Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.109322

Fermentation box cleaning can impair cacao seed fermentation

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Abstract

The quality of the raw material for chocolate manufacture depends on the control of cacao seed fermentation. Practices affect the diversity and metabolic activities of spontaneous yeasts in fermentations. The control of the initial inoculum is important to standardize fermentations and to improve the final seed quality. We conducted fermentations using non-cleaned or cleaned fermentation boxes to evaluate the dynamics of the microorganism populations and their effect in seed composition in two consecutive years. The isolated yeasts were identified by sequencing the D1-domain of the 26S ribosomal genes. Pulp and cotyledon parameters were monitored during fermentations. *Wickerhamomyces anomalus* was the dominant yeast in the fermentations, but the species was less represented in cleaned boxes. In the cleaned-box fermentation, there was a delay in temperature rise of the fermentation, which retarded the suitable moment for seed-mass turning. The reduction in seed-pulp quality caused by restricted rainfall in one year limited the seed-mass heating and voided the seed turning. Thus, under substrate restriction, the initial reduced inoculum caused by cleaning the boxes impaired fermentation, mainly under environmental conditions that compromise the quality of the pulp. However, this variation had little effect on the desired degradation of cotyledon components, substrates for flavor precursors.

Keywords: cocoa; chocolate; *Theobroma cacao*; *Wickerhamomyces anomalus*; yeast.

Practical Application: Natural fermentations conducted in cleaned wooden-boxes should receive yeast starter cultures.

1 Introduction

The spontaneity of the cacao fermentation process favors the establishment of yeast species diversity according to the site, climatic conditions, and fermentation practices adopted (Ardhana & Fleet, 2003; Jespersen et al., 2005; Schwan et al., 1995; Sousa et al., 2016), determining the quality of the resulting chocolate. The source of natural inoculum includes cacao pod surfaces, banana leaves used to cover cacao seed mass, insects, transport container, and particularly the traditional wooden-fermentation box (Fernández Maura et al., 2016). The control of the nature and size of the yeast initial inoculum is important to manage the fermentation process driving the chocolate flavor (Camu et al., 2008).

The yeast protagonism in cacao fermentation have directed the investigation of the effect of the interspecific diversity in the process. The profile of yeast species in cacao fermentation is rarely fixed, varying among the cacao producing regions, that differ for cultivars, fermentation practices and duration (Viesser et al., 2021; Gutierrez et al., 2022). Studies tend to indicate yeast species that dominate the fermentation (Ardhana & Fleet, 2003; Meersman et al., 2013). In natural fermentations conducted in Southern Bahia, the main cacao producing region of Brazil (Cruz et al., 2015; Sousa et al., 2016), the microbiota usually contains a larger number of yeast species, and the reported species occurring included *Candida bombi*, *C. pelliculosa*, *C. rugopelliculosa*, *C. rugosa*, *Kluyveromyces marxianus*, *K. thermotolerans, Kloechera apiculata*, *Lodderomyces elongisporus*, *Pichia fermentans*, *Saccharomyces cerevisiae* and *Torulaspora pretoriensi* (Illeghems et al., 2012; Ozturk & Young, 2017; Serra et al., 2019).

The yeasts degraded the seed-surrounding pulp (Ho et al., 2014; Vuyst & Weckx, 2016) and convert sugars to ethanol, and the production is directly associated with the increase in yeast population. In cacao seed fermentation, the yeast growth interferes in the metabolism of the acetic acid bacteria (AAB) (Schwan & Wheals, 2004). AAB transform ethanol into acetic acid, which is an exothermal reaction and causes the temperature increase of the fermenting pulp-seed mass. During the process, the fermentation temperature gradually raises to values close to 50 °C (Schwan & Wheals, 2004). The production of acetic acid is affected by the conversion of sugars in ethanol, and subsequently, by the rate of ethanol conversion to acetic acid, independently of the size of the AAB population (Pereira et al., 2013).

Received 31 Oct., 2022
Accepted 07 Dec., 2022
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Ethanol and organic acids produced by yeasts, lactic acid bacteria (LAB) and AAB penetrate the seed coat. The acidification of the cotyledons is associated with the rise in temperature, causes cell rupture and death of the embryo (Thompson et al., 2001). The combination of these factors leads to the degradation of seed carbohydrates and storage proteins, generating fundamental precursors (reducing sugars, oligopeptides, amino acids) for the development of the chocolate flavor and aroma upon roasting (Lima et al., 2011; Sousa et al., 2016; Thompson et al., 2001).

The level and duration of the acidification of the cotyledon determine seed flavor (Voigt & Lieberei, 2014). An excessive acidification (pH < 4.5) results in cacao seeds with limited potential flavor, sometimes with an excess of lactic acid, considered an undesirable attribute because it is non-volatile, and it can accumulate even after roasting (Schwan & Wheals, 2004). The cotyledon pH affects the development of the chocolate flavor, with cocoa notes developing at lower pH (pH 4.4 - 5.2) and nutty notes at higher pH (pH 4.8 - 5.6) (Voigt et al., 2018).

Various approaches have been used to standardize the cacao seed fermentation process. All approaches try to provide adequate conditions to the full development of the fermentation to produce ideal seeds for high-quality chocolate. Some strategies use the standardization of the seed volume to be fermented, the stage of harvested pods, or to reduce the amount of seed-pulp before fermentation (Saltini et al., 2013). Other approaches prioritize the nature of the inoculum to favor the resident microbiota of the fermentation environment. The resident microbiota population in the container used for fermentation is considered important for the identity and fermentation quality (Ferreira, 2017). Other approaches try to reduce the initial local inoculum by cleaning the fermentation container and minimizing initial contamination of the seeds (Schwan, 1998; Papalexandratou et al., 2011; Pereira et al., 2013), eliminating resident yeast strains. One practice to control and standardize cacao fermentation consists of the combination of hygienization of the fermentation container and inoculation with starter yeast cultures (Leal et al., 2008). The increase in inoculum pressure aims to accelerate the fermentation, to standardize and to aggregate value to the fermented seeds (Crafack et al., 2014; Figueroa-Hernández et al., 2019).

The natural fermentation of cacao seeds conducted aseptically in stainless steel showed a slower increase in temperature, but sufficient to produce ethanol and acetic acid, containing the same yeast species found in natural fermentations (Pereira et al., 2013). In Brazil and other origins, the use of wooden fermentation box is the conventional fermentation method (Viesser et al., 2021), and therefore, it is necessary to evaluate the practice of cleaning the fermentation box in the nature of the initial inoculum and its effect on the fermentation process.

Therefore, the impact of cleaning the fermentation box in the yeast population and fermentation was evaluated in the present study in two consecutive years. One farm conducted the fermentation in a wooden box without sanitation and the second farm cleaned the boxes before fermentation. The inoculum reduction by cleaning caused a delay in the rise of the temperature of the seed mass, affecting the time of turning the seed mass. However, the change in the initial nature and density of yeast inoculum, and their subsequent effect in the fermentation, were insufficient to change the pattern of carbohydrate degradation in the seeds, one of the precursors of the chocolate flavor, even in the least favorable conditions of substrate for fermentation.

2 Material and methods

2.1 Seed fermentations

The cacao fermentations were conducted in two farms closely located in Bahia, Brazil, during July 2011 and 2012 (1st and 2nd year). The fermentations at the 'Almirante' farm (coordinates -14.719903/-39.368290), located in Barro Preto, BA, were conducted in non-cleaned fermentation box, whereas the ones using cleaned fermentation box were performed at the 'Leão de Ouro' farm (-14.627932/-39.242287) in Uruçuca, BA, 17 km apart. Cleaning the fermentation box is a routine practice at the 'Leão de Ouro' farm (Papalexandratou et al., 2011). The wooden box was superficially disinfected with 70% ethanol on all sides.

In both farms, cacao pods were harvested and broken on the same day. The seeds from both fermentations were depulped to 60% using a mechanical depulper. The seeds (60 kg) were then placed in wooden boxes (54 x 54 x 49 cm), covered with banana leaves. Fermentations lasted 168 h for non-cleaned fermentation box treatment, and 144 h d for the cleaned fermentation box. The seeds mass was turned every 24 h after the seed mass reached around 50 °C. The local temperature and the fermentation temperature were monitored by thermal probes. Local weather data for rainfall was retrieved from an automatic weather station at Ilhéus, BA, from the Brazilian "Instituto Nacional de Meteorologia" (www.inmet.gov.br).

2.2 Seed and box sampling

The surface of the wooden boxes was scraped at both farms, and the samples were placed in sterile plastic bags. Seed samples (with pulp) were collected in triplicates at 0, 24, 48, 72, 96, 120, 144 and 168 h in the non-cleaned fermentation box, whereas sampling stopped at 144 h in the cleaned fermentation box. Samples for microbiological isolation and counting were kept at room temperature until brought to the laboratory, except those for analysis of yeast diversity, that were stored at -20 °C until freeze-dried. Samples for chemical analyses were stored at -20 °C.

2.3 Chemical analyses

Pulp samples were obtained by depulping 25 g seeds (fresh weight) in 225 mL deionized water, homogenized in a blender with a metal disk with holes. A sample of 50 mL was used to determine the pH and titratable acidity. The pulp suspension was diluted in 80 mL of distilled water to determine sugars (sucrose, glucose, and fructose), organic acids (citric, acetic and lactic acids) and ethanol, a sample of 20 mL. An aliquot of 2 mL was filtered through a HA filter 0.45 μM pore size, 25 mm diameter (Millipore; Bedford, MS, USA) for HPLC analysis.

The cotyledon samples were prepared using 5 g (fresh weight) seeds in 100 mL warmed deionized water in a blender with blades. The ground sample was spun at 6,000 g for 10 min. An aliquot the supernatant was used to determine the pH and

acidity. A 1.5 mL aliquot was filtered through a HA Millipore filter and used to determine sugars, organic acids, and ethanol by HPLC LC-10Ai (Shimadzu Corp., Kyoto, Japan) with double detector UV-Visible light (SPD 10Ai) and refraction index detector (RID-10Ai). Samples were separated by an ionic exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm; Shimadzu Corp.) at 30 °C for sugar and alcohols, and at 50 °C for organic acids using perchloric acid (100 mM) as an eluting agent at 0.6 mL min^{-1}. The acids were detected by UV absorbance (210 nm) and sugars and ethanol by RID. The compounds were identified by retention time according to standards, and all samples were estimated in triplicates. External calibration was used to determine sample concentration from calibration curves established by standards at increasing concentrations.

2.4 Culture-based quantification of microorganisms

The samples from the scraps of the fermentation boxes and seed pulp were used in serial dilutions in 0.1% peptone-water for counting and isolating microorganisms. The serial dilutions were inoculated on MYGP medium (0.3% malt extract; 0.3% yeast extract; 1.5% glucose; 0.5% peptone; 2% agar) supplemented with chloramphenicol (100 mg L^{-1}) and chlortetracycline (50 mg L^{-1}), and incubated at 28 ºC for 48 h. Yeasts representing morphotypes visualized on the plates were isolated (Nielsen et al., 2007; Ho et al., 2014), purified by sequential streaking in MYGP medium and stored in 15% glycerol (v/v) at -20 °C for identification.

Serial dilutions from the pulp samples were used to count LAB, AAB and mesophilic bacteria populations. LAB was counted by pour-plate inoculation on MRS agar (De Man et al., 1960) containing 0.1% cycloheximide (v/v) and 0.1% cysteine-HCl (v/v). AAB was counted by surface inoculation on GYC-agar (50 g L^{-1} glucose; 10 g L^{-1} yeast extract; 30 g L^{-1} calcium carbonate; 20 g L^{-1} agar, pH 5.6) supplemented with 0.1% cycloheximide and 50 mg L^{-1} penicillin. Nutrient agar containing 0.1% cycloheximide (v/v) was used as a general medium for viable mesophiles. Plates were incubated at 30 °C for 3–4 d for MYGP, MRS, and Nutrient agar cultures, and at 25 °C for 5–8 d for GYC-agar cultures. The colony-forming units (CFU) were counted only when 30 to 300 colonies were observed per plate.

2.5 Yeast identification by sequencing

The yeast isolates were characterized by sequencing the 26S ribosomal gene (D1/D2 region). The DNA was extracted using PureLink Genomic Plant DNA Purification (Thermo-Fisher Scientific; Waltham, MA, USA). The amplification reactions (25 µL) contained 1 U *Taq* DNA polymerase, 2.5 µL 10X reaction buffer; 2.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.5 µM of each primer (NL-1 GCATATCAATAAGCGGAGGAAAAG; NL-4 GGTCCGTGTTTCAAGACGG) and 25 ng DNA. The amplification cycle started at 94 ºC for 5 min, followed by 30 cycles at 94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min, followed by a final cycle at 72 ºC for 7 min. The amplification product was visualized in 1.5% agarose gel stained using Sybr Green (Thermo-Fisher Scientific). Positive reactions were purified using the ExoProStar 1-Step kit (GE Healthcare; Chicago, IL, USA), and the purified fragments sequenced using BigDye Terminator v3.1 Cycle Sequencing (Thermo-Fisher Scientific) in a 3500 ABI sequencer. Sequencing results were analyzed by blast against the NCBI database.

3 Results

3.1 Initial pulp seed analysis and environmental conditions

In the 1st year, the initial levels of glucose, fructose, and citric acid in the seed pulp and the cotyledons were similar between fermentations (Figure 1). In the 2nd year, the seed pulp contained in general less sugars (sucrose, glucose, and fructose) than in the 1st year. In the cotyledons, the levels of sugars were like the previous year (Figure 1).

In the 1st year, there was more rainfall in the first months of the year, with a total of 667 mm recorded between March and June (Supplementary Material - Figure S1). During the 2nd year, there was less precipitation in the first months of the year, particularly between March and June (total 99 mm), characterizing a dry-spell before pod harvest (Supplementary Material - Figure S1), which may have impacted the pulp quality.

3.2 Seed mass temperature during fermentation and environmental conditions

In the 1st year, the seed mass temperature gradually increased after 48 h in both fermentations (Figure 2). However, there was a delay in temperature rise of the fermentation in the cleaned box, which retarded the moment suitable for seed-mass turning. In the non-cleaned box, the first seed turning occurred at 72 h of fermentation. The temperature in the cleaned box exceeded 50 °C after 72 h, and the first seed turning occurred at 96 h of fermentation. The average temperature of the non-cleaned box and the cleaned box fermentation were 48 and 49.6 ºC, respectively.

During the 2nd year, the seed mass temperature of both fermentations increased after 60 h. There was a discrepancy between the rise in temperature between the fermentations differing for the box cleaning treatment. In the non-cleaned box fermentation, the seed mass took longer to reach 40 ºC (*circa* 80 h), and reached 50 ºC at around 84 h (Figure 2); the first seed turning occurred at 96 h. After the first turning, the seed mass did not recover the temperature, but maintained the temperature at 45 ºC for the next seed turning. Conversely, in the cleaned-box fermentation, the seed mass reached 40 ºC quicker (around 70 h), but afterwards, it reached 50 ºC only at 120 h of fermentation. In the cleaned box fermentation, the first seed turning happened at 120 h of fermentation, and it was the only one that occurred. The temperatures of both fermentations exceeded 50 °C at distinct moments: in the non-cleaned box at 84 h of fermentation, and in the cleaned box at 120 h (Figure 2); the mean temperature was 47.7 and 46.5 ºC, respectively.

3.3 Yeast and bacteria counts

In the 1st year, yeast count peaked during the first 48 h from both fermentations (Figure 3). The peak of the yeast population occurred earlier in the cleaned-box treatment, but the yeast cell count peak was smaller than in the non-cleaned box. The highest yeast count (5.3 x 10^8 CFU g^{-1}) occurred in the non-cleaned box at 48 h of fermentation. In the cleaned box fermentation, the

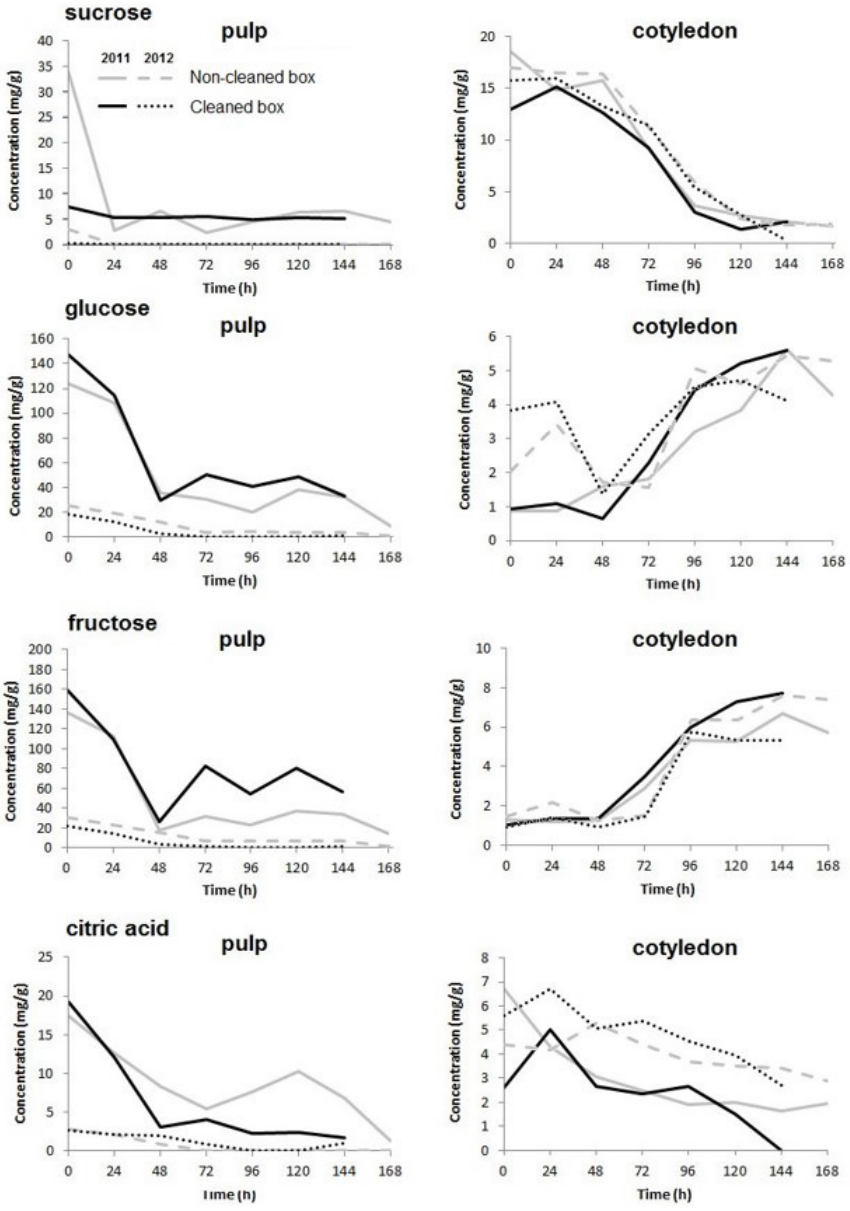

Figure 1. Levels of carbohydrates (sucrose, glucose and fructose) in the cacao seed pulp and cotyledons from seeds from fermentations from the 1st and 2nd year as the non-cleaned box (grey line; 'Almirante' farm) or as cleaned box (black line; 'Leão de Ouro' farm). The samples were collected at 0, 24, 48, 72, 96, 120, and 144 h; 168* h. 168 h was collected only for the non-cleaned box fermentation. The non-cleaned box fermentation lasted 168 h and the cleaned box fermentation 144 h.

highest yeast count was observed at 24 h (0.21 x 10^8 CFU g^{-1}). In both fermentations, the yeast count reduced after 72 h and kept stable up to the end of the fermentation.

In the 2nd year, both fermentations showed an increase in yeast count during the first 48 h, without peaking as in the previous year. The yeast count in the cleaned-box fermentation continued elevated until 120 h (Figure 3), possibly because the lower seed-mass temperatures favor the maintenance of the yeast population for a longer period.

The cleaned-box practice did not affect the counts of bacteria. The counts of AAB in the 1st year was similar for both fermentations (Figure 3). The increase in AAB count occurred between 24 and 48 h, followed by a reduction between 72 and 96 h. A second count peak happened between 120 h or at the end of the fermentation (Figure 3). In the 2nd year, the AAB count increased in the first 72 h, followed by a reduction at 96 h and a second increase at the end of the fermentation. The LAB count in the 1st year showed a growth pattern similar to the AAB (Figure 3), with an initial increase, followed by a reduction and second increase. In the 2nd year, the LAB count pattern was similar, with large counts at 72 h. The counts of mesophiles kept the same levels in the 2nd year, with fewer changes than observed in the 1st year (Figure 3).

3.4 Identification of yeast species

From the fermentation boxes during the 1st year, thirteen yeast isolates were obtained, 10 from the non-cleaned box and three from the cleaned box. The species common to each box was *Wickerhamomyces anomalus* (two isolates from each box). The species *Candida parapsilosis* (one isolate), *Pichia manshurica* (two isolates), *Saccharomyces cerevisiae* (six isolates) were exclusive to the non-cleaned box, whereas *Torulaspora delbrueckii* was exclusive to the cleaned box (one isolate).

From the seed pulp, 116 isolates were identified, with 48 isolates from the non-cleaned box and 68 isolates from the cleaned box (Table 1). The most frequent species in both fermentations was *W. anomalus*, but it representativeness was lower in fermentations conducted on cleaned box. Other species identified at both fermentations were *C. intermedia*, *C. quercitrusa*, *Candida* sp., *P. galeiformis*, *P. manshurica*, *S. cerevisiae*, *T. delbrueckii*, and *Wickerhamomyces* sp.

The yeasts isolated from boxes and seed mass in the experiment conducted in the 2nd year were not identified due to sample storage problems.

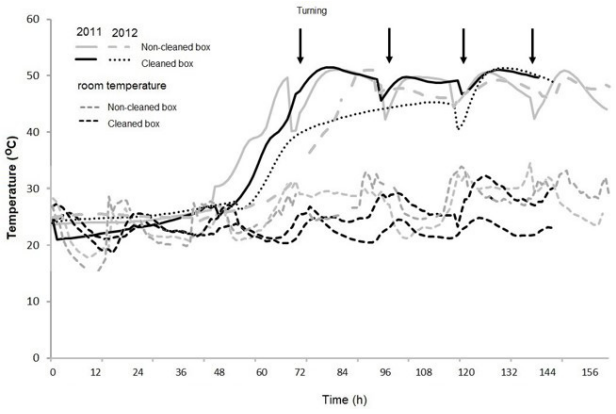

Figure 2. Temperature (ºC) inside the fermenting seed mass in the 1st (filled line) and 2nd year (dashed line). The room temperature is represented by the dashed line. The cacao seed fermentations were carried out as the non-cleaned box (grey line; 'Almirante' farm) and as cleaned box (black line; 'Leão de Ouro' farm). The non-cleaned box fermentation lasted 168 h and the cleaned box fermentation 144 h. Temperatures in the seed mass were monitored by thermal probes. The arrows indicate the moment of seed-mass revolving.

3.5 Seed pH during fermentation

In the 1st year, the initial pH of the seed pulp in the non-cleaned box and cleaned box was 4.1 and 3.8, respectively, and the initial pH of the cotyledons was 6.5 and 6.3. In both fermentations, the cotyledon pH started to decrease after 72 h and reaching at 96 h values below 5.0. The final pH of the seed pulp and cotyledon was similar between fermentations (non-cleaned box: 4.7 and 4.6; cleaned box: 4.9 and 4.8).

In the 2nd year, the initial pH of the seed pulp in the non-cleaned box and cleaned box was 3.8 and 3.9. The initial

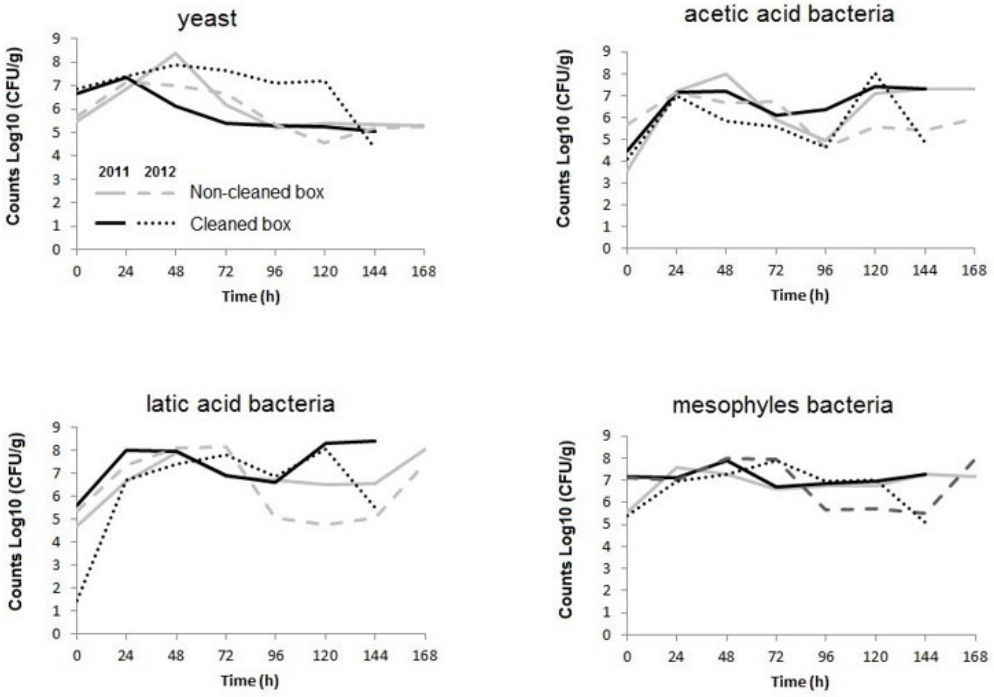

Figure 3. Population growth dynamic of Yeast, Acetic Acid Bacteria (AAB), Lactic Acid Bacteria (LAB), Mesophile bacteria in cacao seed fermentations conducted as the non-cleaned box (grey line; 'Almirante' farm) and as cleaned box (black line; 'Leão de Ouro' farm). The Unity Forming Colonies (UFC) were determined by culture and direct counting of serial dilution. The samples were collected at 0, 24, 48, 72, 96, 120, and 144 h; 168* h. 168 h was collected only for the non-cleaned box fermentation. The non-cleaned box fermentation lasted 168 h and cleaned box fermentation 144 h.

Table 1. Yeast species, number of isolates and relative frequency obtained during the 1st year in the pulp of fermented seeds from the non-cleaned and cleaned fermentation box.

Yeast species	non-cleaned box		cleaned box	
	Total	%	Total	%
*Wickerhamomyces.anomalus**	26	54.2	23	33.8
Candida quercitrusa	6	12.5	5	7.4
Saccharomyces cerevisiae	5	10.4	4	5.9
Candida sp.	4	8.3	7	10.3
Wickerhamomyces sp.	2	4.2	4	5.9
Candida intermedia	1	2.1	13	19.1
Pichia manshurica	1	2.1	4	5.9
Torulaspora delbrueckii	1	2.1	4	5.9
Pichia galeiformis	1	2.1	1	1.5
Meyerozyma guilliermondii	1	2.1	0	0.0
Pichia kluyveri	0	0.0	1	1.5
Candida natalensis	0	0.0	1	1.5
Saccharomyces chevalieri	0	0.0	1	1.5
Total	48	100.0	68	100

* *Wickerhamomyces anomalus* is a synonym of *Candida pelliculosa*, *Pichia anomala*, *Hansenula anomala*.

pH of the cotyledons was 6.7 for each fermentation. In both fermentations, the pH of the cotyledons decreased drastically. The values reduced to 5.0 at 96 h. The final pH of the seed pulp and cotyledon was dissimilar between fermentations. The final pH of the seed pulp in the non-cleaned box and cleaned box was 5.8 and 4.6 and 5.0 and 4.7 for the cotyledons.

3.6 Sugars, ethanol, and organic acids in seed pulp and cotyledons

In the 1st year, sucrose, glucose, fructose, and citric acid levels in the seed pulp decreased during the first 48 h (Figure 1). In the fermentation conducted in the cleaned box, the production of ethanol (at 24 h) and acetic acid (at 48 h) occurred earlier and more intense (Figure 4). The presence of acetic acid in the pulp corresponded to titratable acidity (Figure 5). The level of lactic acid increased during the process (Figure 4).

In the 2nd year, the glucose, fructose, and citric acid levels in the pulp was smaller and they were reduced to even minor values at 72 h (Figure 1); the production of ethanol and acetic acid was smaller. The production of ethanol reached its maximum level at different moments (Figure 4), at 48 h (cleaned box) and 72 h (non-cleaned box). The acetic acid level in the seed pulp raised at 96 h for both fermentations. The seed pulp acidity (Figure 5) followed the accumulation of acetic acid, whereas the production of lactic acid was smaller.

In the cotyledons at the 1st year, the sucrose level started to reduce at 72 h of fermentation, reaching the lowest levels at 96 h for both fermentations (Figure 1). The level of glucose and fructose increased at 96 h and stabilized (Figure 1). The level of citric acid decreased and remained stable up to 96 h (Figure 1). The peak of ethanol levels appeared in the cotyledons at 48 h (Figure 4), and the levels of acetic acid increased at 96 h. The cotyledon titratable acidity (Figure 5) followed the acetic acid level. The level of lactic acid in the cotyledons increased during fermentation.

In the 2nd year, the sucrose, glucose, and fructose level in the cotyledons were similar to the previous year. The sucrose level reached the lowest levels at 120 h at both fermentations (Figure 1). The levels of glucose and fructose increased at 96 h and stabilized. The citric acid level started to fall at 96 h of fermentation (Figure 1). The peak of ethanol appeared in the cotyledons at 72 h (Figure 4), and the level of acetic acid increased at 96 h. The level of acetic acid was higher in the cleaned box fermentation. The seed titratable acidity did not follow the determination of acetic acid in the cotyledons (Figure 5). The level of lactic acid increased at 72 h of fermentation in the non-cleaned box and 120 h in the cleaned box fermentation.

4 Discussion

The nature and size of the initial inoculum can be important in controlling cacao seed fermentation (Camu et al., 2008). Practices and seasonal variation in spontaneous fermentation of cacao seeds can affect the diversity and metabolic activities of yeasts in the fermentation. Controlling the fermentation process to drive chocolate flavor development (Camu et al., 2008) could be achieved by managing the nature and size of the inoculum with the adoption of hygienization of the fermentation container. Here, our study analyzed the effect of box-cleaning on the dynamics of the fermentation process. Fermentation conducted in cleaned box affected the rise of temperature of the seed mass, interfering with the timing of seed-mass revolving, mainly when the pulp substrate had less sugars.

The fermentations were performed using large amount of cacao seeds. The volume of seeds may affect the fermentation process, and the best fermentations are believed to occur in volumes of 55 to 60 kg (Saltini et al., 2013). To ferment large volumes of cacao seeds demands sufficient ripe pods, limiting the possibility to test many practices at once (Sousa et al., 2016). Considering these limitations, the experiments described here were performed at two sites (each with a box fermentation

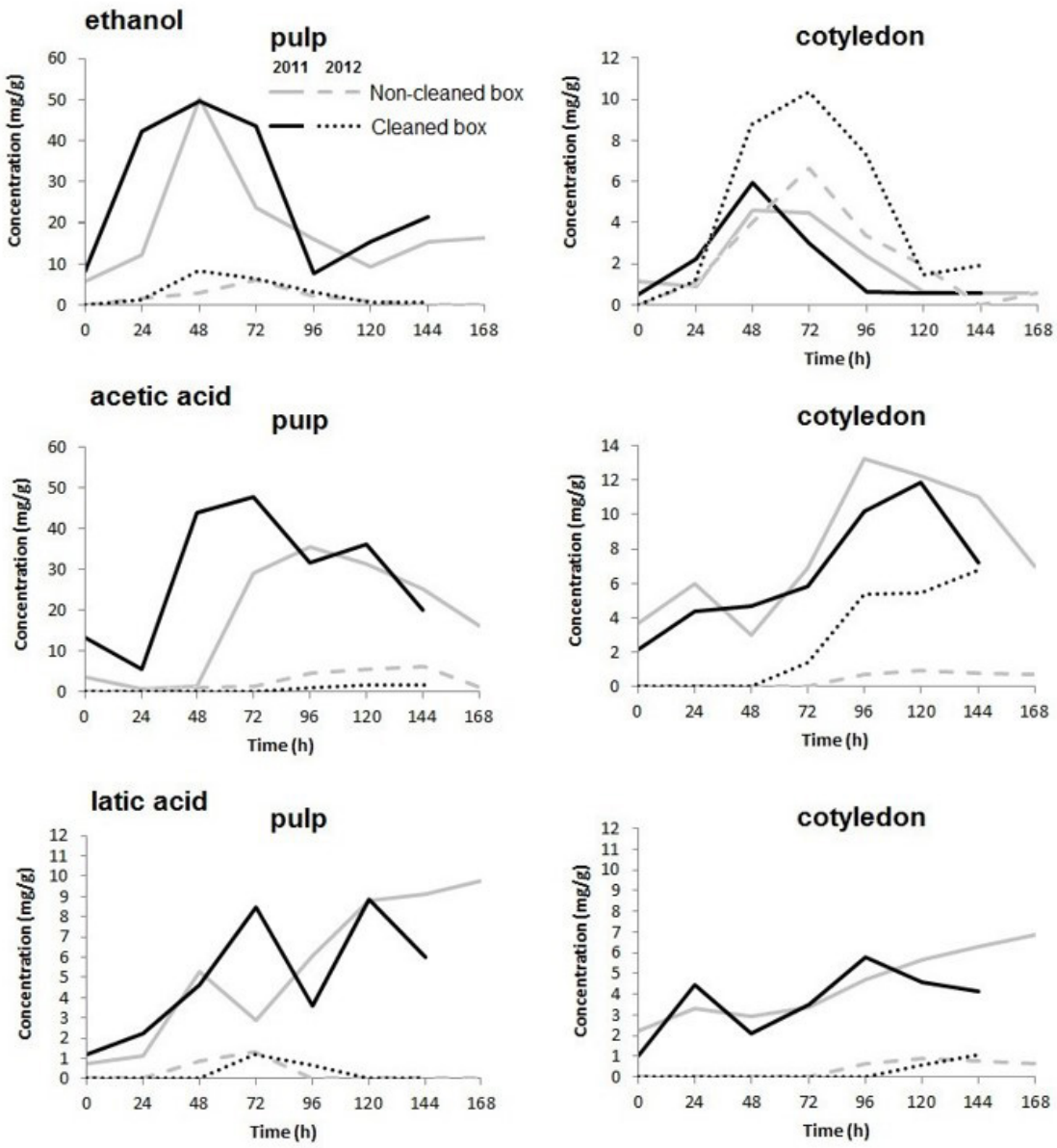

Figure 4. Levels of ethanol, acetic acid, lactic acid detected in the cacao seed pulp and cotyledons from seeds from fermentations from the 1st and 2nd year as the non-cleaned box (grey line; 'Almirante' farm) or as the cleaned box (black line; 'Leão de Ouro' farm). The samples were collected at 0, 24, 48, 72, 96, 120, and 144 h; 168* h. 168 h was collected only for the non-cleaned box fermentation. The non-cleaned box fermentation lasted 168 h and the cleaned box fermentation 144 h.

management) during two years at the same farms and season to verify the observation consistency. Both farms were close (*ca.* 17 km), and the microbiota tends to be similar in the same region (Serra et al., 2019). There was climatic variation between years, with the 2nd year showing a dry spell. The reduction in the pulp quality in the 2nd year can be explained by the reduction of the rainfall in May and June (Supplementary Material - Figure S1). The carbohydrate levels in the cotyledons was similar between years, and the seed components displayed little variation under stress conditions (Niether et al., 2017).

According to the initial and final seed temperature and pH parameters, all fermentations can be considered adequate (Vuyst & Weckx, 2016). Both types of fermentation displayed similar species in the pulp. *Wickerhamomyces anomalus* (syn. *Candida pelliculosa*, *Pichia anomala*, *Hansenula anomala*) was the most frequent in the fermentations. There are reports of the low frequency occurrence of *W. anomalus* in fermentations in Brazil and Ivory Coast, but the species is important for the formation of cocoa flavor (Illeghems et al., 2012; Koné et al., 2016; Papalexandratou et al., 2011). The most common yeast species reported in cacao seed fermentations are *Hanseniaspora* spp., *H. uvarum*, *H. opuntiae*, *S. cerevisiae* and *P. kudriavzevii* (Papalexandratou et al., 2011; Illeghems et al., 2012).

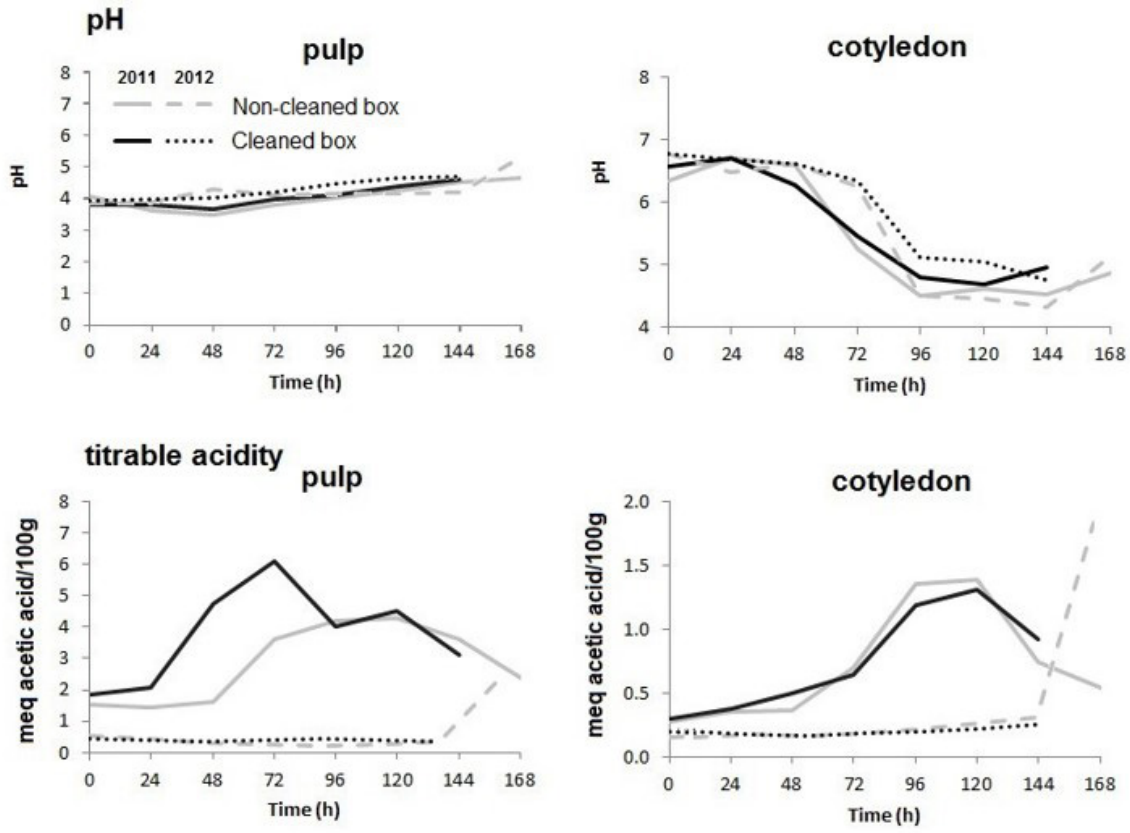

Figure 5. pH and titratable acidity detected in the fermenting cacao seed pulp and cotyledons from fermentations from the non-cleaned box (grey line; 'Almirante' farm) or from the cleaned box (black line; 'Leão de Ouro' farm). The samples were collected at 0, 24, 48, 72, 96, 120, and 144 h; 168* h. 168 h was collected only for the non-cleaned box fermentation. The non-cleaned box fermentation lasted 168 h and cleaned box fermentation 144 h.

Although *S. cerevisiae* is more representative of the species identified in the non-cleaned fermentation box, the dominance of *W. anomalus* in our cacao seed fermentations might be attributed to the killer toxin produced by *W. anomalus* that can inhibit the growth of *S. cerevisiae* (Fredlund et al., 2002), and it might contribute to its dominance during the fermentation. The adequate fermentation performed by *W. anomalus* make it a potential candidate to be used as an initial inoculum to standardize the final chocolate flavor, as occurred in the wine industry (Izquierdo Cañas et al., 2014).

The number and diversity of isolated yeast species from the cleaned box was smaller than those from non-cleaned box (three from the cleaned box and10 from the non-cleaned box). Probably, the restriction imposed by box cleaning the reminiscent yeast inoculum seemed to cause a change in the yeast species frequencies. Cleaning the fermentation box might be the cause of the reduction of frequency of *W. anomalus* (33.8%), favoring the local diversity of the inoculum (Izquierdo Cañas et al., 2014; Vuyst & Weckx, 2016) and making the yeast species composition in the fermentation more heterogeneous. The use of non-cleaned fermentation boxes might favor the selection of yeasts more adapted to the process, acting as a source of inoculum and promoting a more uniform fermentation. Further, the presence of a resident inoculum, adapted to local fermentation could determine geographic identity of the fermented seeds (Ferreira, 2017; Gutierrez et al., 2022). Conversely, non-cleaned boxes can be a deterrent for using a starter yeast inoculum.

Cleaning the fermentation box probably reduced the density of the yeast inoculum and it resulted in a smaller yeast count during the fermentation. The reduction in the density of the natural inoculum by fermentation box cleaning caused a delay in rising of the seed-mass temperature. Fermentation carried out in cleaned boxes reached the temperature to allow seed-mass turning later than fermentations in non-cleaned boxes. Fermentation conducted in the cleaned box in the 2nd year showed a slower rise in temperature, with a different temperature rising pattern from the previous year.

The poor pulp quality in the 2nd year amplified the differences between the fermentation practices. Pulp quality is essential for yeast growth (Afoakwa et al., 2008; Ho et al., 2015; Lefeber et al., 2010; Meersman et al., 2013). The level of carbohydrates is affected by environmental factors (Vuyst & Weckx, 2016), such as the limited rainfall that occurred in the months previous to pod harvest in the 2nd year.

The poor pulp quality was enough to maintain the heating ramp (35 to 40 °C in 48 h) and to reach the maximum temperature (50 °C), attesting the occurrence of an adequate fermentation

(Vuyst & Weckx, 2016). However, the practice of seed-mass turning for aeration was compromised, being possible only once at the end of the fermentation. Turning is a practice that accelerates fermentation and can influence the final quality of the chocolate, especially in fermentations with large seed mass (Camu et al., 2008; Hamdouche et al., 2019). Thus, the reduction in inoculum size by box cleaning presents a negative aspect, mainly in fermentations using depulped seeds and pulp with lower sugar levels.

In the 1st year, the cleaning anticipated the peak of yeast count, and consequently, the ethanol and acetic acid production. Even though the yeast population was smaller, there was more production of ethanol. Thus, cleaning the fermentation box might have reduced the inoculum of less efficient yeast, leaving less inoculum, but potentially with more ability for ethanol production. A similar change in ethanol production was reported for a yeast population in an aseptic fermentation conducted in stainless steel tank (Pereira et al., 2013).

The low-quality substrate for the fermentations in the 2nd year may have determined the absence of the microbial peak counts in both fermentations, and the lower production of ethanol in the pulp. Despite the absence of the peak in yeast-count, ethanol was produced in the pulp demonstrating the increase in yeast metabolic activity. Again, the ethanol production was anticipated in the clean box fermentation. However, the ethanol contents in the pulp and the moment of detection in the cotyledons were not associated with the contents in the pulp for all the fermentations.

In all the fermentations reported here, the acetic acid levels in the pulp and cotyledons varied widely between years, but the final pH of the cotyledons was surprisingly similar among them. The uptake of acetic acid produced by AAB is known to reduce the cotyledon pH from the initial 6.3–7.0 to 4.0–5.5 (Vuyst & Weckx, 2016). The timing of pH reduction was different between years, but the first detection of acetic acid and acidification of the cotyledons occurred at the same time, at 96 h. Probably, the intrinsic characteristics of the seeds determine the moment of acetic acid penetration into the cotyledons (Vuyst & Weckx, 2016).

The pH reduction in the 1st year started at 72 h, and in the 2nd year at 96 h. The periods of pH reduction coincided with the rising of the seed mass temperature above 40 °C. The temperature appears to affect linearly the pH response of the cotyledons (John et al., 2020). Perhaps, the direct inverse association between pH and temperature in the fermentations can explain the observed cotyledon pH values. The fermentation that took longer to reach the maximum temperature showed a higher pH between 96 h and 144 h, despite the accumulation of acetic acid in the cotyledons. The reduction in pH is associated with the decrease in seed viability (Nagel et al., 2019), and increasing temperature is recognized as one of the factors that cause the death of the cacao seed during fermentation.

Despite the variation in the timing of pH and temperature changes, the degradation of seed carbohydrates was similar between fermentations. The acid and enzymatic hydrolysis of sucrose and proteins in the cotyledons, generating reducing sugars, amino acids, and oligopeptides, is affect by pH levels (Amin et al., 1997; Sousa et al., 2016; Thompson et al., 2001; Voigt et al., 2018). The pattern of carbohydrate degradation observed was similar to other cacao seed fermentations reported (Crafack et al., 2013).

Although the time-interval with low cotyledon pH varied between years, the degradation of seed carbohydrates was similar. The longest time-interval with low cotyledon pH improves the sucrose and protein degradation (Biehl & Voigt, 1999; Thompson et al., 2001). Invertases have constitutive activity that is interrupted when fermentation temperature reaches 50 °C (Voigt & Lieberei, 2014). The stability in the degradation of carbohydrates suggests the importance of the physiology and genetics of the seed (Mejía et al., 2021) in the formation of flavor precursors (Ramos et al., 2014). Therefore, the intrinsic seed physiology might partially contribute to flavor development as it affects the pH levels and carbohydrate degradation in cotyledons.

The models for cacao seed fermentation consider two types of variables (López-Pérez et al., 2018; Moreno-Zambrano et al., 2018): the state variables (levels of glucose, fructose, ethanol, lactic acid, acetic acid) and the microbial population size (yeasts, AAB, LAB).

Here, the box cleaning practice limited the initial density of yeast population, and it turned the process less resilient to external conditions, like climatic conditions that affect levels of glucose and fructose in the seed pulp. Therefore, the practice of cleaning the fermentation box for standardization of fermentation must be followed by inoculation with a starter culture to assure the stability for the fermentation, particularly upon seasonal conditions that reduce the quality of the seed pulp.

Before adopting the use of cleaning fermentation boxes, it should be considered the quality and amount of available cacao seed pulp or the use of inoculum complementation to avoid limitations in the fermentations.

5 Conclusion

The initial reduced inoculum derived from cleaning the boxes impaired fermentation.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Funding

This work was supported by the Brazilian National Council for Scientific and Technological Development (CNPq, Portuguese: Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq 560710/2010-0 and 310645/2021-2).

Acknowledgements

We would like to thank the staff from the farms 'Almirante' and 'Leão de Ouro' for the dedicated assistance. The support from the Mars Center for Cocoa Sciences in conducting the experiment and the physicochemical analysis was greatly appreciated.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. Rainfall data (mm) at the Ilhéus station in Southern Bahia, Brazil from January to December 2011 and 2012. Data retrieved from the weather database from the 'Instituto Nacional de Climatologia' (http://www.inmet.gov.br) from the automatic station A410, Ilhéus, Bahia, Brazil.

This material is available as part of the online article from https://doi.org/10.1590/fst.109322