International Journal of Food Microbiology 279 (2018) 43–56

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Unravelling the contribution of lactic acid bacteria and acetic acid bacteria to cocoa fermentation using inoculated organisms

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ARTICLE INFO

Keywords:
Cocoa bean fermentation
Yeasts
Lactic acid bacteria
Acetic acid bacteria
Chocolate flavour

ABSTRACT

Cocoa beans (*Theobroma cacao* L.) are the raw material for chocolate production. Fermentation of the bean pulp by microorganisms is essential for developing the precursors of chocolate flavour. Currently, the cocoa fermentation is still conducted by an uncontrolled traditional process via a consortium of indigenous species of yeasts, lactic acid bacteria and acetic acid bacteria. Although the essential contribution of yeasts to the production of good quality beans and, typical chocolate character is generally agreed, the roles of lactic acid bacteria and acetic acid bacteria are less certain. The objective of this study was to investigate the contribution of LAB and AAB in cocoa bean fermentation by conducting small scale laboratory fermentations under aseptic conditions, inoculated with different groups of microorganisms previously isolated from spontaneous cocoa fermentations. The inoculation protocols were: (1) four yeasts *Hanseniaspora guilliermondii*, *Pichia kudriavzevii*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*; (2) four yeasts plus the lactic acid bacteria *Lactobacillus plantarum* and *Lactobacillus fermentum*; (3) four yeasts plus the acetic acid bacteria *Acetobacter pasteurianus* and *Gluconobacter frateuri* and (4) four yeasts plus two lactic acid bacteria and two acetic acid bacteria. Only the inoculated species were detected in the microbiota of their respective fermentations. Beans from the inoculated fermentations showed no significant differences in colour, shell weights and concentrations of residual sugars, alcohols and esters ($p > 0.05$), but they were slightly different in contents of lactic acid and acetic acid ($p < 0.05$). All beans were fully brown and free of mould. Residual sugar levels were less than 2.6 mg/g while the shell contents and ethanol were in the range of 11–13.4% and 4.8–7 mg/g, respectively. Beans fermented in the presence of LAB contained higher levels of lactic acid (0.6-1.2 mg/g) whereas higher concentrations of acetic acid (1.8–2.2 mg/g) were detected in beans inoculated with AAB.

Triangle and hedonic sensory evaluations of chocolates prepared from beans taken from the three fermentations showed no significant differences ($p > 0.05$). It was concluded that the growth of lactic acid bacteria and acetic acid bacteria may not be essential for the fermentation of cocoa beans.

1. Introduction

Cocoa beans (*Theobroma cacao* L.) are the major raw material for chocolate production. Fermentation of the beans is essential for removing the bean pulps and developing chocolate flavour (Fowler, 2009; Thompson, Miller, & Lopez, 2013). The microbial ecology of cocoa bean fermentation is complex and involves the successional growth of various species of yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and, possibly, species of *Bacillus*, other bacteria and filamentous fungi (De De Vuyst, Lefeber, Papalexandratou, & Camu, 2010; Schwan & Wheals, 2004). The essential contribution of yeasts to cocoa fermentation and development of chocolate flavour has been well demonstrated by numerous studies, including a recent study of ours (Ho, Zhao, & Fleet, 2014) and is generally agreed upon among researchers with little controversy. Divergence of opinions, however, exists with regard to the contribution of LAB to bean quality and chocolate flavour. On the one hand, LAB are believed to play a critical role in bean fermentation because they ferment pulp sugars to mainly lactic acid and utilise citric acid within the pulp to produce lactic acid, acetic acid and several volatile compounds (Camu et al., 2008; Camu et al., 2007; Lefeber, Gobert, Vrancken, Camu, & De Vuyst, 2011a). On the other hand, there is also evidence that contributions of LAB may not be necessary for the production of high quality beans that give typical chocolate character (Barel, 1998; De Vuyst et al., 2010; Ho, Zhao, & Fleet, 2015). Similarly, the contribution of AAB to bean and chocolate quality is also not clear. AAB are believed to be involved in utilising pulp sugars

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https://doi.org/10.1016/j.ijfoodmicro.2018.04.040
Received 7 September 2017; Received in revised form 19 April 2018; Accepted 25 April 2018
Available online 27 April 2018
0168-1605/

and organic acids, oxidising ethanol to acetic acid and increasing the temperature of the bean mass. These bacteria are able to produce aldehydes, ketones and other products as metabolites (Drysdale & Fleet, 1988; Raspor & Goranovic, 2008) that could impact on the formation of flavour compounds in chocolate, while acetic acid may contribute excessive acidity to cocoa beans and chocolate (Holm, Aston, & Douglas, 1993; Jinap, 1994; Jinap & Zeslinda, 1995). Further research is needed to define the contribution of AAB more precisely.

There are three basic approaches that can be used in the study of microbial ecology of cocoa fermentation: spontaneous fermentation, controlled fermentation in which the growth of specific groups of microorganisms is suppressed and controlled fermentation with inoculated organisms. Each approach has its advantages and drawbacks. Spontaneous fermentation is the method by which cocoa fermentation is conducted in the industry and, therefore, this approach represents the closest approximation of the true ecosystem of cocoa fermentation. However, because different microorganisms grow simultaneously in spontaneous fermentation, it is often difficult to separate the contribution of individual groups of organisms to the fermentation and, ultimately, bean and chocolate quality. This drawback can be overcome, to some extent, by controlled fermentation in which the growth of a specific group of organisms (e.g., yeasts) is suppressed by using preservatives that specifically act on the target group (e.g., Natamycin on yeasts). This approach, however, has its own limitations, one of which being that it is not always easy to find preservatives that would just suppress the growth of the target organisms. For example, it is rather difficult to find a preservative that would only suppress the growth of LAB without affecting the growth of other bacteria. This drawback can be overcome by controlled fermentation with inoculated organisms, where sterile beans are inoculated with organisms of interest and, thus, their specific contribution to cocoa fermentation can be evaluated.

A number of researchers have conducted cocoa fermentations with inoculated organisms under natural or aseptic conditions. Early such studies are aimed mainly at accelerating the fermentation process and, thus, only yeasts, especially pulp degrading, pectinolytic strains, are used. Inoculated fermentations were fast with good pulp breakdown, and yielded chocolate with sensory characteristics similar to those of the control, uninoculated fermentation (Buamah, Dzogbefia, & Oldham, 1997; Dzogbefia, Buamah, & Oldham, 1999; Sanchez, Daguenet, Guiraud, Vincent, & Galzy, 1985; Sanchez, Guiraud, & Galzy, 1984). Other studies have attempted to mimic natural fermentation but achieve better control of the process (Crafack et al., 2013; Dircks, 2009; Lefeber, Papalexandratou, Gobert, Camu, & De Vuyst, 2012; Schwan, 1998). The fermentations are usually conducted with a mixture of dominant yeast, LAB and, in some studies, AAB strains isolated from natural spontaneous fermentations. The microbial succession, speed of process, quality of beans and chocolate for the inoculated fermentations were generally similar to those of a spontaneous control fermentation, thereby demonstrating that a controlled inoculated process could mimic a traditional process. These studies generally conclude that yeasts are needed in order to obtain acceptable chocolate; however, the roles of the bacteria, especially those of the LAB and AAB, are still unclear.

The objective of this study was to gain further insight into the roles of LAB and AAB in cocoa bean fermentation by conducting small scale laboratory fermentations under aseptic conditions, inoculated with combinations of yeasts, LAB and AAB.

2. Materials and methods

2.1. Inoculated fermentation of cocoa beans

2.1.1. Preparation of microbial starter cultures for inoculation

The cultures used for inoculated fermentations were previously isolated and identified as the predominant species during spontaneous fermentations of cocoa beans from Queensland, Australia. They were: the yeasts *Hanseniaspora guilliermondii* (Accession No. FJ491945.1), *Pichia kudriavzevii* (formerly *Issatchenkia orientalis*; Accession No. EU798698.1), *Kluyveromyces marxianus* (Accession No. DQ249190.1) and *Saccharomyces cerevisiae* (Accession No. KX023222.1); the LAB *Lactobacillus plantarum* (Accession No. HQ117897.1) and *Lactobacillus fermentum* (Accession No. FJ462686.1); and the AAB *Acetobacter pasteurianus* (Accession No. GQ240639.1) and *Gluconobacter frateurii* (Accession No. JF794021.1). The purified isolates were preserved in sterilised 40 % v/v glycerol and stored at –80 °C. Before use as starter cultures, the microbial isolates were checked for purity by streak culture on Malt Extract Agar (MEA) (Oxoid) for the yeasts and de Man Rogosa Sharpe (MRS) Agar (Oxoid) for the bacteria. Colonies from these plate cultures were used to inoculate Malt Extract Broth (Oxoid) and MRS Broth (Oxoid) for the yeasts and bacteria, respectively. After culture at 25 °C for 24–36 h for the yeasts and at 30 °C for 24–36 h for the bacteria, microbial biomass for each organism was aseptically harvested and washed with sterilised water by centrifugation at ~ 18200 ×*g* for 15 min at room temperature in an EBA 12 Centrifuge (Hettich, Newport Pagnell, Buckinghamshire, UK). The cell pellet was then resuspended in sterilised water prior to being inoculated into the cocoa mass.

2.1.2. Cocoa bean fermentations by inoculation with starter cultures

Cocoa pods (*Trinitario* variety) were harvested from plantations in North Queensland, Australia and transported to the University of New South Wales, Sydney. Upon arrival, cocoa pods were stored for 7–10 days from harvest at 20–25 °C. Only undamaged, ripe and physically intact pods were selected for subsequent use. The outer surfaces of these pods were cleaned by washing with warm water (40 °C) and a soft kitchen brush. The pods were then completely immersed in 0.5% sodium hypochlorite solution for 30 min to kill natural microbial flora on the pod surface and then washed twice with sterilised water. After washing, cocoa pods were soaked in 70% ethanol for 1 min. After evaporation of the ethanol from the surface, cocoa pods were cut open with a stainless steel knife and the beans with attached pulp were aseptically removed. The beans were aseptically transferred to a sterile plastic container (12.5 L) where they were mixed and then divided into five plastic boxes (17 × 17 × 20 cm), each containing 3.5 kg of beans. The plastic containers had drilled holes on the sides and the base to facilitate juice drainage and aeration.

Cocoa beans were aseptically inoculated with about 10 mL of the cultures of yeasts and bacteria prepared as described in Section 2.1.1 according to the protocol given in Table 1. The cultures were inoculated into the beans by pipetting and mixing using sterile gloves. The initial population of each species of yeast and bacteria was approx. 10^5–10^6 cfu/g. The fermentation boxes were covered with sterilised lids, wrapped with aluminium foil to prevent external contamination, and incubated at 25 °C (0–12 h), 30 °C (12–24 h), 35 °C (24–36 h), 40 °C (36–48 h), 45 °C (48–72 h) and 48 °C (72–144 h) to simulate the temperature evolution of commercial cocoa fermentations. The fermenting beans were aseptically mixed every 48 h. Samples of beans were taken daily for microbiological and chemical analyses. Samples for microbiological analysis were used immediately while those for chemical analysis were stored at − 20 °C until examined. The fermentations were terminated at day 6 and the beans were spread onto the trays for drying at 30 °C and relative humidity 70 % for 5 days.

The fermentations were carried out twice, once using cocoa beans harvested in August 2013 and again with beans harvested in December 2013. Previously, several preliminary fermentations were done to establish an aseptic process for conducting the inoculated fermentations.

2.2. Microbiological analyses

Cocoa samples taken during fermentations were analyzed for their populations of yeasts, LAB and AAB according to the methods given in

Table 1
Experimental protocol for the inoculated fermentations of cocoa beans

Fermentation	Starter cultures inoculated
1	*Control aseptic fermentation, beans removed from pods under aseptic conditions and not inoculated*
2	*Hanseniaspora guilliermondii*, *Pichia kudriavzevii*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* (*yeasts only*)
3	*Hanseniaspora guilliermondii*, *Pichia kudriavzevii*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae, Lactobacillus plantarum* and *Lactobacillus fermentum* (*yeasts + LAB*)
4	*Hanseniaspora guilliermondii*, *Pichia kudriavzevii*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae, Acetobacter pasteurianus* and *Gluconobacter frateurii* (*yeasts + AAB*)
5	*Hanseniaspora guilliermondii*, *Pichia kudriavzevii*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae, Lactobacillus plantarum*, *Lactobacillus fermentum, Acetobacter pasteurianus* and *Gluconobacter frateurii* (*yeasts + LAB + AAB*)
6	*Control indigenous fermentation, beans removed from unwashed pods under non-aseptic conditions and brought into contact with the pod surfaces to provide a source of indigenous contamination*

Ho et al. (2014). The enumeration of AAB during fermentations was done simultaneously with two different media, Wallerstein Laboratories Nutrient Agar (WLNA) and Glucose Yeast Extract Agar (GYEA) to increase the reliability of data. Population data reported are the means of duplicate analyses. Representatives of each colony type were isolated from each sampling time, purified by re-streaking on their appropriate media, and used for identification to genus and species level by a combination of phenotypic and molecular methods as described in Ho et al. (2014). Briefly, the Polymerase Chain Reaction (PCR) was performed to amplify the 5.8S-Internally Transcribed Spacer rRNA gene region of yeast isolates and the 16S rRNA gene of bacterial isolates. DNA amplifications were done with the primers ITS1 and ITS4 for yeasts, 27F and 1495R for LAB and 16Sd and 16Sr for AAB. PCR products were then used for identification of yeasts and bacteria to species by a combination of restriction fragment length polymorphism (RFLP) and sequence analyses. The procedures and conditions of DNA extraction, PCR, RFLP and sequence analyses, including the use of reference cultures, were done as described in Ho et al. (2014).

2.3. Chemical analyses

The measurement of pH and analyses of sugar, ethanol, glycerol, mannitol and organic acid concentrations were determined on cocoa pulp and nib fractions according to methods described in Ho et al. (2014). The pH of the cocoa samples was measured every 24 h while the concentrations of sugars, ethanol, glycerol and organic acids were analyzed every 72 h. The relative levels of volatile compounds were determined on cocoa nib fractions every 72 h according to the SPME-GC-MS method described in Ho et al. (2014).

2.4. Quality evaluation of cocoa beans and chocolates

The cut test and chocolate manufacture were performed as described in Ho et al. (2014). Chocolates were evaluated for sensory quality using the triangle taste test and the affective or hedonic tests (Carpenter, Lyon, & Hasdell, 2000; Lawless & Heymann, 2010).

In the triangle test, the panellists were given three different samples of chocolates made from beans inoculated with only yeasts or yeasts + LAB or yeasts + AAB, each labelled with a three-digit code. Two samples were the same and one was different. The six possible order combinations were randomised across the panellists (25–30 for each pair of samples). They assessed each sample in the order provided, from left to right, and were asked to select the sample that was different from the other two.

The hedonic test consisted of one sample of chocolate prepared from the control, indigenous fermented beans and two samples made from inoculated beans (with only yeasts and with yeasts + LAB + AAB). A panel of 30 assessors rated their degree of liking for the chocolate flavour and overall liking of each sample using a seven-point hedonic scale where a score of 1 indicated “dislike very much”, a score of 7 indicated “like very much” while a score of 4 indicated “neither dislike nor like”.

2.5. Statistical analysis

One-way single factor analysis of variance and *t*-test were used to determine significant differences between means using Microsoft Excel. Significant differences in the concentrations of sugars, organic acids, volatile compounds and shell content were considered when $p < 0.05$.

3. Results

3.1. Microbial ecology of cocoa bean fermentations inoculated with starter cultures

3.1.1. Growth of yeasts

Fig. 1 shows the growth of total yeasts during the fermentation of control and inoculated cocoa beans harvested in August 2013 (Fig. 1a) and December 2013 (Fig. 1b). The growth of individual yeast species is shown in Fig. 2 and 3.

The control, aseptically prepared beans showed no detectable yeasts ($< 10^2$ cfu/g) until 72 h, after which they grew to populations of approx. 10^7 cfu/g (Fig. 1a, b). For the August beans, the growth of *S. cerevisiae*, *K. marxianus* and *P. kudriavzevii* occurred, before the latter died off (Fig. 2a). For the December beans, only *K. marxianus* grew (Fig. 3a).

For the inoculated fermentations, the initial yeast population was 10^5–10^6 cfu/g as expected (Fig. 1a, b). For the yeast only fermentation, all four inoculated yeast species grew to 10^7–10^8 cfu/g during the next 24 h. Thereafter, *H. guilliermondii* died off while *S. cerevisiae*, *K. marxianus* and *P. kudriavzevii* remained at these levels before slowly declining towards the end of fermentation (Figs. 2b, 3b). The decline was greater for the December fermentation where all yeasts died off except for *K. marxianus*. This pattern of behavior for the yeast species was the same for the yeast + LAB inoculated fermentations suggesting that the LAB did not impact on yeast growth (Figs. 2c, 3c). For the yeast + AAB and yeast + LAB + AAB inoculated fermentations, all the yeast species grew during the first 24 h to maximum populations but these were 5–10 fold less than for the yeast only and yeast + LAB fermentations (Figs. 2c, d; 3c, d). This was particularly notable for *H. guilliermondii*. Also, there was a faster dying off of the other yeast species. These data suggested a negative impact of the AAB on the growth and survival of yeasts.

For the control, indigenous fermentation, yeasts were not detectable in freshly extracted beans for the August fermentations (Fig. 1a) but were present at 10^4–10^5 cfu/g for the freshly extracted December beans (Fig. 1b). These fermentations were characterised by the successional growth of *H. guilliermondii*, *P. kudriavzevii* and *K. marxianus* (Figs. 2f, 3f). Other yeast species detected in the control, indigenous fermentations were *S. cerevisiae* for the August beans and *Pichia guilliermondii* and *Cryptococcus luteolus* for the December beans. The *Cryptococcus luteolus* died off within the first 24 h (Fig. 3f).

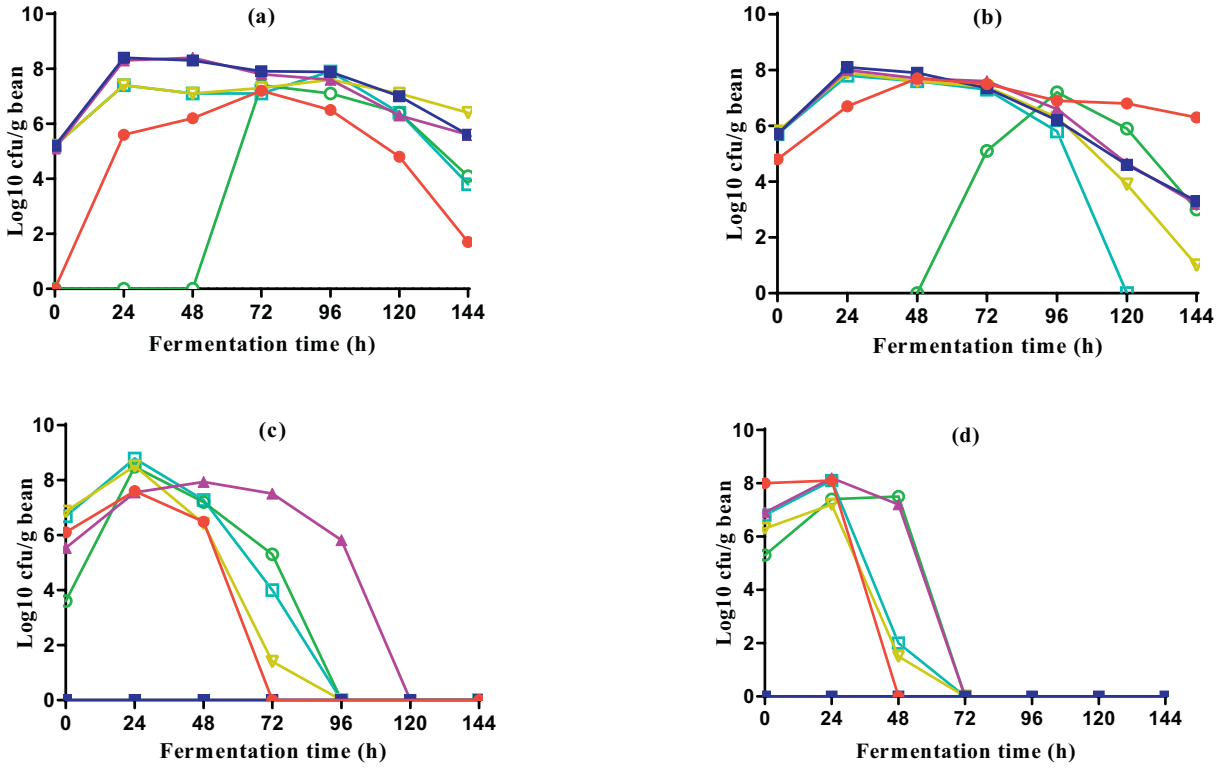

Fig. 1. Total counts of yeasts (a, b) and bacteria (c, d) during fermentations of beans harvested in August 2013 (a, c) and in December 2013 (b, d); control, aseptic fermentation (AS) (○); AS inoculated with yeasts (■); AS inoculated with yeasts and lactic acid bacteria (▲); AS inoculated with yeasts and acetic acid bacteria (▽), AS inoculated with yeasts, lactic acid bacteria and acetic acid bacteria (□) and control, indigenous fermentation (●).

3.1.2. Growth of bacteria

Fig. 1 also shows the growth of total bacteria during the control and inoculated fermentations for beans harvested in August 2013 (Fig. 1c) and December 2013 (Fig. 1d). The growth of individual bacterial species is given in Fig. 4 and 5.

For the control aseptic fermentations, bacteria were detected in freshly extracted beans at approx. 10^4–10^5 cfu/g which grew to 10^7–10^9 cfu/g during the next 48 h before declining to undetectable levels ($< 10^2$ cfu/g) by 72 h (Fig. 1c, d). This growth was due to an *Asaia* sp. for the August beans (Fig. 4a) and *Pantoea agglomerans* for the December beans (Fig. 5a).

No bacteria were detected in the yeast only inoculated beans for both the August and December fermentations. The same result was also found for a third preliminary trial conducted with beans harvested in June 2012 (data not presented). For the yeast + LAB inoculated fermentations, *L. plantarum* and *L. fermentum* grew by 10–100 fold during the first 24 h of fermentation. Thereafter, *L. fermentum* quickly died off by 48 h for the December fermentation and 72 h for the August fermentation. *L. plantarum* persisted longer in both fermentations before declining (Figs. 4b and 5b). For the fermentations inoculated with yeasts + AAB, *A. pasteurianus* and *G. frateurii* grew 10–100 fold during the first 24 h and then died off. For the August beans, *G. frateurii* was not detectable after 72 h and *A. pasteurianus* after 96 h. The decline was more rapid in the December fermentation, where both species were not detectable at 48 h. For the August fermentation inoculated with yeasts + LAB + AAB, all bacteria grew by 10–100 fold during the first 24 h, before dying off. *L. fermentum* and *G. frateurii* died off faster (by 72 h) than *L. plantarum* and *A. pasteurianus* (by 96 h) (Fig. 4d). For the equivalent December fermentation, *L. fermentum* and *G. frateurii* did not grow and the bacteria died off by 24 h. However, both *L. plantarum* and *A. pasteurianus* grew during the first 24 h before dying off by 48–72 h (Fig. 5d).

In the control, indigenous August fermentation, *A. pasteurianus* and *G. frateurii* were present in freshly extracted beans and grew by about 100 fold during the first 24 h before dying off by 72 h (Fig. 4e). An unidentified *Asaia* spp. was also dominant in the freshly extracted beans but it did not grow and died off by 24 h. No LAB were detected in fermentations of the August beans. For the indigenous December fermentation, only *P. aggromelans* and *L. plantarum* were detected in freshly extracted beans. Neither of these species grew before dying off (Fig. 5e). Weak growth of *G. frateurii* was detected at 24 h, but it died off by 48 h.

3.2. Chemical changes during the inoculated fermentations of cocoa beans

3.2.1. pH changes

Fig. 6 shows changes in the pH of the pulp and nibs during fermentations of beans harvested in August 2013. The unfermented pulp had an initial pH of 3.9 which progressively increased to a final value of 4.9–5.1 for beans inoculated with only yeasts or with yeasts + LAB. The pulp pH of beans inoculated with yeasts + AAB or with yeasts + LAB + AAB decreased to 3.4 after 24 h and then gradually increased to about 4.3 by the end of fermentation (Fig. 6a), suggesting an impact of the inoculated AAB on the pH. The pulp pH of the beans undergoing control indigenous fermentation was consistently lower than that of beans from all inoculated fermentations. It decreased to around 3.1 at 24 h and then gradually increased to 3.6 by the end of fermentation (Fig. 6a).

The pH of unfermented nibs was 6.4 that decreased to a final value of 5.7 for fermentation inoculated with yeasts or yeasts + LAB (Fig. 6b). The nib pH of beans inoculated with yeasts + AAB or yeasts + LAB + AAB declined to 5.2 at 48 h, and then increased to 5.4 by the end of fermentation. Nibs of the control, indigenous fermented beans showed similar pH changes (Fig. 6b).

Unfermented December beans had a lower pulp pH (3.7) and higher nib pH (6.6) compared with the August beans, but their kinetics of

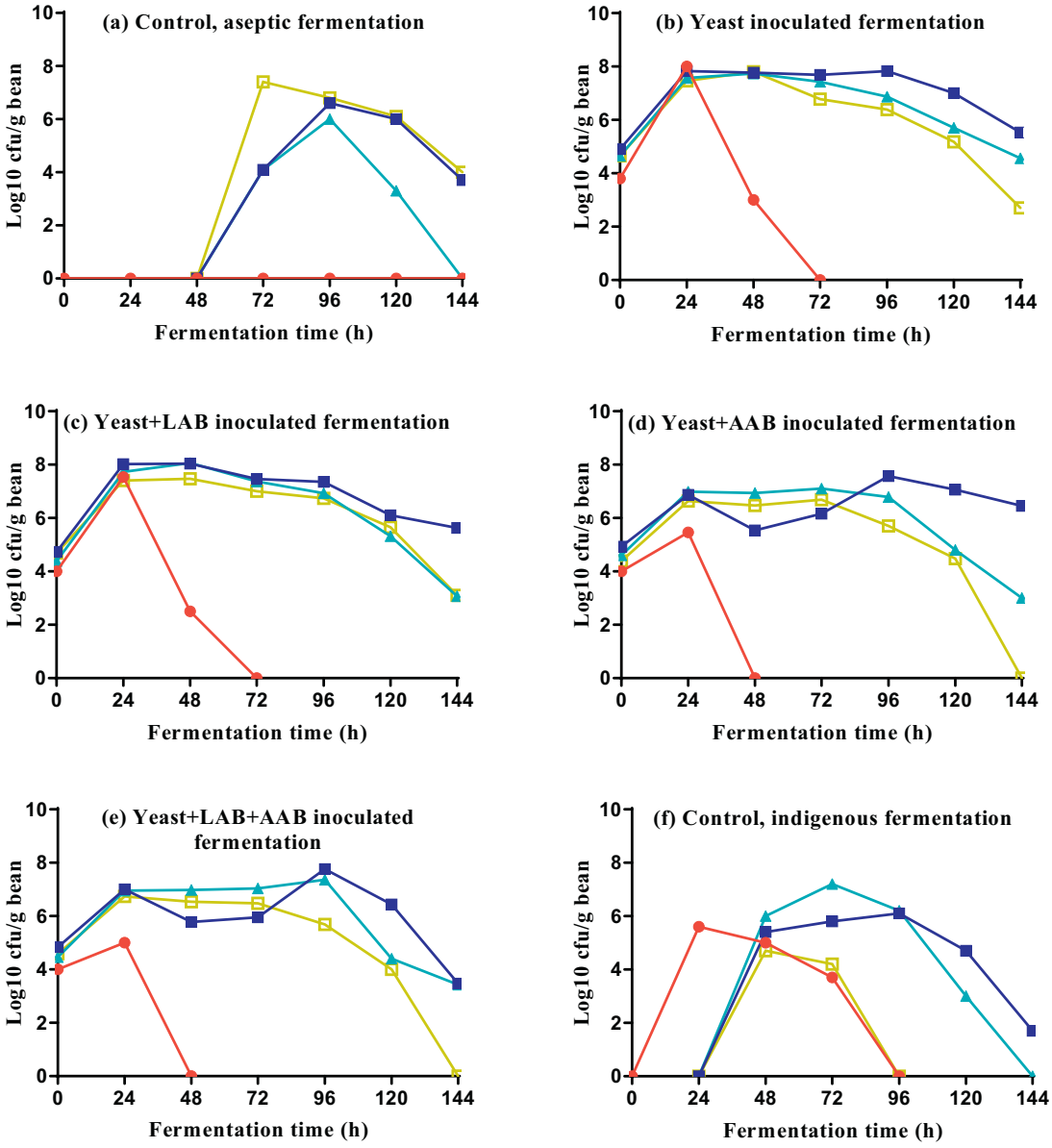

Fig. 2. The growth of yeasts *Hanseniaspora guilliermondii* (●), *Kluyveromyces marxianus* (■), *Pichia kudriavzevii* (▲) and *Saccharomyces cerevisiae* (□) during fermentations of beans harvested in August 2013.

change were similar to those of the August fermentations (data not shown).

3.2.2. Changes in the concentrations of sugars and ethanol

Table 2 shows changes in the concentrations of sugars and ethanol in the cocoa pulp and nibs. Data of chemical analyses including sugars, ethanol, organic acids and volatile compounds are not presented for the control aseptic fermentations as the ecological results showed them to be atypical of a normal process.

For both the August and December trials, glucose (41–47 mg/g) and fructose (45–52 mg/g) were the main sugars of the cocoa pulp before fermentation. These were largely used up throughout all fermentations with the inoculated cultures, giving final concentrations less than 2.6 mg/g. While there were some minor variations in utilisation of glucose and fructose for the different inoculation protocols (Table 2), these differences were not statistically significant ($p > 0.05$). Similar trends were found for the control indigenous fermentations although the rate of sugar utilisation was slower. Sugar utilisation in the pulp correlated with the production of ethanol which peaked in the middle stages of fermentation and then decreased (Table 2). At the end of fermentation, higher concentrations of ethanol were found in the pulp of August beans compared with those of December and this correlated with the higher initial concentration of sugars in the pulp. For the August fermentations, higher concentrations of ethanol were found for the yeast only and yeast + LAB fermentations, but this was not the case for the December fermentations. For both the August and December trials, significantly higher ethanol concentrations ($p < 0.05$) were found in the pulp for the inoculated fermentations than in the indigenous control (Table 2).

Sucrose was the main sugar in the nibs (12.6–15 mg/g) that decreased to 0.4–1.5 mg/g during fermentation (Table 2). Small amounts of glucose (1.2–2.6 mg/g) and fructose (1–2.15 mg/g) occurred in unfermented nibs that increased by about 2 fold during fermentation. These trends were similar for all inoculated fermentations and the control indigenous fermentation ($p > 0.05$). No ethanol was found in unfermented nibs, but it increased during fermentation to maximum levels at mid fermentation, thereafter decreasing to final levels of about 6–7 mg/g and 4.8–5.5 mg/g for the August and December trials,

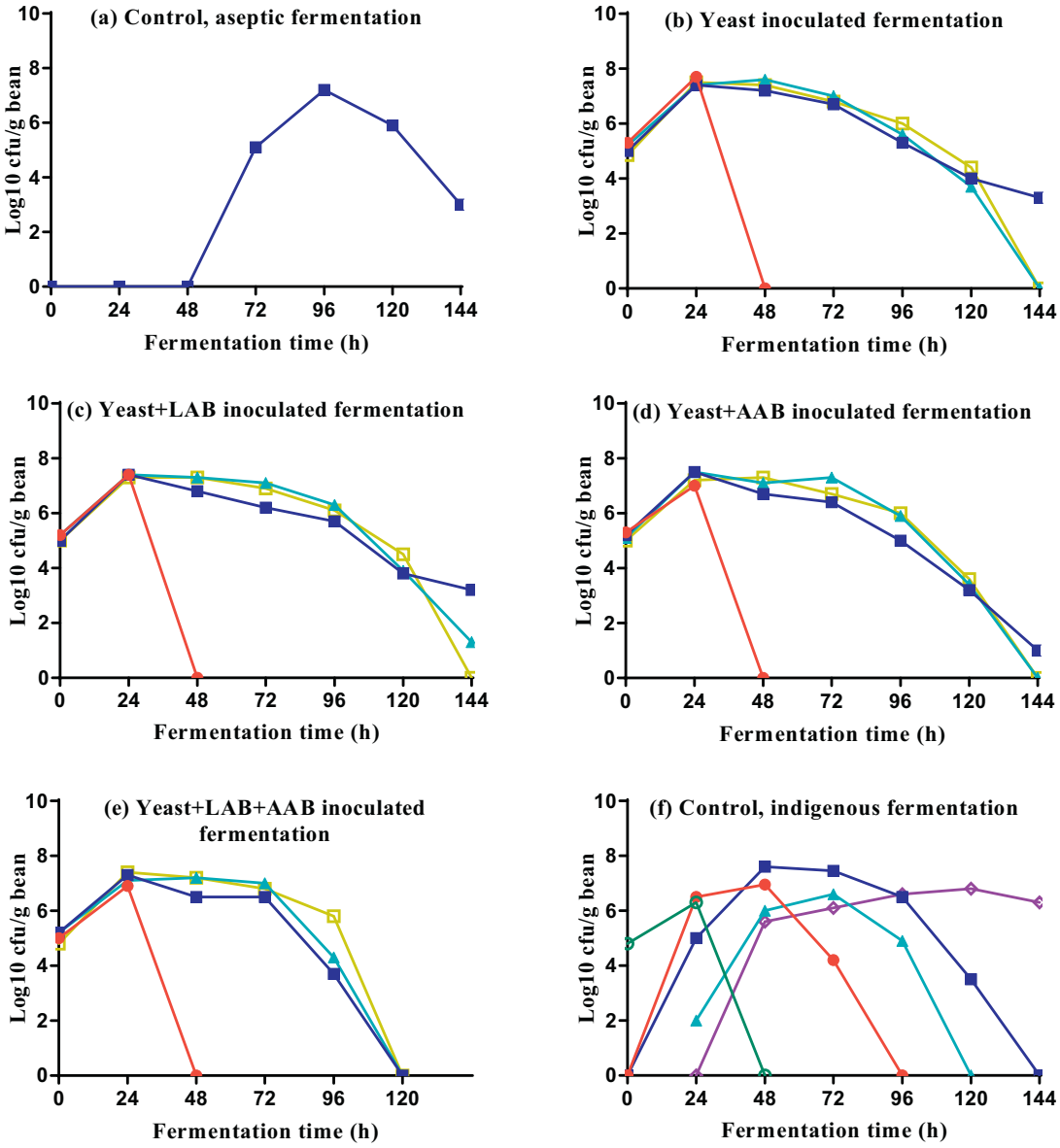

Fig. 3. The growth of yeasts *Hanseniaspora guilliermondii* (●), *Kluyveromyces marxianus* (■), *Pichia kudriavzevii* (▲), *Saccharomyces cerevisiae* (□), *Pichia guilliermondii* (◇) and *Cryptococcus luteolus* (○) during fermentations of beans harvested in December 2013.

respectively (Table 2). These changes were similar for all inoculated fermentations ($p > 0.05$), but ethanol concentrations in the control indigenous fermented beans were significantly lower by about two fold ($p < 0.05$) (Table 2).

Glycerol was not detected in either pulp or nibs of unfermented beans but it was produced in the pulp during fermentations at maximum concentrations of 0.33–1.1 mg/g which transferred to the nibs at 0.06–0.4 mg/g. The kinetics of glycerol production were similar for all inoculated fermentations but its concentration in these beans was significantly higher by about 2–3 fold than that in the control indigenous beans (data not shown). Mannitol was not found in the pulp or nibs of freshly extracted and fermenting beans in both trials.

3.2.3. Changes in the concentration of organic acids

Table 3 shows changes in the concentrations of citric, lactic and acetic acids of the August and December fermentations.

Citric acid (6.5–6.7 mg/g) was the main acid in the cocoa pulp. Its concentration increased to 6.2–7.7 mg/g in inoculated fermentations of the yeast only and the yeast + AAB while it was 5.9–7.0 mg/g during fermentations inoculated with the yeast + LAB and the yeast + LAB + AAB (Table 3).

The unfermented nibs of the August beans had 5.0 mg/g of citric acid that decreased to 2–2.5 mg/g during all fermentations. This change was not affected by the inoculation of yeasts, LAB or AAB (Table 3). This same conclusion was also obtained for the December beans where the initial concentration of citric acid in the nibs was 3.6 mg/g and decreased to 1.5–1.9 mg/g (Table 3).

No lactic acid was detected in the unfermented pulp. It increased to about 1 mg/g for fermentations inoculated with yeasts only and yeasts + AAB for both trials. However, for the yeast + LAB and yeast + LAB + AAB fermentations, it increased to higher amounts of 2.1–2.3 mg/g for the August beans and 2.5–3.4 mg/g for the December beans (Table 3), demonstrating an impact of the LAB on its production. Lactic acid was also produced in the pulp for the control indigenous fermentations, but generally at lower amounts than for the inoculated fermentation (Table 3). No lactic acid was detected in the nibs of unfermented beans but it was found in the nibs of all fermented beans and at higher levels for those where LAB were inoculated (Table 3). The

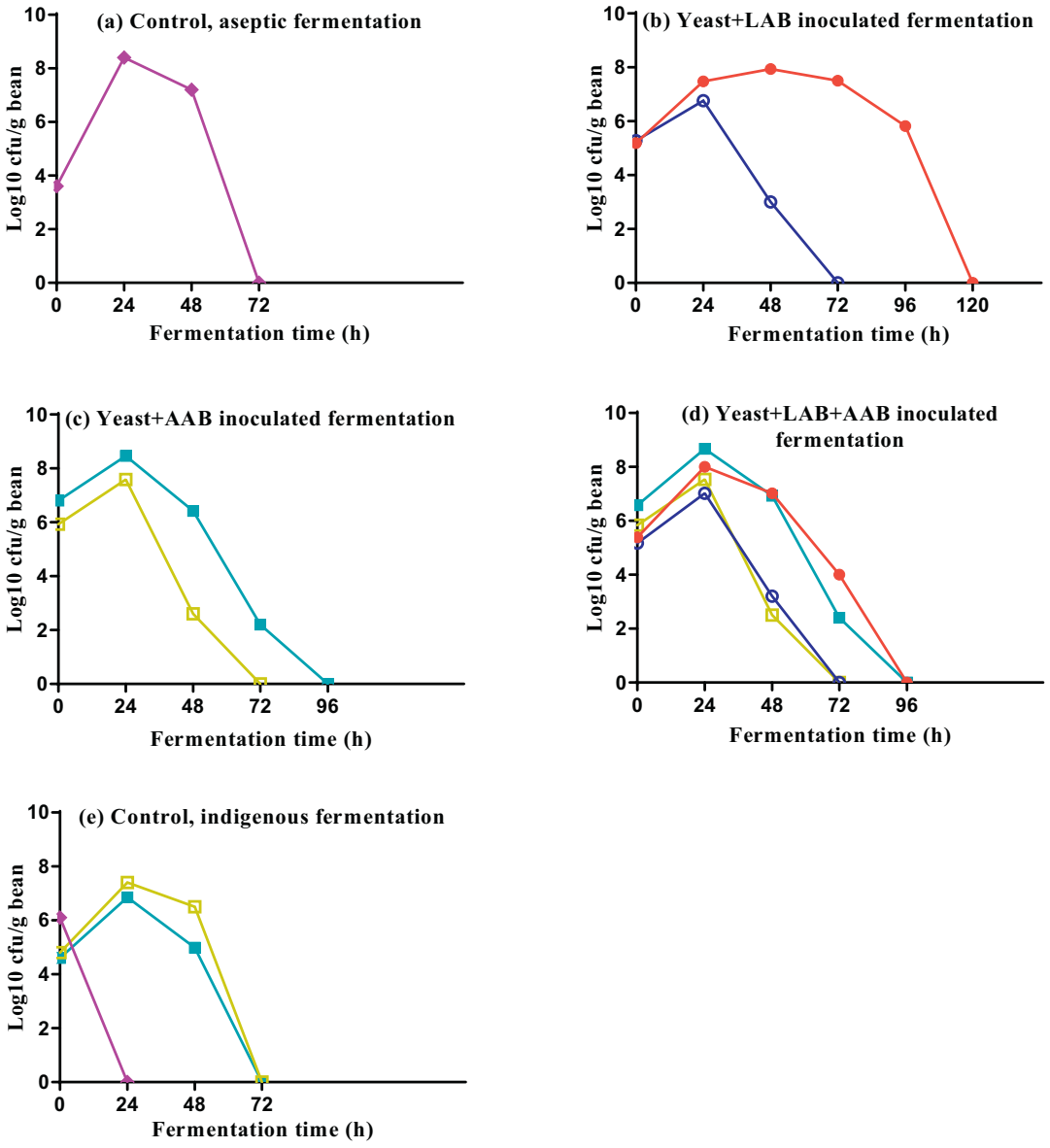

Fig. 4. The growth of bacteria *Lactobacillus plantarum* (●), *Lactobacillus fermentum* (○), *Gluconobacter frateurii* (□), *Acetobacter pasteurianus* (■) and *Asaia* sp. (◆) during fermentations of beans harvested in August 2013.

very low levels of lactic acid in the control indigenous fermentation of the August beans correlated with the absence of LAB in this fermentation (Fig. 4e).

Acetic acid was not found in the pulp of freshly extracted beans. It increased to about 0.8–1.1 mg/g for fermentations inoculated with yeasts only and yeast + LAB for the August beans whereas these values for the yeast + AAB and yeast + LAB + AAB fermentations were 1.45–1.6 mg/g (Table 3), suggesting an effect of the AAB growth on its production. In the December fermentations, the levels of acetic acid in the pulp of all inoculated beans were about 0.75–1 mg/g and the impact of AAB on its production was not evident, possibly due to the weaker growth of the inoculated AAB. No acetic acid was found in unfermented nibs but it was detected during all fermentations of inoculated beans. For the August fermentation, the concentration of acetic acid was higher ($p < 0.05$) in the nibs of beans inoculated with AAB (1.8–2.2 mg/g) than in those without AAB in the inoculum (1–1.4 mg/g). Acetic acid (0.9–1.0 mg/g) was detected in the nibs of all inoculated beans for the December fermentation (Table 3). In the control, indigenous fermentations, it was found in the pulp (1.0–1.1 mg/g) and the nibs (1.2–1.3 mg/g) for both trials (Table 3).

3.2.4. Production of volatile compounds

Fig. 7 shows the concentrations of total higher alcohols, esters, aldehydes and ketones in the cocoa nibs at 0, 72 and 144 h of fermentations and after roasting.

3.2.4.1. Higher alcohols. For both trials, low levels of total higher alcohols were found in unfermented nibs. These levels increased by as much as 30 fold during fermentation and then decreased after roasting (Fig. 7a, b). Similar levels of higher alcohols were found for the yeast only inoculated fermentations and the yeast + bacteria inoculated fermentations ($p > 0.05$), suggesting that the bacteria contributed little to their production. The concentrations of higher alcohols were generally less in the control, indigenous fermentations than in the inoculated fermentations but such differences were not significant after roasting. Phenylethyl alcohol (47–70%, 57–64% of total higher alcohols), isoamyl alcohol (13–36%, 22–27%) and 2-methyl-1-butanol (11–14%, 9–12%) were the main alcohols detected in the nibs before and after roasting. The data in parenthesis show the range of relative amounts found in the nibs of the August and December beans, respectively, after roasting. Other higher alcohols detected in

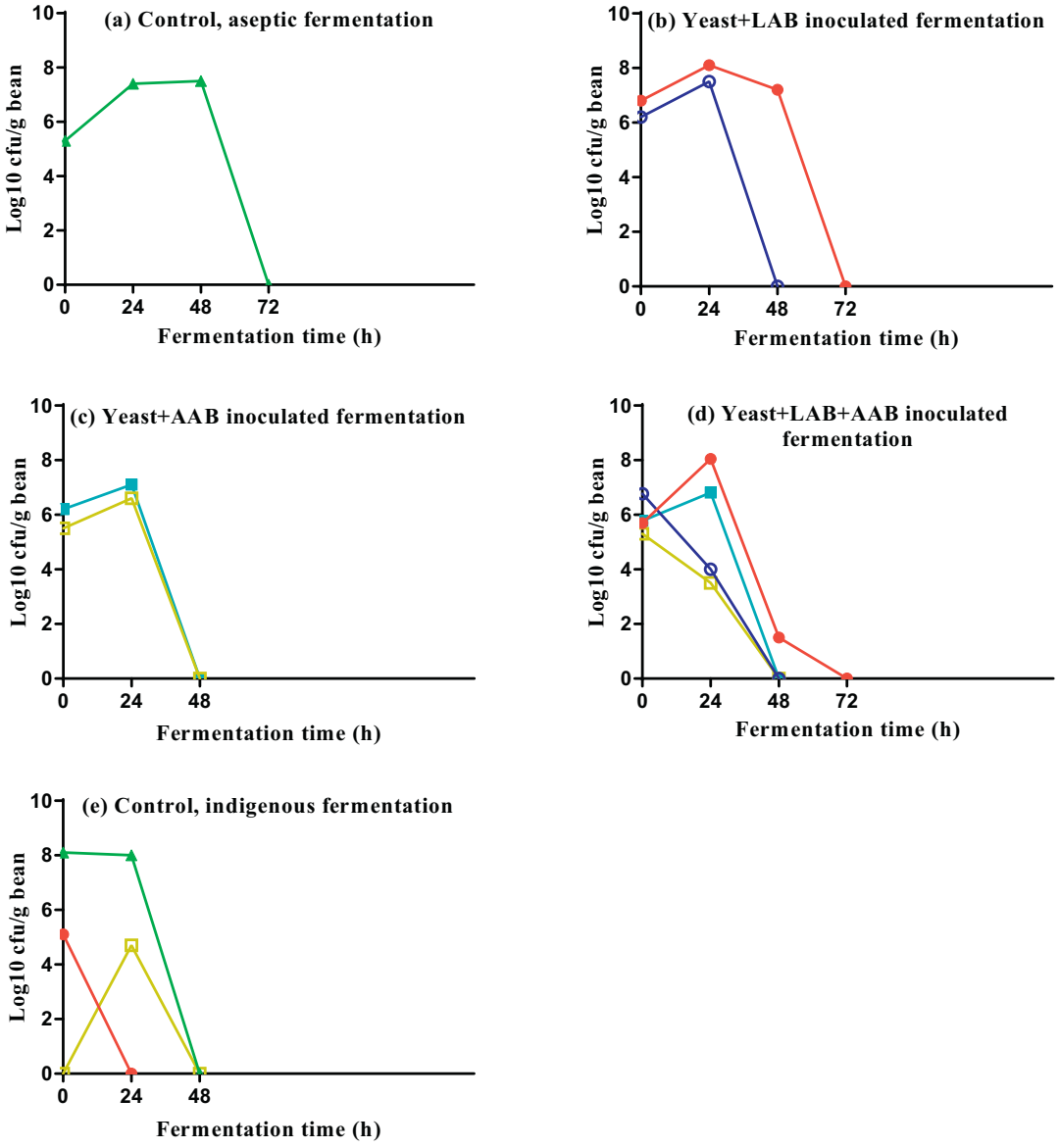

Fig. 5. The growth of bacteria *Lactobacillus plantarum* (●), *Lactobacillus fermentum* (○), *Gluconobacter frateurii* (□), *Acetobacter pasteurianus* (■) and *Pantoea aggromelans* (▲) during fermentations of beans harvested in December 2013.

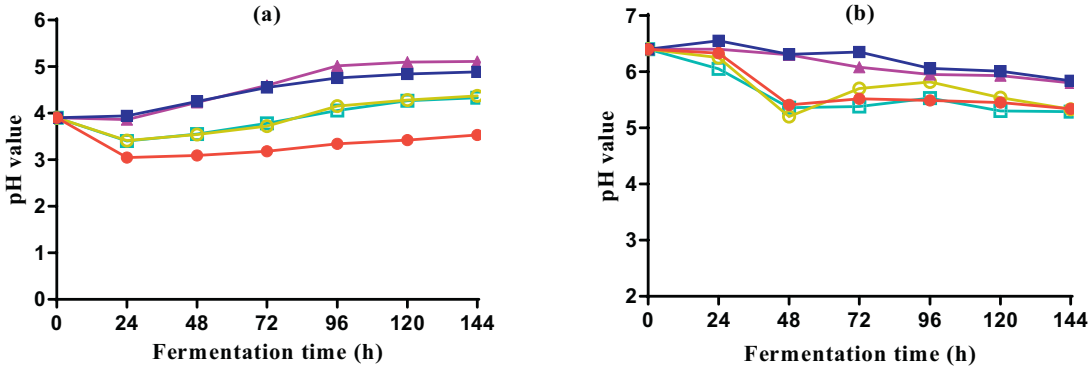

Fig. 6. The changes in pulp (a) and nib (b) pH during the August fermentations of beans inoculated with only yeasts (■), yeasts + LAB (▲), yeasts + AAB (○) and yeasts + LAB + AAB (□) and the control beans (●). Data are the means of duplicate analyses ± 0.05.

Table 2
Changes in the concentration of sugars and ethanol during fermentations of beans harvested in August (a) and December (b) 2013

		Pulp (mg/g)*						Nib (mg/g)*					
Bean fermentation		0h		72 h		144 h		0 h		72 h		144 h	
		a	***b***	a	***b***	a	***b***	a	***b***	a	***b***	a	***b***
	Sucrose	–		–	–	–	–	15.0	***12.6***	3.3	***3.5***	1.5	***0.4***
	Glucose	47.0	***41.0***	7.6	***3.3***	2.4	***1.3***	2.6	***1.2***	4.2	***2.3***	5.6	***2.8***
Control indigenous fermentation	Fructose	52.0	***45.0***	2.2	***1.7***	1.0	***0.8***	2.2	***1.1***	3.7	***2.1***	5.4	***2.7***
	Ethanol	0.0	***0.0***	7.1	***6.6***	4.2	***4.1***	0.0	***0.0***	5.9	***5.6***	3.1	***2.2***
	Sucrose	–	–	–	–	–	–	15.0	***12.6***	2.1	***3.8***	1.7	***0.4***
	Glucose	47.0	***41.0***	1.8	***1.1***	1.6	***0.1***	2.6	***1.2***	4.9	***2.6***	5.3	***3.4***
Yeasts	Fructose	52.0	***45.0***	1.1	***1.9***	1.0	***0.4***	2.2	***1.1***	4.6	***2.7***	5.1	***3.2***
	Ethanol	0.0	***0.0***	12.4	***9.2***	8.6	***6.3***	0.0	***0.0***	9.0	***8.6***	6.8	***5.4***
	Sucrose	–	–	–	–	–	–	15.0	***12.6***	2.6	***3.3***	1.7	***0.6***
	Glucose	47.0	***41.0***	2.0	***1.2***	1.7	***0.9***	2.6	***1.2***	4.4	***2.4***	4.9	***3.3***
Yeasts + LAB	Fructose	52.0	***45.0***	1.1	***1.4***	1.4	***0.8***	2.2	***1.1***	4.2	***1.5***	5.3	***3.2***
	Ethanol	0.0	***0.0***	12.5	***9.5***	9.0	***6.6***	0.0	***0.0***	10.1	***9.1***	6.9	***5.6***
	Sucrose	–	–	–	–	–	–	15.0	***12.6***	2.1	***3.5***	1.7	***0.2***
	Glucose	47.0	***41.0***	3.5	***2.2***	1.9	***0.5***	2.6	***1.2***	4.9	***1.1***	5.5	***2.4***
Yeasts + AAB	Fructose	52.0	***45.0***	3.2	***1.7***	2.7	***1.1***	2.2	***1.1***	4.9	***1.6***	5.5	***2.3***
	Ethanol	0.0	***0.0***	9.2	***9.0***	7.2	***5.9***	0.0	***0.0***	8.5	***8.8***	6.3	***4.8***
	Sucrose	–	–	–	–	–	–	15.0	***12.6***	2.4	***2.7***	1.7	***0.3***
	Glucose	47.0	***41.0***	5.0	***1.5***	2.6	***0.2***	2.6	***1.2***	5.1	***1.0***	5.4	***2.1***
Yeasts + LAB + AAB	Fructose	52.0	***45.0***	2.3	***1.6***	2.0	***0.5***	2.2	***1.1***	5.1	***1.6***	5.6	***2.3***
	Ethanol	0.0	***0.0***	8.1	***10.0***	7.7	***6.6***	0.0	***0.0***	6.9	***8.6***	6.7	***5.3***

* Each value is the mean of duplicate analyses of samples ± 10%.

lesser amounts were linalool, benzyl alcohol and 1-phenylethanol. The qualitative profile of alcohols was the same for all inoculated fermentations in both trials.

3.2.4.2. Esters. Very little esters were detected in the unfermented nibs of the August and December beans. During fermentation, drying and roasting, total ester concentrations increased by as much as 72 fold for the August beans (Fig. 7c) and 150 fold for the December beans (Fig. 7d). While there is some variation with the 72 h data for the August fermentation, similar levels of total esters were found for the yeast only and yeast + bacteria inoculations, suggesting no impact of bacteria on ester production. The main esters found in roasted beans from all fermentations were isoamyl acetate (23–32%, 21–28% of total amount), ethyl acetate (25–28%, 24–31%), 2-methylbutyl acetate (11–15%, 10–16%) and ethyl hexanoate (6–11%, 8–17%), with the values in brackets corresponding to the levels in the nibs of the August and December beans, respectively. Other esters found in lesser amounts were phenylethyl acetate, ethyl isovalerate, ethyl phenylacetate and ethyl octanoate. The same qualitative profiles were found in beans from all the fermentations.

3.2.4.3. Aldehydes. Significant amounts of aldehydes were found in unfermented beans. For the December beans, they increased slightly during fermentation and decreased on roasting ($p > 0.05$) and there were no differences between the five fermentation protocols (Fig. 7f). For the August beans, there were significant increases ($p < 0.05$) in total aldehydes during fermentations inoculated with yeasts + AAB and with yeasts + LAB + AAB, but these differences were not significant after roasting ($p > 0.05$) (Fig. 7e). In addition, the control, indigenous fermentation gave increased aldehydes. Four main aldehydes were detected in the nibs before and after roasting and these were benzaldehyde (35–60%, 52–70% of total), hexanal (20–34%, 18–35%), phenyl acetaldehyde (5–10%, 4–6%) and 2-phenyl-2-butenal (5–10%, 5–12%), with the relative values given being those for the August and December beans, respectively.

3.2.4.4. Ketones. Unfermented nibs of the August beans contained considerable levels of ketones which decreased 2–3 fold through fermentation and roasting. The kinetics of ketone change were similar for all inoculated fermentations as well as the indigenous control ($p > 0.05$) (Fig. 7g). Lower amounts of ketones were detected in the

Table 3
Changes in the concentration of organic acids during fermentations of beans harvested in August (a) and December (b) 2013

		Pulp (mg/g)*						Nib (mg/g)*					
Bean fermentation		0 h		72 h		144 h		0 h		72 h		144 h	
		a	***b***	a	***b***	a	***b***	a	***b***	a	***b***	a	***b***
	Citric	6.7	***6.5***	7.8	***6.0***	8.4	***6.4***	5.0	***3.6***	4.1	***2.9***	2.3	***1.7***
Control indigenous fermentation	Lactic	–	–	0.2	***1.1***	0.4	***1.3***	–	–	–	***0.5***	–	***0.6***
	Acetic	–	–	0.6	***0.4***	1.0	***1.1***	–	–	0.8	***1.1***	1.2	***1.3***
	Citric	6.7	***6.5***	7.4	***6.8***	7.3	***7.5***	5.0	***3.6***	3.0	***2.1***	2.3	***1.6***
Yeasts	Lactic	–	–	0.5	***0.5***	1.1	***1.0***	–	–	0.4	***0.5***	0.5	***0.6***
	Acetic	–	–	0.5	***0.3***	0.8	***0.8***	–	–	0.6	***0.6***	1.0	***0.9***
	Citric	6.7	***6.5***	5.6	***6.3***	6.4	***7.0***	5.0	***3.6***	3.8	***2.5***	1.9	***1.9***
Yeasts + LAB	Lactic	–	–	0.9	***1.2***	2.3	***3.4***	–	–	0.5	***1.1***	0.7	***1.2***
	Acetic	–	–	0.6	***0.5***	1.1	***0.8***	–	–	1.1	***0.5***	1.4	***0.9***
	Citric	6.7	***6.5***	6.8	***6.3***	6.2	***7.7***	5.0	***3.6***	3.2	***2.6***	2.3	***1.6***
Yeasts + AAB	Lactic	–	–	0.4	***0.5***	1.1	***0.9***	–	–	0.4	***0.5***	0.4	***0.6***
	Acetic	–	–	0.8	***0.4***	1.5	***0.7***	–	–	1.6	***0.9***	1.8	***1.0***
	Citric	6.7	***6.5***	6.4	***6.6***	6.0	***6.8***	5.0	***3.6***	3.8	***2.0***	2.6	***1.5***
Yeasts + LAB + AAB	Lactic	–	–	0.9	***1.2***	2.1	***2.5***	–	–	0.5	***0.5***	0.6	***0.5***
	Acetic	–	–	1.3	***0.5***	1.6	***1.0***	–	–	1.9	***0.8***	2.2	***0.9***

*Each value is the mean of duplicate analyses of samples ± 10%.

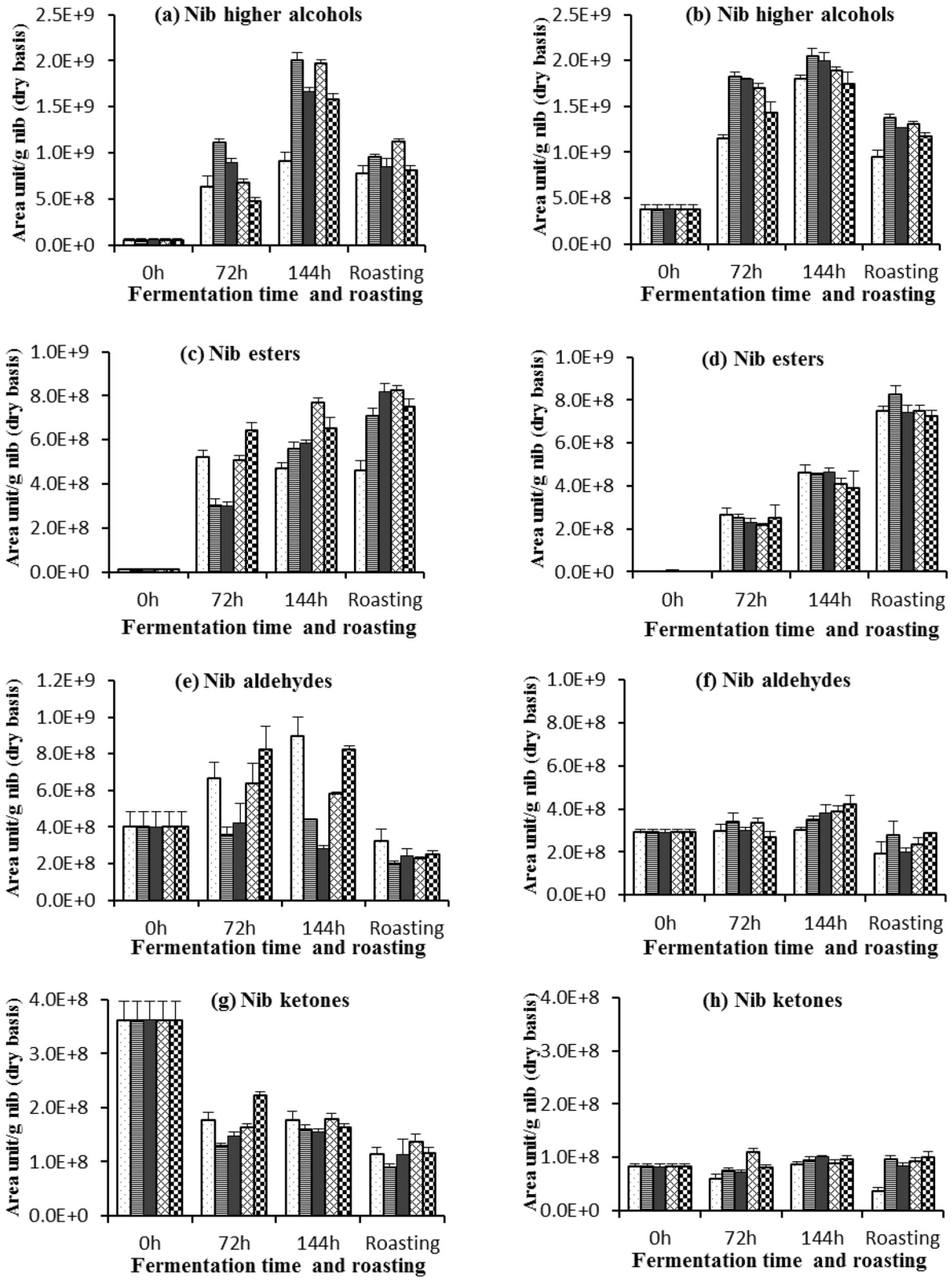

Fig. 7. Changes in the concentration of higher alcohols, esters, aldehydes and ketones, in the nibs of beans inoculated with only yeasts (▤), yeasts + LAB (■), yeasts + AAB (▨) and yeasts + LAB + AAB (▩) and control beans (□) harvested in August 2013 (a, c, e, f) and December 2013 (b, d, f, h).

December beans and their total concentration did not significantly change during fermentation and roasting, regardless of fermentation protocol (Fig. 7h). The main ketones found in the nibs before and after roasting were 2-heptanone (43–54%, 36–49% of total), acetophenone (26–39%, 15–25%), 2-pentanone (10–24%, 7–20%) and 2-nonanone (4–14%, 18–37%), with the values in brackets corresponding to the levels in the nibs of the August and December beans, respectively.

3.2.4.5. Pyrazines. Pyrazines were not found in unfermented and fermenting cocoa nibs but were produced during roasting of the nibs.

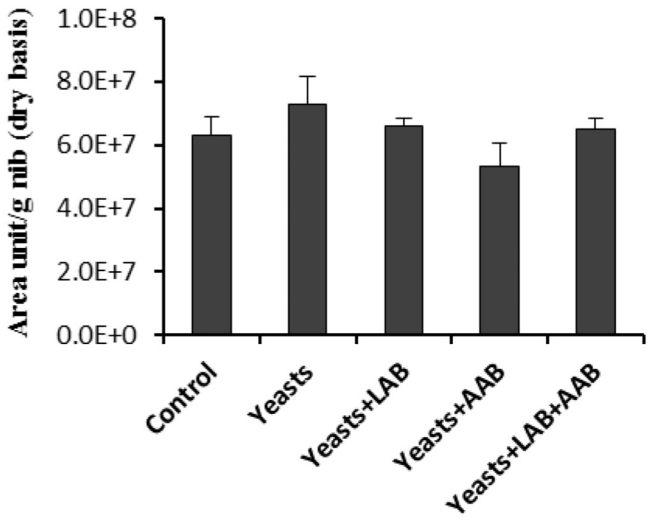

Fig. 8. Pyrazine concentrations in roasted cocoa nibs from the December beans fermented with combinations of yeasts and bacteria

Six pyrazine compounds detected were 2,3,5,6-tetramethylpyrazine (31–57 % of total), 2,3-dimethylpyrazine (29–40 %), 2,5-dimethylpyrazine (14–20 %), 2-methylpyrazine (9–16%), 2-ethy-6-methylpyrazine (8–14 %) and 2-ethyl-3,5-dimethylpyrazine (3–6 %). The kinetics of pyrazine production were statistically similar for beans inoculated with yeasts only, yeasts + LAB, yeasts + AAB and yeasts + LAB + AAB (Fig. 8).

3.3. Quality evaluation of cocoa beans

The cut test revealed that all dried, fermented beans were fully brown and free of visible mould. The shell contents of those beans are in the range of 12.2–13.4% and 11–12% for the August and December beans, respectively (Table 4). In both trials, the inoculated fermentations produced beans with less shell contents than those from the control, indigenous fermentations; however, the shell weights obtained for the various fermentation protocols were not statistically different ($p > 0.05$).

The results of the sensory triangle test are presented in Table 5. No significant differences were found in the flavour of chocolate made from beans inoculated with only yeasts compared to chocolates prepared from beans inoculated with yeasts + LAB or yeasts + AAB ($p > 0.05$).

The results of the hedonic test show that all three samples of chocolate made from the control beans and the beans inoculated with only yeasts or with yeasts + LAB + AAB were acceptable in terms of chocolate flavour and overall liking. Statistical analysis (*t*-test) of the mean liking scores shows that the flavour and overall liking of those chocolates were not significantly different ($p > 0.05$) (data not shown).

Table 4
Percentage of shell weight of dried, fermented beans

Sample	August beans*	December beans*
Control indigenous fermentation	13.4% ± 0.5%	12.0% ± 1.0%
Yeast inoculation	13.1% ± 1.0%	11.5% ± 0.6%
Yeast + LAB inoculation	12.5% ± 0.1%	10.9% ± 0.3%
Yeast + AAB inoculation	12.4% ± 0.3%	11.4% ± 0.5%
Yeast + LAB + AAB inoculation	12.2% ± 0.4 %	11.0% ± 0.6%

*The data are the mean of duplicate measurements of 20 beans of each batch; they are not significantly different ($p > 0.05$).

4. Discussion

Data in previous studies (Ho et al., 2014, 2015) suggested that yeasts were necessary for the successful fermentation of cocoa beans and LAB were not essential for the process. These conclusions were further tested in the current study by conducting controlled, inoculated fermentations using aseptically extracted beans where the growth of indigenous species was prevented or minimised.

4.1. Microbial ecology of fermentations

The aseptically extracted and fermented beans did not show growth of the main species of LAB or AAB usually associated with cocoa bean fermentations (Figs. 4a, 5a). However, they did show the presence of an *Asaia* spp or *P. agglomerans*, both of which grew but died off by 72 h. Contamination of controlled fermentations of "aseptically" extracted cocoa beans with indigenous microorganisms seems very difficult to avoid and this has also been the experience of other researchers (Crafack et al., 2013; Dircks, 2009; Sanchez et al., 1985; Schwan, Rose, & Board, 1995). However, for the fermentation studies presented in this study, the impact of indigenous microbiota did not occur or was sufficiently minimised, because the relevant yeasts, LAB or AAB were not detected in the freshly extracted beans and were overwhelmed by the inoculated species at 10^5–10^7 cfu/g.

Overall, the microbial ecology of the inoculated fermentations and indigenous fermentations followed the population and species profiles broadly described in the literature (Lima, Almeida, Nout, & Zwietering, 2011; Schwan & Wheals, 2004; Thompson et al., 2013). This consisted of the sequential development of the key yeast species *H. guilliermondii*, *P. kudriavzevii*, *K. marxianus* and *S. cerevisiae*, and growth of the key species of LAB, namely, *L. plantarum* and *L. fermentum* and the AAB, *A. pasteurianus*, *G. frateruii*. Nevertheless, some points are worthy of mention. Yeast growth was not affected by LAB, however, in the presence of AAB, less growth of *H. guilliermondii* occurred and there was a faster dying off for the yeast population. The antagonism of AAB to yeasts has been observed in wine fermentations (Fleet, 2003). *L. fermentum* was more sensitive than *L. plantarum* to the yeast inoculum and died off sooner which contrasts to many observations that this species is usually more dominant at the end of fermentations (Camu et al., 2008a; Camu et al., 2007; Nielsen et al., 2007; Pereira, Magalhaes, de Almeida, Coelho, & Schwan, 2013). The control indigenous fermentations of the beans harvested in August did not show the presence of LAB, but did have strong contributions from AAB while those of December beans had a very weak presence of both LAB and AAB.

4.2. Chemical changes during fermentation

The concentrations of glucose (41–47 mg/g) and fructose (45–52 mg/g) in the pulp, their utilisation during fermentation and the parallel production of ethanol (8–12.5 mg/g) are consistent with what has been published previously (Camu et al., 2007; Crafack et al., 2013; Galvez, Loiseau, Paredes, Barel, & Guiraud, 2007). For the nibs, sucrose (12–15 mg/g) was the main sugar found and it was hydrolysed to glucose and fructose. Ethanol diffused into the nibs giving final concentrations of 2–7 mg/g. These data are also consistent with previous works and the findings of Ho et al. (2014, 2015). For the inoculated fermentations, similar sugar utilisation and ethanol production were obtained, except for some utilisation of ethanol by the AAB. The same trends were also noted for the control indigenous fermentations, although the inoculated fermentations were faster and there was a greater utilisation of pulp sugars and higher ethanol production. The faster progress of inoculated fermentations has been known for a long time (Dircks, 2009; Knapp, 1924; Nicholls, 1913) and adds to the merits of using starter culture technology in the cocoa industry. The data for the August fermentation (Table 2) show the presence of less ethanol when AAB are present ($p < 0.05$) which is consistent with their ability to

Table 5
Triangle test for sensory evaluation of chocolates prepared from beans fermented with different combinations of yeasts and bacteria.

Batch	Sample	Number of judges	Number of correct judgements	Correct judgements needed for significance* (95% confidence level)
August	Yeasts vs Yeasts + LAB	30	10	15
2013	Yeasts vs Yeasts + AAB	30	11	15
December	Yeasts vs Yeasts + LAB	25	7	13
2013	Yeasts vs Yeasts + AAB	25	9	13

*Minimum number of correct judgements needed to declare for two samples to taste significantly different from one another (Adapted from critical value table for triangle test in Lawless & Heymann, 2010).

oxidise this substrate to acetic acid (Schwan & Wheals, 2004; Thompson et al., 2013). Such changes were not so evident for the December fermentations and might be explained by the weaker growth of AAB in this trial. In the absence of yeast growth, it was possible to show that LAB and AAB are capable of utilising pulp sugars, although not completely (see Ho et al., 2014).

The fermentation of pulp components, especially sugars, leads to the production of secondary metabolites, many of which have flavour impacts that could diffuse into the nibs to affect chocolate character (Afoakwa, Paterson, Fowler, & Ryan, 2008; Rodriguez-Campos et al., 2012). Different groups and species of yeasts, LAB and AAB are capable of producing different profiles of such metabolites and, therefore, could have unique influences on chocolate properties (De Vuyst & Weckx, 2016; Pereira, Miguel, Ramos, & Schwan, 2012). In this study, the cocoa nibs were screened for an array of some 50 fermentation volatiles, encompassing higher alcohols, esters, aldehydes and ketones. Fermented nibs contain a diverse range of higher alcohols and esters that are not found in unfermented nibs. They are produced during fermentation and diffuse from the pulp into the nibs. As reported in Ho et al. (2014, 2015), these higher alcohols and esters are mainly produced by yeasts. These findings are confirmed here, demonstrating that yeasts are the main sources of these flavour components. The same conclusions can be applied to the presence of aldehydes and ketones, but interpretation is more complex since unfermented beans contain significant levels of these components that were observed to vary for different batches of beans.

The production and significance of lactic acid during cocoa fermentations is mainly related to the contributions of LAB and their fermentation of pulp sugars and has been discussed by Camu et al. (2007), De Vuyst and Weckx (2016), Holm et al. (1993) and (Jinap & Zeslinda, 1995). Lactic acid produced by this mechanism diffuses into the nibs where it would contribute to the decrease in their pH to values around 5.25–5.75 that are best suited to the activity of endogenous enzymes needed to generate chocolate flavour precursors (Biehl, Brunner, Passern, Quesnel, & Adomako, 1985; Biehl & Voigt, 1999; Hansen, del Olmo, & Burri, 1998). However, too much of this acid (e.g. nib concentrations exceeding 5 mg/g dry weight) is thought to give undesirable acid properties to the chocolate (Holm et al., 1993; Jinap & Dimick, 1990; Jinap & Zeslinda, 1995). Fermentations with yeasts only showed the production of lactic acid which diffused into the beans, and confirmed the conclusion in Ho et al. (2015), that yeasts can also be a source of this acid, as would be expected from their metabolic properties (Plessas et al., 2008; Radler, 1993). Maximum concentrations of lactic acid (1.1 mg/g) produced in these fermentations were at the lower end of the range (0.9–9 mg/g) reported in the literature for spontaneous cocoa fermentation (Crafack et al., 2013; Moreira, Miguel, Duarte, Dias, & Schwan, 2013; Schwan, 1998). Nevertheless, when LAB were added to the fermentation with yeasts, their populations increased to about 10^8 cfu/g and higher levels of lactic acid were produced that diffused into the beans. However, such increased lactic acid did not significantly decrease the pH of the beans (Fig. 6b). Final concentrations of lactic acid produced in the inoculated fermentations with LAB were about 2–4 mg/g (Table 3). These values were consistent with the study by Lefeber, Janssens, Moens, Gobert, and De Vuyst (2011b), where cocoa fermentations inoculated with LAB produced lactic acid at final levels of 2–10 mg/g, depending on bean origin, fermentation methods and starter culture composition.

The production of acetic acid during cocoa fermentations is generally associated with the activity of AAB and their oxidation of the ethanol produced by yeasts (De Vuyst & Weckx, 2016; Lima et al., 2011; Schwan & Wheals, 2004). In Ho et al. (2014), it was clearly demonstrated that acetic acid was produced in fermentations where no yeasts grew and very little ethanol was produced. This suggested that LAB could also be a significant source of this acid, as originally suggested by Roelofsen (1958), and more recently proposed through their heterofermentative metabolism of hexose sugars and metabolism of citric acid (Adler, Bolten, Dohnt, Hansen, & Wittmann, 2013; Camu et al., 2008; Camu et al., 2007). The diffusion of acetic acid into the beans is considered to have three outcomes: it is significant in leading to bean death (Quesnel, 1965); it contributes to the decrease in nib pH as needed for optimum activity of endogenous enzymes (Biehl et al., 1985; Hansen et al., 1998); and it may contribute excessive acidity to the beans and chocolate at concentrations exceeding about 10 mg/g (Holm et al., 1993; Jinap, 1994; Jinap, Dimick, & Hollender, 1995). Yeasts are well known for their ability to produce acetic acid from hexose fermentation (Radler, 1993; Swiegers, Bartowsky, Henschke, & Pretorius, 2005) and this was demonstrated in bean fermentations with yeasts alone where pulp and nib concentrations of about 1 mg/g were found (Table 3). Similar values were obtained for fermentations when LAB were added to the yeasts, suggesting that these bacteria were not significant producers of acetic acid under these conditions. For the August fermentations where AAB were added to the yeasts, increased acetic acid production was observed and transferred to the nibs (about 2–2.5 mg/g) and this correlated with the strong growth of AAB (10^9 cfu/g) in these fermentations (Fig. 4). The higher production of acetic acid in fermentations where AAB were added was reflected in the lower pH of the nibs obtained from these fermentations (Fig. 6). Such increases were not observed for the December fermentations where the overall growth of AAB was 100 fold less (10^7 cfu/g). The final levels of acetic acid (1–2.5 mg/g) detected in the pulp and nibs were similar to those values reported by Schwan (1998).

The data on citric acid are more problematic to interpret. For the nibs, the concentrations found and subsequent decrease during fermentation are consistent with findings by other researchers (Ardhana & Fleet, 2003; Camu et al., 2008; Dircks, 2009; Pereira et al., 2012). These changes were not impacted by the yeast or bacterial ecology despite different effects of the ecology on pulp composition. According to many previous reports, pulp citric acid is utilised during fermentation, which leads to an increase in pulp pH (Papalexandratou, Camu, Falony, & De Vuyst, 2011a; Papalexandratou et al., 2011b; Pereira et al., 2013). While some species of yeasts and LAB associated with the fermentation are known to utilise citric acid, the data show strongest utilisation by strains of *L. fermentum* (Moreira et al., 2013; Ouattara et al., 2017). Fermentations in the presence of yeasts alone, gave an increase in pulp citric acid (Table 3) as was also observed for the August indigenous fermentation and other indigenous fermentations reported in Ho et al. (2015). These results may be explained by the fact that various yeast species are known to produce citric acid (Abou-Zeid & Ashy, 1984; Anastassiadis, Aivasidis, & Wandrey, 2002; Anastassiadis & Rehm, 2006). When LAB were added to the fermentations, there was evidence

of weak citric acid utilisation in the early stages of fermentation, followed by increases in its concentration, again probably due to yeast production of this acid. Strain variation in the utilisation of citric acid by species of LAB has been reported, and this property was not determined for the strains used in this study. The changes in pulp pH for the various fermentations examined (Fig. 6) follow the general trend reported in previous research, namely a slight decrease in pH during the first 24–48 h, followed by an increase (Dircks, 2009; Nielsen et al., 2007). Consistent with the ecological and organic acid data, greater initial pH decreases were observed for the fermentations where AAB were inoculated and higher concentrations of acetic acid were found (Fig. 6). However, the subsequent increase in pulp pH does not accord with the fact that this is caused by citric acid utilisation (Lima et al., 2011; Schwan & Wheals, 2004), because this was not observed and, in contrast, its production was found. Further research is needed to resolve this anomaly.

With regard to volatile compounds, four major groups of higher alcohols, esters, aldehydes and ketones detected throughout fermentation, drying and roasting and the formation of pyrazines during roasting were generally similar for all inoculated and uninoculated fermentations. The main volatiles of each group were consistent with those found in Ho et al. (2014, 2015). These results indicate that the presence or absence of LAB or AAB does not appear to have a significant impact on the formation of volatile compounds during cocoa fermentation whereas the growth of yeasts is essential for developing desired chocolate flavour. These findings are in accordance with the early studies of Knapp (1924), Nicholls (1913), Preyer (1913), Roelofsen and Giesberger (1947) and Roelofsen (1958) where it is proposed that only yeasts are necessary to give proper cocoa fermentation while the contribution of bacteria is likely to produce undesirable flavour characteristics.

4.3. Quality of cocoa beans and chocolate

Indigenous fermentations and aseptic fermentations inoculated with selected yeasts and bacteria produced dried beans that were fully fermented, and had acceptable appearances and shell weights (Afoakwa, 2010; Fowler, 2009; Wood & Lass, 1985). Beans from indigenous fermentations and inoculated fermentations with yeasts only or yeasts + LAB + AAB gave chocolates that were equally scored for chocolate taste and overall liking. Further sensory evaluation by the triangle test clearly demonstrated that there were no significant differences ($p > 0.05$) between chocolates made from beans fermented with yeasts only, yeasts + LAB and yeasts + AAB. Therefore, it may be concluded that acceptable quality chocolate can be obtained from beans where yeasts are the main agents of fermentation and that LAB and AAB are not necessary for the fermentation process. This conclusion is consistent with the findings in Ho et al. (2015) where acceptable quality chocolate was obtained from beans indigenously fermented in the absence or restricted growth of LAB. These sensory data also support the conclusion in Ho et al. (2014) that yeasts are essential for the cocoa bean fermentation process and the statement of Nicholls (1913), over 100 years ago, that “yeasts, and these only, are all the organisms which are required for the production of a proper fermentation in cacao”.

In summary, the results of the present study as well as our previous publication (Ho et al., 2015) appear to indicate that yeasts only are necessary for a successful cocoa fermentation. This proposition needs to be understood in terms of how they would create the environmental conditions necessary to cause bean death and the endogenous biochemical reactions that give the precursors of chocolate flavour. Three factors are considered to contribute to bean death during the fermentation process: (i) acetic acid at concentrations around 0.4 %; (ii) ethanol concentrations about 4% and; (iii) an increase in temperature to 45–50 °C (Griffiths, 1959; Lehrian & Paterson, 1983; Quesnel, 1965; Roelofsen, 1958). These factors work synergistically meaning that, when applied together, lesser values apply (e.g. much less ethanol than 4% is needed to kill the beans at 45 °C than at 37 °C). Although these properties are often mentioned in the literature, sound, reliable data as to what values and what combinations cause bean death are lacking. While acetic acid was once thought to be the major contributor, recent evidence suggests that ethanol may be the driving property (Thompson et al., 2013). Yeasts alone produce ethanol and acetic acid as demonstrated in this study and these two compounds could account for bean death. Yeasts also metabolise ethanol, especially when sugars are depleted (Geurt, De Kok, & Roels, 1980; Jespersen, Nielsen, Honholt, & Jakobsen, 2005; Raamsdonk et al., 2001) and this could lead to temperature increases, although in the present study the temperature increases were purposely managed by controlled incubation due to the low mass of beans used in the fermentations. More studies are needed to better understand the underlying mechanisms of bean death. However, good quality chocolate was obtained from the yeast only fermentations, so it may be assumed that the chemical changes they cause are sufficient to induce this bean death and associated endogenous reactions that lead to chocolate character. Notably, the dried nibs had pH values around 5.5–5.8 which falls in the range for the production of good chocolate (Jinap, 1994; Jinap et al., 1995). Because of the importance of yeasts in the process, more research is required to determine the roles and significance of various species. At this stage, it is not known how different, individual yeast species might contribute to the fermentation and whether or not a single species would be sufficient or a consortium is required. Further, controlled aseptic cocoa fermentations with these species individually and in mixtures will be needed to determine if they are essential to the process and what specific impacts they may have on chocolate flavour. Such information will then provide a better platform of knowledge for developing cocoa fermentation processes using defined starter cultures and transforming them into a controlled industrial operation.

4.4. Conclusion

This study found that fermentation of sterile cocoa beans by inoculating them with yeasts, yeasts + LAB, or yeasts + LAB and AAB produced beans with no significant differences in colour, shell weights and concentrations of residual sugars, alcohols and esters ($p > 0.05$). But they were slightly different in contents of lactic acid and acetic acid ($p < 0.05$) with beans fermented in the presence of LAB and AAB contained higher levels of lactic and acetic acids, respectively. All beans were fully brown and free of mould. Triangle and hedonic sensory evaluations of chocolates prepared from beans taken from the three fermentations showed no significant differences ($p > 0.05$) in flavour and overall liking. It was concluded that the growth of lactic acid bacteria and acetic acid bacteria may not be essential for the fermentation of cocoa beans while yeasts only are necessary for a successful cocoa fermentation. Further research is needed to confirm the conclusion and to determine the contribution of yeast species individually and in mixtures to cocoa fermentation and chocolate flavour – a necessary step in the development of starter cultures for cocoa fermentation.

ACKNOWLEDGEMENTS

We would like to dedicate this work to the late Professor Graham Harold Fleet, who was not only the academic supervisor for both of us in our PhD studies, some 20 years apart, but also a mentor and great friend. We also gratefully acknowledge Mr. Yan Diczbalis, Department of Agriculture and Forestry, Queensland, Australia and Mr. Alan Mortimer, the Australian Blending Company for assisting in supplying cocoa beans for this project. Ms. Van Ho thanks the Department of Education, Employment and Workplace Relations, Australian Government for providing an Endeavour PhD Scholarship to conduct this research.

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