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**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**

**Authors:** Simonetta Visintin<sup>1</sup>, Valentina Alessandria<sup>1</sup>, Antonio Valente<sup>2</sup>, Paola Dolci<sup>1</sup>, Luca Cocolin<sup>1\*</sup>

<sup>1</sup>University of Torino, Department of Agriculture, Forest and Food Sciences, Largo Paolo Braccini 2, 10095 Grugliasco (Torino) Italy

<sup>2</sup>SOREMARTEC ITALIA S.r.l., Piazza Ferrero 1 - 12051 Alba (Cuneo) Italy

**\*Corresponding Author:** Luca Cocolin, Largo Paolo Braccini 2, 10095 Grugliasco (Torino) Italy, Tel.: +39 011 670 8553; fax: +39 011 670 8549, E-mail address: [lucasimone.cocolin@unito.it](mailto:lucasimone.cocolin@unito.it)

**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

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

## **2.2 Sampling procedure**

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

## **2.3 Statistical analysis**

The data obtained from the pH, temperatures and yeasts, as well as the LAB and AAB counts of the 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)<sub>5</sub> (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**

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

176

### 177 **3. Results**

#### 178 **3.1 Microbiological analysis of the box and heap fermentations**

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

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

#### 186 **3.2 Identification of isolates and species dynamics during fermentations.**

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

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

### 3.3 Intraspecies biodiversity

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

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

### 3.4 Growth in stressful conditions

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

percentage was determined by comparing each isolate grown in the media with and without any modification, 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 ratio >10% were considered as not grown. In some cases, the data showed an increased growth value compared to the reference conditions, which means values higher than 100% (Suppl. Tab. 1, 2 and 3).

Only a few strains of *C. ethanolica*, *P. kudriavzevii*, *P. manshurica*, *H. opuntiae*, *S. cerevisiae* and *Sch. pombe* were able to grow at 45°C (Suppl. Tab. 1). The yeasts were able to grow at pH 3.5 and 4.5, while the growth at pH 2.5 did not on average, exceed 66%, with respect to the reference condition. *S. cerevisiae*, *C. ethanolica*, *H. uvarum* and *P. manshurica* were able to cope with the pH stress at pH 2.5. Fifty-four and 38% of the total isolates were able to grow at 8, 10 and 12% of ethanol, respectively. *S. cerevisiae* showed the highest growth at 12% (Suppl. Tab. 1). All the yeast species grew well at 5, 15 and 30 % of glucose and fructose. Among the strains that were able to grow with 30% of sugars, *C. ethanolica*, *H. uvarum*, *P. manshurica*, *S. cerevisiae*, *Sch. pombe* and *T. delbrueckii* showed the highest growths.

LAB growth was, on average, higher at 37°C than at 30° or 45°C (Suppl. Tab. 2). Only a few strains of *Lb. fermentum* (55%), *Lb. plantarum* (41%) and *Lb. rhamnosus* (4%) exceeded 50% of the growth ratio at pH 3.5 (Suppl. Tab. 2), and the average growth reached a maximum of 36% at pH 2.5. In the presence of 8% and 10% ethanol the average growth of yeast isolates was close to 40%; the *Lb. plantarum*, *Lb. fermentum* and *Leuc. pseudomesenteroides* species were able to grow with 12% of ethanol. At this latter concentration, 36% of the *Lb. plantarum* strains reached more than 90% of growth, compared to the reference condition (Suppl. Tab. 2). Almost all of the isolates were able to grow with 30% of glucose and fructose (Suppl. Tab. 2). Eighty-five percent of the LAB strains were able to grow at 1% of lactic acid, but the average growth did not reach more than 38%, except for a few isolates of *Lb. plantarum* and *Lb. fermentum*, which were able to grow with a maximum value of 4% of lactic acid (Suppl. Tab. 2). As far as acetic acid tolerance is concerned, 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. pasteurianus*, 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. syzygii* (Suppl. Tab. 3). With 30% of fructose, *A. pasteurianus* (65%), *A. lovaniensis* (20%), *A. syzygii* (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. syzygii* 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. syzygii* 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.

### 274 3.6 Enzymatic activities

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

285

### 286 4. Discussion

287 The ecology of heap and box fermentation methods has already been investigated (Camu et al.  
288 2007; Daniel et al. 2009; De Melo Pereira et al. 2013; Jespersen et al. 2005; Lagunes Gálvez et al.  
289 2007; Meersman et al. 2013; Nielsen et al. 2006, 2007; Papalexandratou et al. 2011, 2013;),  
290 however, only a few studies have made a comparison of the microbial diversity of these two  
291 traditional methods.

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

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

304 The molecular identification of the isolates pointed out a major complexity of the yeast population  
305 with respect to the LAB and AAB groups. In box fermentation, the predominance of *S. cerevisiae* in  
306 the first phase of the process was more evident with respect to the heap fermentation. This species  
307 has already been noted to be involved in box (Ardhana and Fleet 2003; De Melo Pereira et al. 2013;  
308 Meersman et al. 2013; Nielsen et al. 2007; Papalexandratou et al. 2011, 2013) and heap  
309 fermentations (Daniel et al. 2009; Jespersen et al. 2003; Meersman et al. 2013; Nielsen et al. 2005,  
310 2007; Papalexandratou et al. 2011). The presence of *P. kudriavzevii*, *T. delbrueckii* and *H. opuntiae*  
311 has also been recorded during box fermentations (Daniel et al. 2009; Hamdouche et al. 2015; Ho et  
312 al. 2014; Meersman et al. 2013; Papalexandratou et al. 2013), but not during heap one. Heap  
313 fermentation was dominated by different species: first *H. uvarum* that was seen as the most  
314 abundant species at starting point, then, *S. cerevisiae* until 48 h followed by *H. guilliermondii*  
315 together with *C. ethanolica*. Here, *C. ethanolica* was dominant also in the last phase of box  
316 fermentation while, in other studies, it was only found during heap fermentations (De Melo Pereira  
317 et al. 2013; Nielsen et al. 2007).

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

323 *A. pasteurianus* was the most important species in the AAB group and its presence has already been  
324 reported in other studies (Camu et al. 2007; Cleenwerck et al. 2008; Lefeber et al. 2012; Nielsen  
325 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 tested 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  $\beta$ -glucosidase activities were tested. Forty-five % showed glycosidase activity, most of which belongs to *S. cerevisiae*, and this was followed by *H. guilliermondii*, *H. uvarum* and *C. ethanolica*. Almost all the strains positive for  $\beta$ -glucosidase activity were distributed over the isolated species, but mainly *S. cerevisiae*, *H. uvarum* and *H. guilliermondii* 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

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

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**Figure legends.**

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

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

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

Figure 1

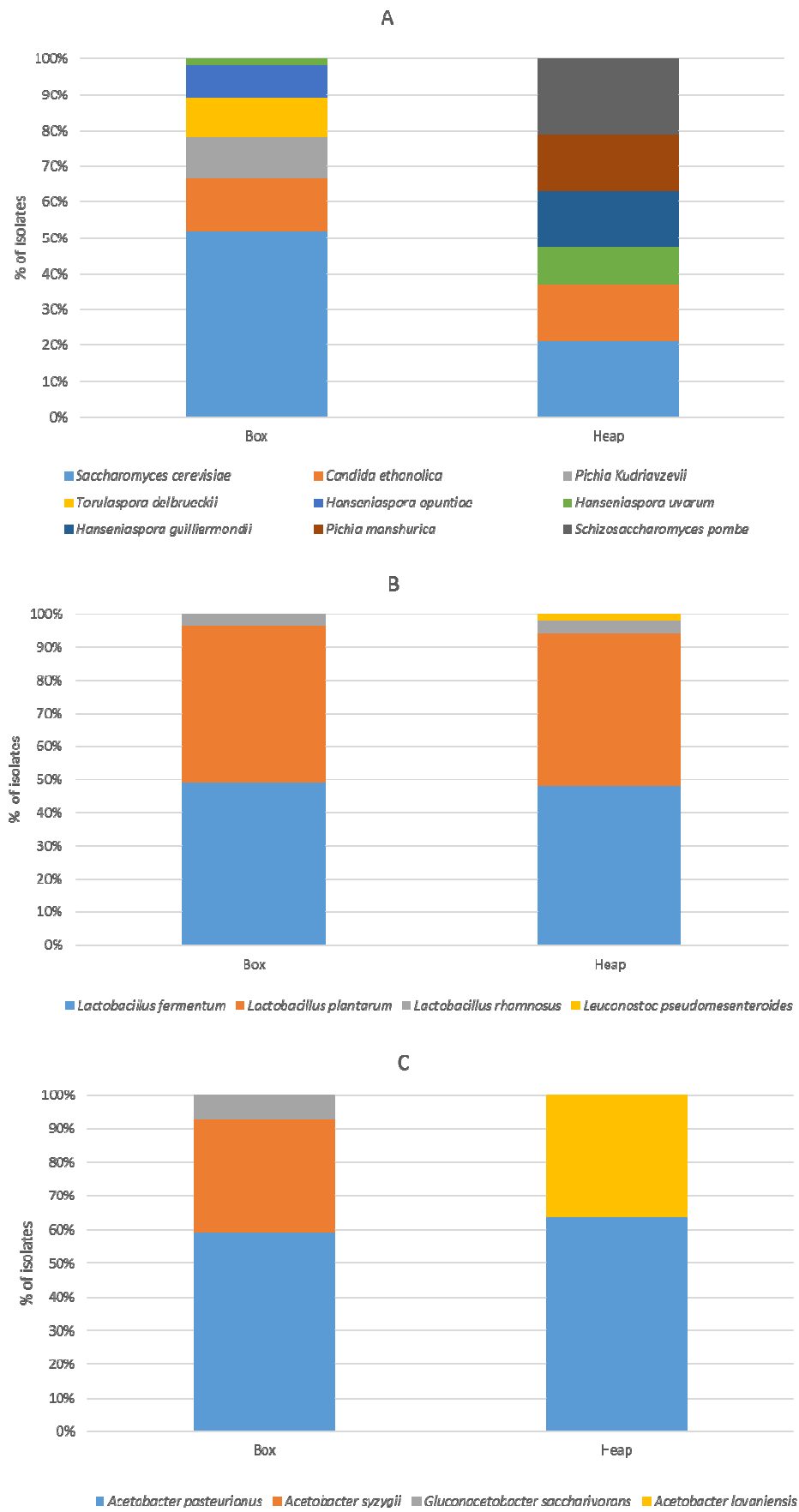
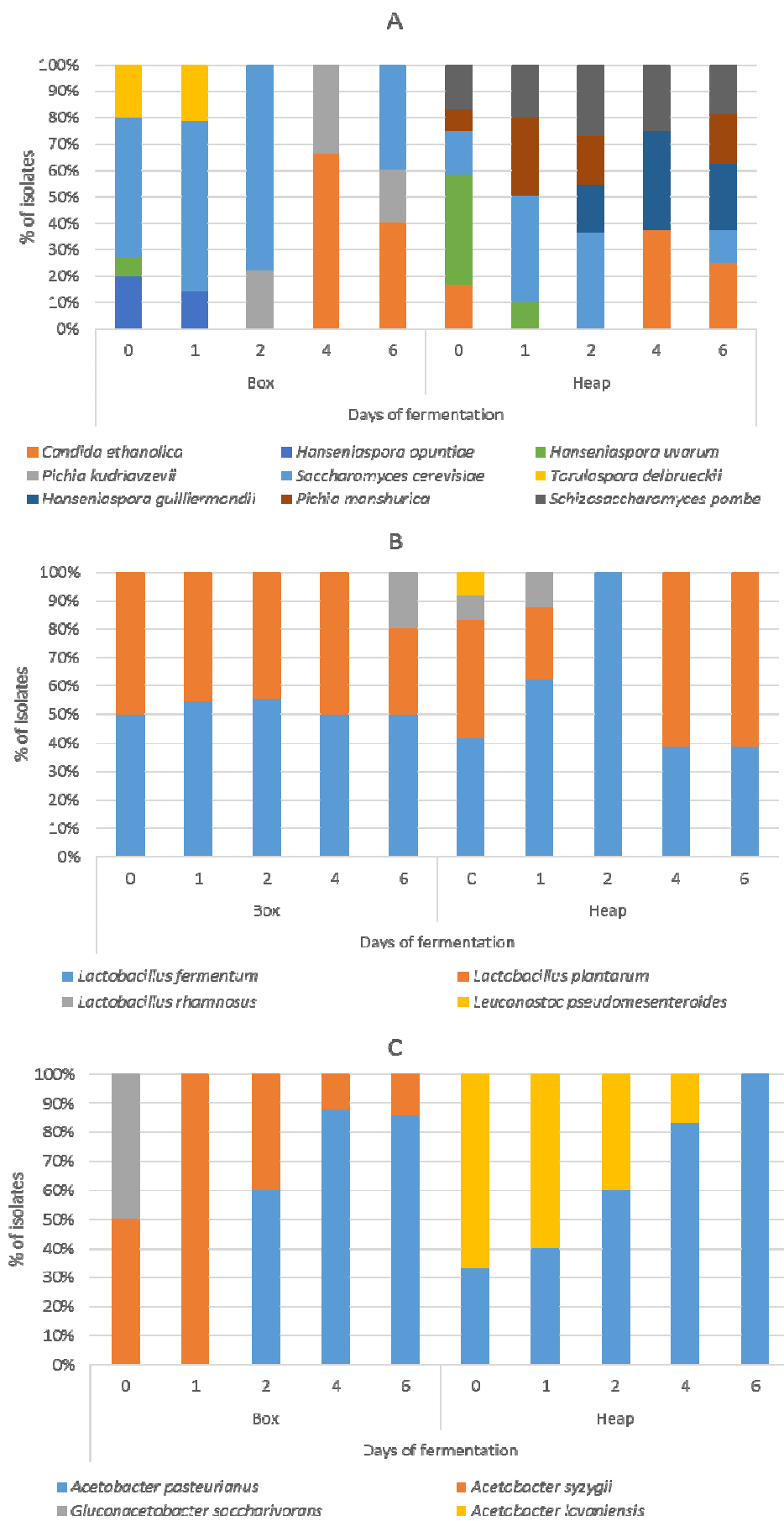
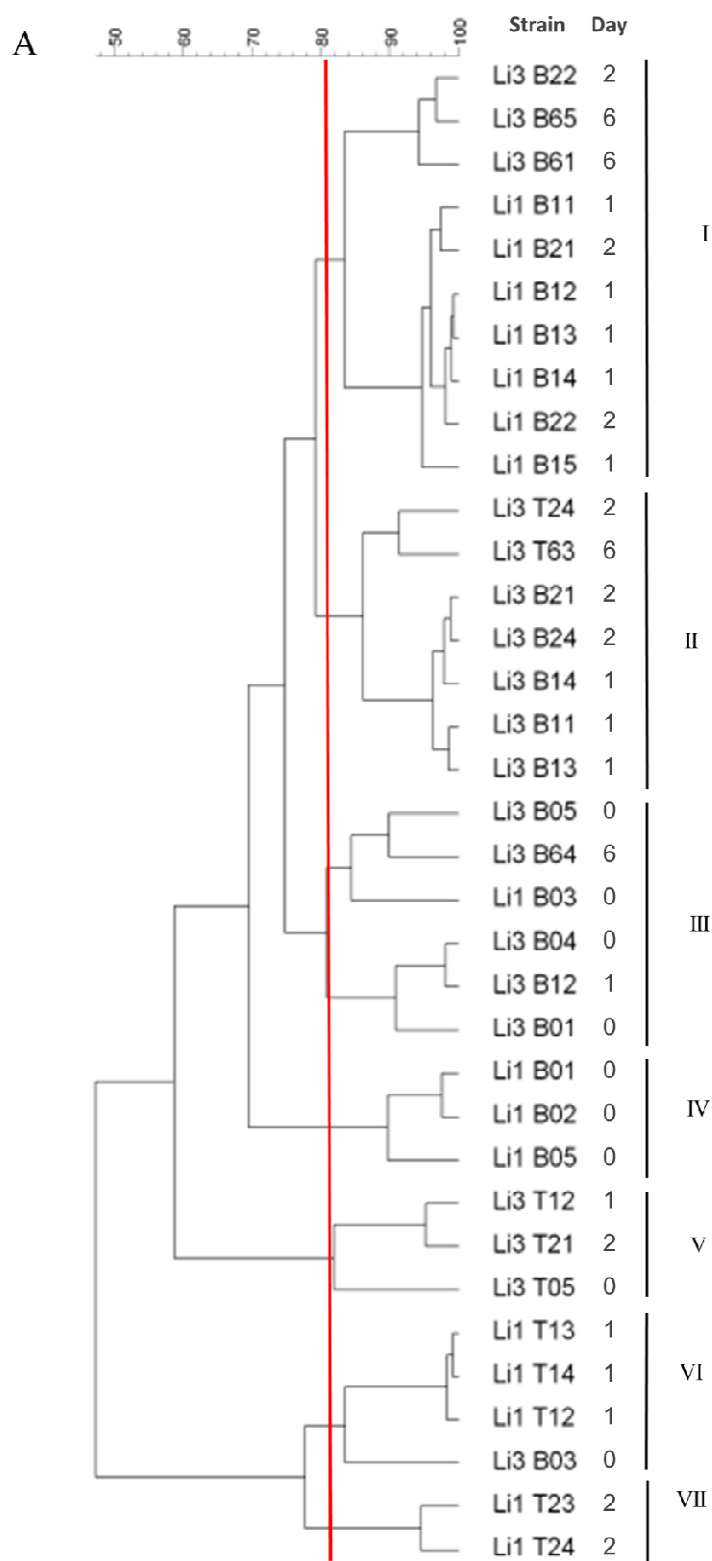


Figure 2

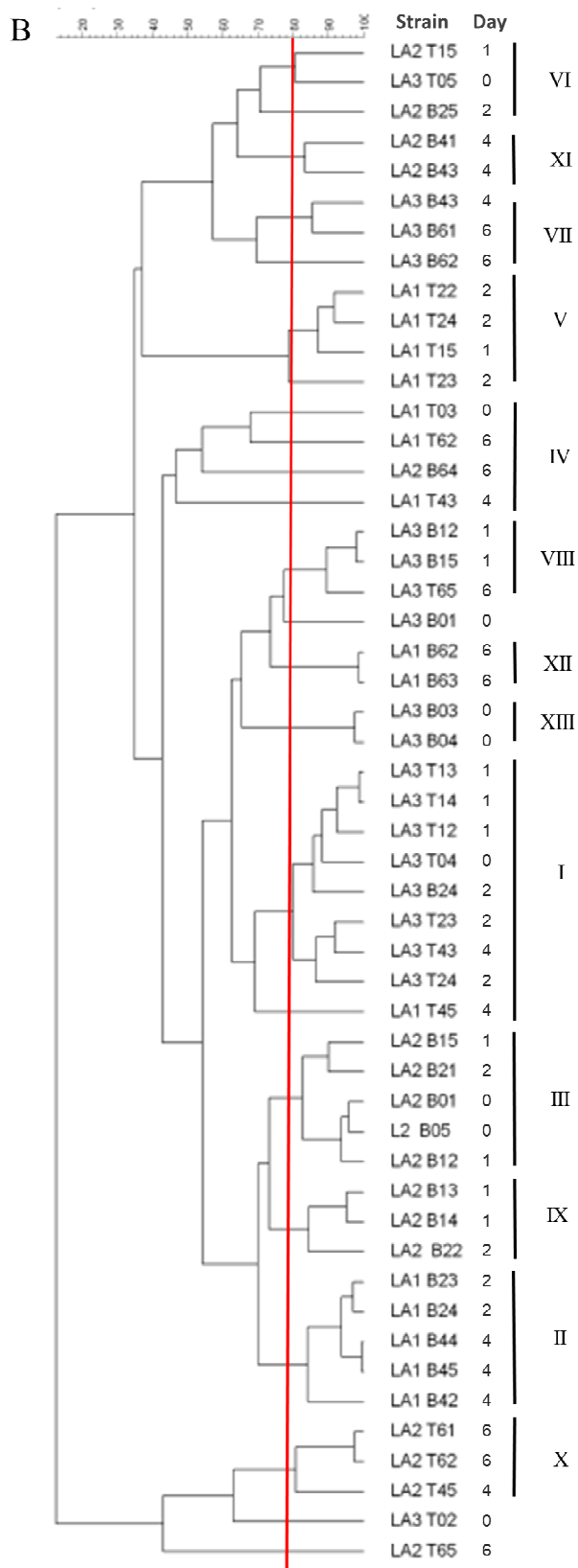


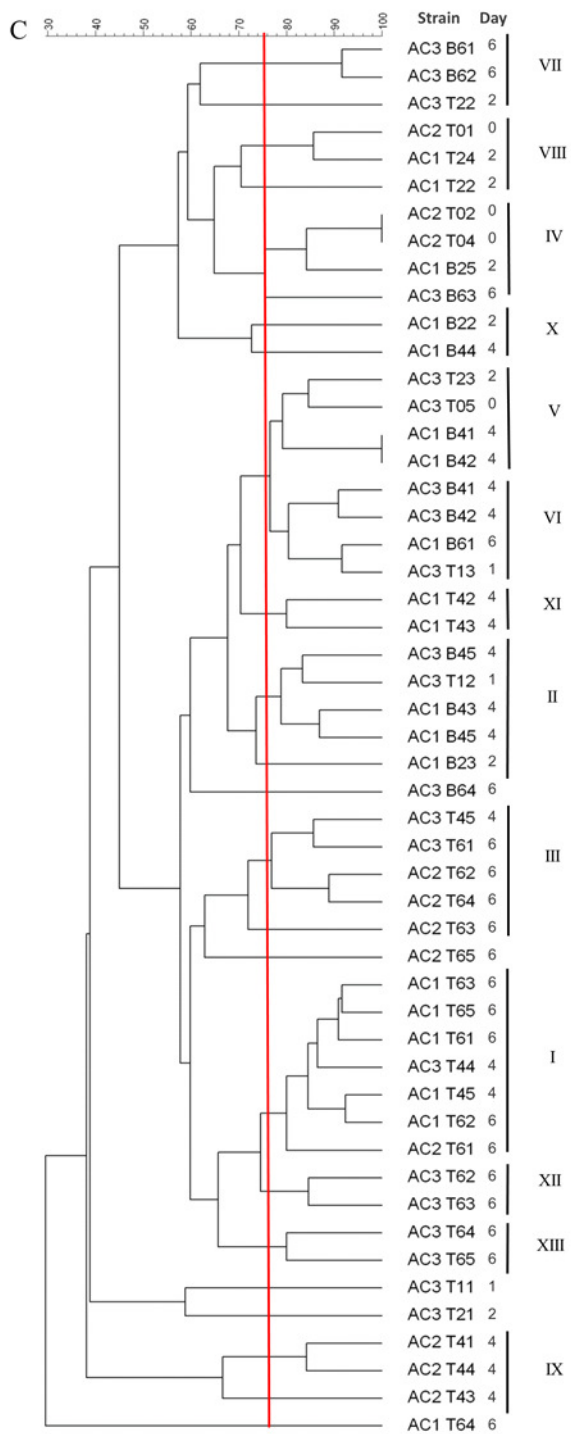
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Figure 3



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**Table 1.** pH and temperature measurements, yeast, LAB and AAB counts during box (B) and heap (H) fermentations. The values of pH, T°C and CFU are the mean  $\pm$  standard deviation of three independent box and heap fermentations. Statistical data are expressed as \*,\*\*,\*\*\* and NS that indicate significance at P<0.05, P<0.01, P<0.0001 and no significant difference, respectively.

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

602 **Supplementary Tables**

603

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

610

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

617

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

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