chosen in order to evaluate if strains,
338 coming from a stressful environment, developed resistance. *S. cerevisiae* demonstrated the ability to
339 withstand all stressful conditions, and several strains were able to grow at 45°C, in contrast with the
340 result of Daniel et al. (2009). This thermo-tolerance property was noticed also for *P. kudriavzevii*,
341 as reported by Pereira et al. (2012), together with a few strains of *C. ethanolica*, thereby explaining
342 their presence mainly in the last phase of box and heap fermentations. *Sch. pombe* was able to grow
343 at pH 2.5, in presence of 30% of sugars as recorded by Gomes et al. (2002) and at 45°C. Those
344 resistances may explain its presence during heap fermentations, and make this species interesting
345 for further studies since not much literature, concerning cocoa beans process, focus on this species
346 to the best of our knowledge. *T. delbrueckii*, which was isolated only in the first 48 h of box
347 fermentations, demonstrated to be resistant to high concentrations of sugars, as already been
348 described (Bely et al., 2008; Ciani et al., 2006). The same behaviour was also observed for *H.*
349 *uvarum*, which showed the best growths among all the yeast strains at pH 2.5. However, it was not
350 resistant to high temperature, explaining thereof its absence in the second phase of the processes.

351 The LAB showed the main differences in growth behavior at pH 2.5, 12% of ethanol and for a rise
352 in the sugar concentration, which strongly inhibited growth. Generally, strains of *Lb. fermentum* and
353 *Lb. plantarum* have been found to have similar stress resistance behavior that helps them to
354 withstand the fermentation process, which supports their dominance in both, box and heap methods.
355 Their capability to be acid tolerant has already been confirmed in other studies (Pereira et al., 2012;
356 Ramos et al., 2013), further, few strains of *Lb. fermentum* in particular have shown a very good
357 growth ability (see Suppl. Tab. 2). The lactic acid parameter has resulted to be stressful for the
358 LAB, but a few strains of *Lb. plantarum* and *Lb. fermentum* were able to grow in the tests
359 conducted at the higher concentrations (max value of 4%), although these species have shown the
360 capability to grow until 5% in other studies (Pereira et al., 2012). The present results underline how
361 the ability to grow in the presence of different acetic acid concentrations is strain-dependent,
362 especially in the case of *Lb. fermentum* and *Lb. plantarum*. The different pH values, and the
363 acidification activity of LAB, have been correlated to the quality of the process (Schwan and
364 Wheals 2004), because of their influence on the enzymatic activities and on the microbial
365 succession. In the present study, *Lb. rhamnosus* and *Leuc. pseudomesenteroides* have shown a
366 higher acidification effect than *Lb. plantarum* and *Lb. fermentum* a result that has also been reported
367 in a recent study by Zuo et al. (2014), this may be correlated to the different pH values observed at
368 48 h within the two methods.
369 Although the optimal growth temperature of AAB is between 25 and 30°C, most of the tested
370 isolates were able to grow at 45°C, this being a condition that occurs naturally during cocoa bean
371 fermentations (Schwan and Wheals 2004). High osmotic stress conditions (30%) reduced AAB
372 growth, especially for high levels of glucose, as already reported (Gullo and Giudici 2008), rather
373 than fructose. Resistance of AAB to acetic acid was strain dependent, this acid is the main product
374 of AAB ethanol oxidation, but is also a limiting factor for their growth. The same behavior was
375 detected in the presence of lactic acid, which inhibited the AAB to a great extent; variability among
376 strains of the same species was observed. *A. syzygii* showed high grow capability until 15% of

sugar, and at 12% of ethanol, but did not show good ability at 45°C, these characteristics meet the behavior highlighted in box fermentation in our data.

Ho et al. (2014) has recently highlighted the importance of yeasts role for cocoa beans fermentation revealing that, beans fermented without yeasts, had a higher content of shell material suggesting that the pulp was not fully degraded, and remained attached to the testa. Concerning pulp degradation yeast strains were tasted for pectinase activities and *S. cerevisiae* showed the best result, followed by *C. ethanolica* as previously reported (Blanco et al., 1999). Twenty percent of *S. cerevisiae* strains were also positive to protease activity, although protease activity is normally correlated to non-*Saccharomyces* species (Charoenchai et al. 1997; Englezos et al. 2015; Strauss et al. 2001). Endo and exo protease activities influence the concentration of free amino acids within the beans (Ho et al., 2014), contributing to chocolate flavour due to the formation of pyrazines during roasting. Polysaccharides are fermented together with sugars during cocoa bean fermentations, thereby glycosidase and ß-glucosidase activities were tested. Forty-five % showed glycosidase activity, most of which belongs to *S. cerevisiae,* and this was followed by *H. guilliermondi, H. uvarum* and *C. ethanolica*. Almost all the strains positive for ß-glucosidase activity were distributed over the isolated species, but mainly *S. cerevisiae*, *H. uvarum* and *H. guillermondii* were involved, as indicated in other studies (Fia et al., 2005; Hernández et al., 2003). The fermentation of pulp sugars by yeasts produces a vast array of volatile metabolites (e.g., higher alcohols, organic acids, esters, aldehydes, ketones, etc.). Those are known for their aromatic properties and they are produced both, in the pulp and into the beans, and they may be transferred from the pulp to the nibs during fermentation, as recently reported by Ho et al. (2014).

Strains isolated from cocoa bean fermentations require a full physiological characterization in order to have a better understanding of their ability to adapt to complex environmental conditions. The results obtained in this study can help contribute to the knowledge of microbial dynamics of cocoa bean fermentations and the phenotypic characterization has clearly shown that the strains were either able or not to withstand different stresses. Moreover additional information about enzymatic

activities can improve and complete their physiologic profile in order to understand their potential impact on the fermentation process. To improve the existing knowledge, the resistance to stress conditions, together with the presence of specific enzymatic activities, should be considered when choosing the optimal strains for suitable cocoa bean fermentations starter culture formulations.

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563

564 **Figure legends.**

565

566 **Figure 1.** Comparison of yeast (A), LAB (B) and AAB (C) populations isolated from box and heap
567 fermentations. Data are expressed as percentages of the total isolated strains.

568

569 **Figure 2.** Microbial succession of yeasts (A), LAB (B) and AAB (C), during box and heap
570 fermentations. Data are expressed as percentages of the total isolated strains.

571

572 **Figure 3.** Intraspecies characterization of 35 isolates of *Saccharomyces cerevisiae* (A), 51 isolates
573 of *Lactobacillus fermentum* (B) and 51 isolates of *Acetobacter pasteurianus* (C). The groups,
574 obtained by means of cluster analysis of the Rep-PCR profiles, were calculated using the Pearson
575 correlation Index and are indicated in roman numerals. Strain codes with the respective day of
576 isolation are reported. B/T in the strain codes refer to Box/Heap fermentations.

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Figure 1

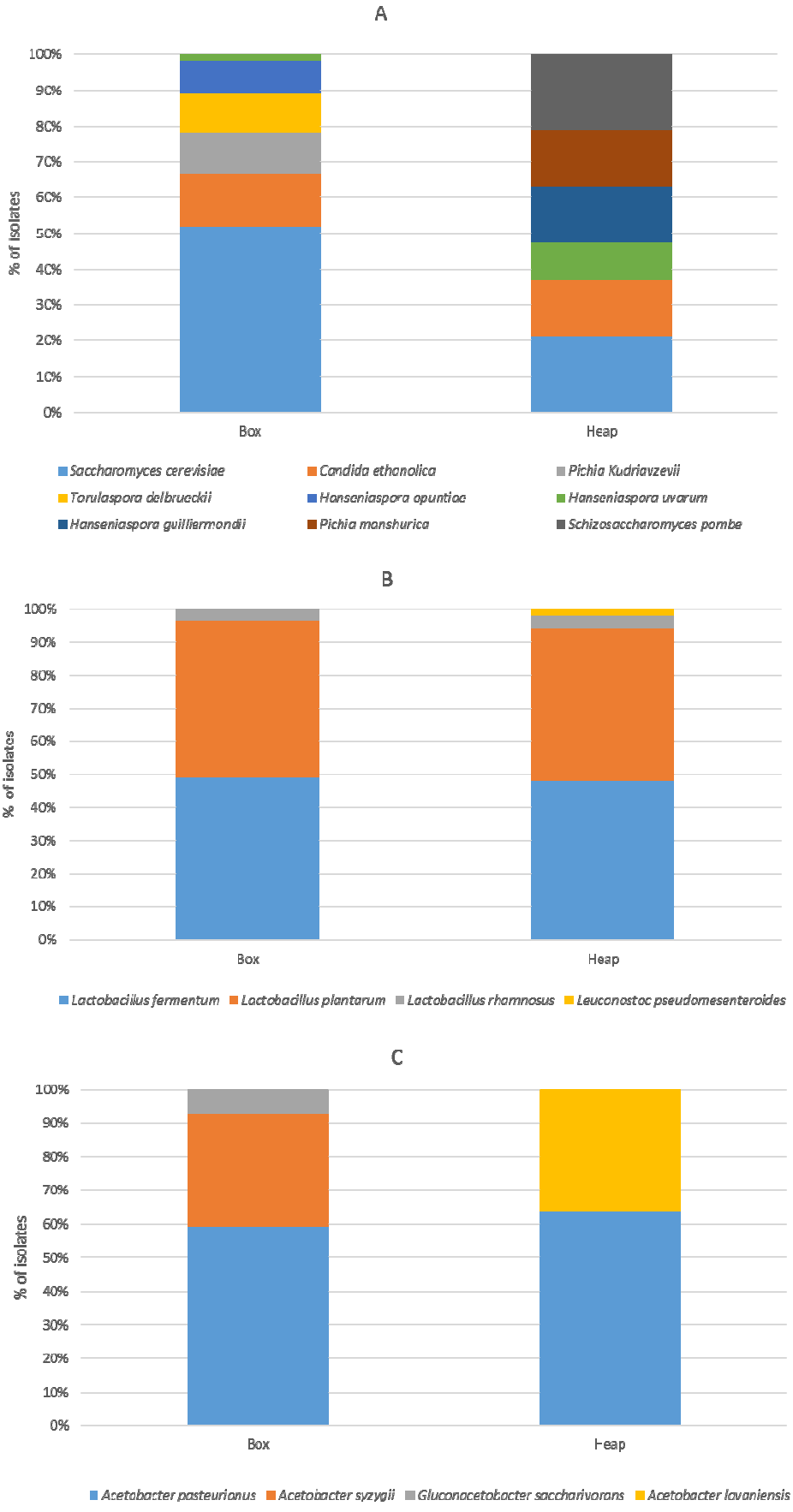

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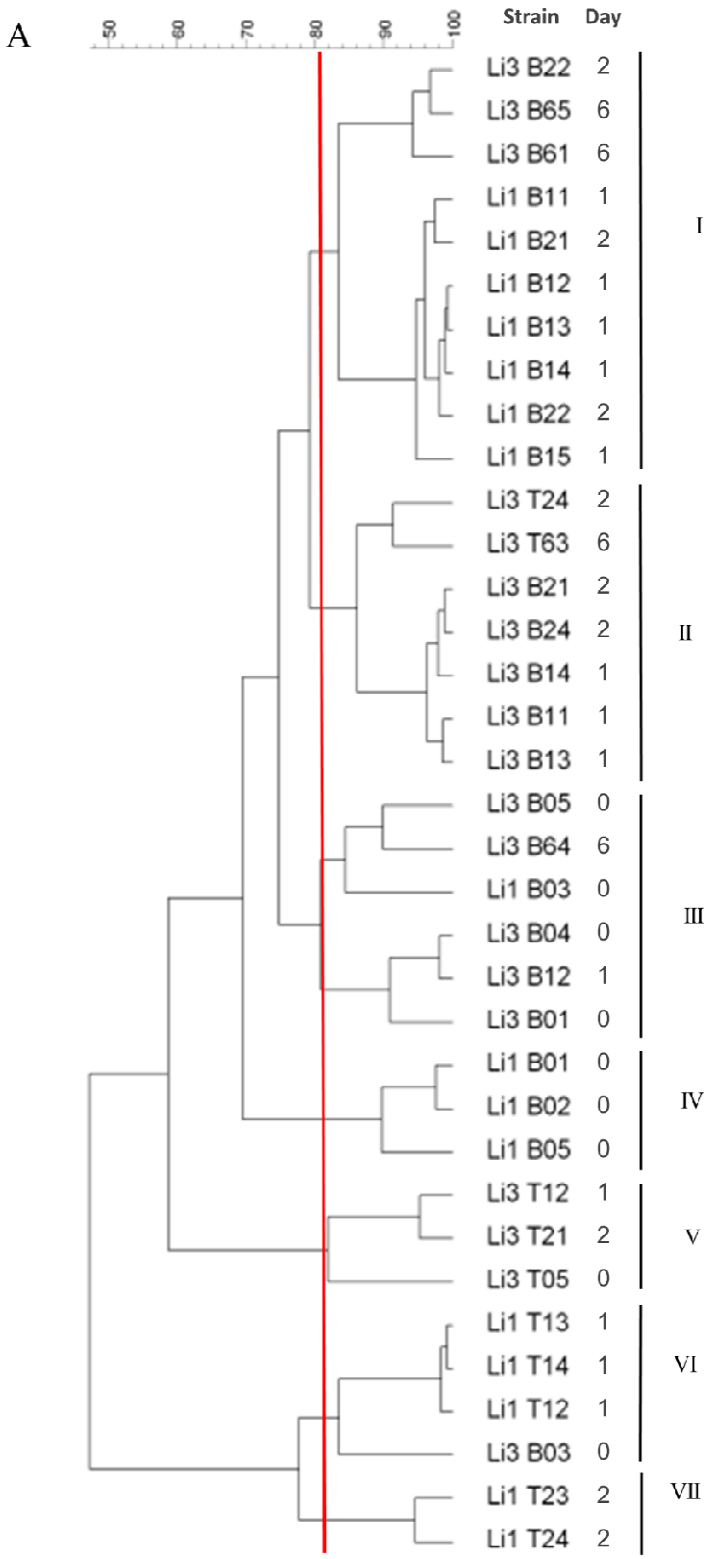
Figure 2

587

588 **Figure 3**

589



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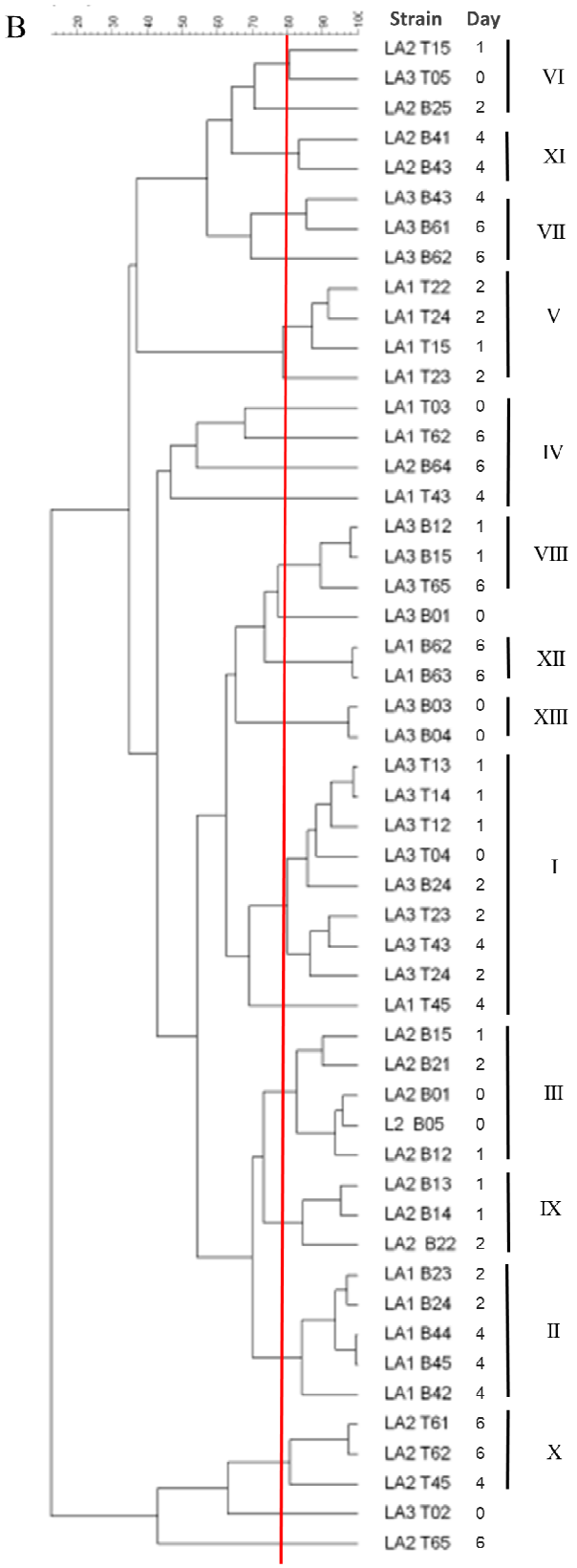
B
Strain
Day
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LA3 T05 0
LA2 B25 2
LA2 B41 4
LA2 B43 4
LA3 B43 4
LA3 B61 6
LA3 B62 6
LA1 T22 2
LA1 T24 2
LA1 T15 1
LA1 T23 2
LA1 T03 0
LA1 T62 6
LA2 B64 6
LA1 T43 4
LA3 B12 1
LA3 B15 1
LA3 T65 6
LA3 B01 0
LA1 B62 6
LA1 B63 6
LA3 B03 0
LA3 B04 0
LA3 T13 1
LA3 T14 1
LA3 T12 1
LA3 T04 0
LA3 B24 2
LA3 T23 2
LA3 T43 4
LA3 T24 2
LA1 T45 4
LA2 B15 1
LA2 B21 2
LA2 B01 0
L2 B05 0
LA2 B12 1
LA2 B13 1
LA2 B14 1
LA2 B22 2
LA1 B23 2
LA1 B24 2
LA1 B44 4
LA1 B45 4
LA1 B42 4
LA2 T61 6
LA2 T62 6
LA2 T45 4
LA3 T02 0
LA2 T65 6
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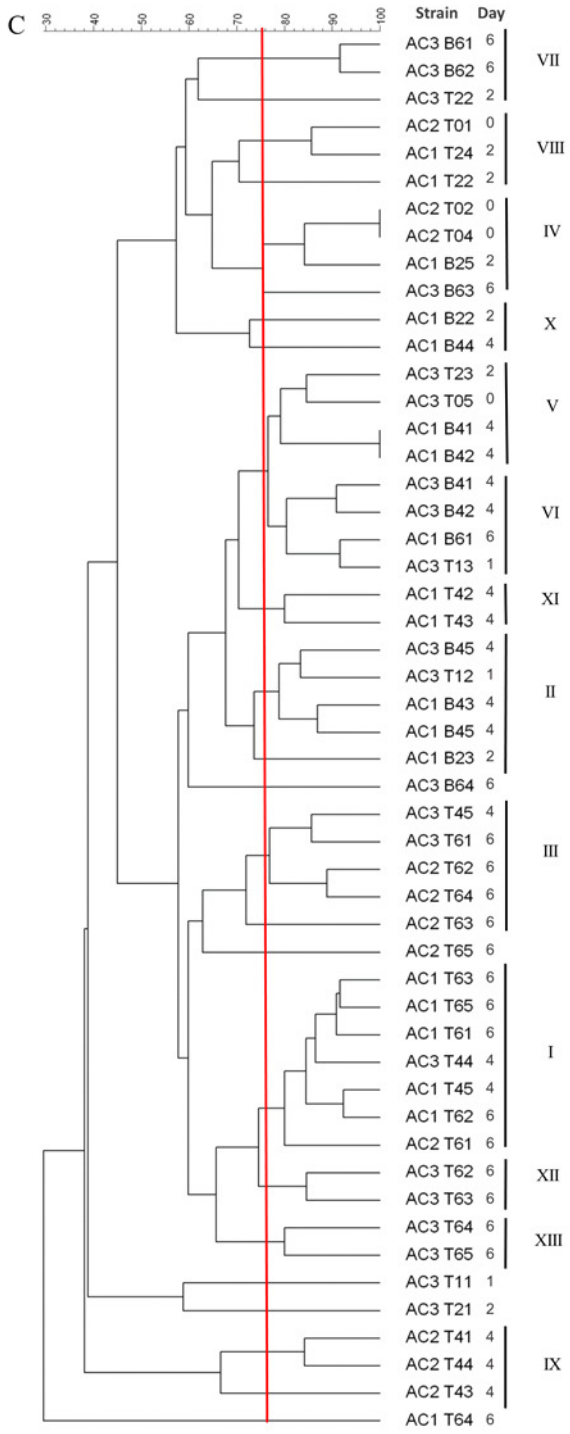
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Strain Day
AC3 B61 6
AC3 B62 6
AC3 T22 2
VII
AC2 T01 0
AC1 T24 2
AC1 T22 2
VIII
AC2 T02 0
AC2 T04 0
AC1 B25 2
AC3 B63 6
IV
AC1 B22 2
AC1 B44 4
X
AC3 T23 2
AC3 T05 0
AC1 B41 4
AC1 B42 4
V
AC3 B41 4
AC3 B42 4
AC1 B61 6
AC3 T13 1
VI
AC1 T42 4
AC1 T43 4
XI
AC3 B45 4
AC3 T12 1
AC1 B43 4
AC1 B45 4
AC1 B23 2
II
AC3 B64 6
AC3 T45 4
AC3 T61 6
AC2 T62 6
AC2 T64 6
AC2 T63 6
III
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AC1 T63 6
AC1 T65 6
AC1 T61 6
AC3 T44 4
AC1 T45 4
AC1 T62 6
AC2 T61 6
I
AC3 T62 6
AC3 T63 6
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AC2 T43 4
IX
AC1 T64 6

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595 **Table 1.** pH and temperature measurements, yeast, LAB and AAB counts during box (B) and heap
596 (H) fermentations. The values of pH, T°C and CFU are the mean ± standard deviation of three
597 independent box and heap fermentations.
598 Statistical data are expressed as *,**,*** and NS that indicate significance at P<0.05, P<0,01,
599 P<0,0001 and no significant difference, respectively.
600

B/H		Fermentation time (h)				
		0	24	48	96	144
pH	B	4.3±0.2	3.9±0.4	3.8±0.5	3.7±0.3	3.7±0.4
	H	4.1±0.6	3.9±0.3	3.3±0.3	3.7±0.3	3.9±0.3
	Sig.	NS	NS	NS	NS	NS
Temperature °C	B	31.3±0.3	36.4±0.1	39.1±0.6	39.4±0.3	41.9±0.6
	H	29.5±0.6	32.6±0.6	36.1±0.8	38.7±0.2	39.7±0.4
	Sig.	**	***	**	*	**
YEASTS, LOG_{10} CFU/G	B	7.7±0.6	6.5±0.3	6.4±0.3	7.5±0.4	6.4±0.3
	H	7.6±0.6	6.8±0.2	7.2±0.3	8.5±0.2	7.6±0.6
	Sig.	NS	NS	*	*	*
LAB, LOG_{10} CFU/G	B	6.6±0.4	8.1±0.4	7.9±0.4	7.0±0.3	5.6±0.5
	H	6.6±0.4	7.9±0.3	6.9±0.4	8.1±0.3	8.1±0.8
	Sig.	NS	NS	*	*	*
AAB, LOG_{10} CFU/G	B	6.3±0.4	7.4±0.6	6.8±0.5	7.6±0.7	6.7±0.2
	H	6.4±0.2	7.2±0.2	7.3±0.6	8.8±0.2	7.9±0.8
	Sig.	NS	NS	NS	*	NS

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Supplementary Tables

Supplementary Table 1. Results obtained from the physiological characterization of the yeast isolates. Data are expressed as positive/negative for the enzymatic activities. Physiological characterization results are expressed as the ratio between the growth of the isolates in the media with and without modification (reference condition), where 100% means that the isolate grew in the same way as in the reference condition. Isolates that did not exceed a percentage of growth of <10% were considered as not grown. The values reported are the means of triplicate experiments.

Supplementary Table 2. Results obtained from the physiological characterization of the LAB isolates. Physiological characterization results are expressed as the ratio between the growth of the isolates in the media with and without modification (reference condition), where 100% means that the isolate grew in the same way as in the reference condition. Isolates that did not exceed a percentage of growth of <10% were considered as not grown. The values reported are the means of triplicate experiments.

Supplementary Table 3. Results obtained from the physiological characterization of the AAB isolates. Physiological characterization results are expressed as the ratio between the growth of the isolates in the media with and without modification (reference condition), where 100% means that the isolate grew in the same way as in the reference condition. Isolates that did not exceed a percentage of growth of <10% were considered as not grown. The values reported are the means of triplicate experiments.