

Fermentation of cocoa bean with addition of lactic acid bacteria

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ABSTRACT: Natural fermentation of cocoa beans is a complex process and thanks to wild microflora in the materials, mainly yeasts, lactic acid bacteria, acetic acid bacteria and others. Addition of some useful microorganisms such as yeasts and/or certain bacterial species has showed to be very promising to improve the fermented bean quality for chocolate production. This research was to study the effect of adding naturally isolated lactic acid bacteria during cocoa fermentation in small boxes on the bean quality. A lactic acid bacteria product containing at least 2.3×10^7 CFU of *Lactobacillus fermentum* per g was used at different ratios to the bean mass, namely 0% (for comparison), 2%, 4% and 6% (w/w) from start; and/or 2% after 24-hour intervals of fermentation, which made 16 different regimes all together. Fermentation was last up to 7 days and mixing of the beans was done after 48 and 96 hours of fermentation. Samples were taken every 24 hours for enumeration of yeasts, lactic acid bacteria, and acetic acid bacteria and for pH measurements. A cut test was performed on fermented beans (after being sun dried) for their quality assessment. It was found that the ratios of bacteria product used and adding times significantly influenced the quality of fermented beans. The samples added 6% bacteria product at start and 2% bacteria at day 2, day 3 and day 4 of fermentation yielded the best results. In these samples, the counts of lactic acid bacteria and acetic acid bacteria at the end of fermentation were also the highest in comparison with other samples. The results showed that addition of lactic acid bacteria at certain concentrations and time intervals gave positive effect on the bean quality.

Keywords: Cocoa; fermentation; lactic acid bacteria

Introduction

Natural fermentation of cocoa beans is a complex process and thanks to wild microflora in the materials. During fermentation, microorganisms remove the pulp surrounding the fresh beans and produce indispensable metabolites (Schwan and Wheals, 2004). The microbial succession in the fermentation process has been well understood (Schwan et al., 1995). Yeasts, dominating the fermentation for the first 24 h, are responsible for pectin depolymerisation, anaerobic fermentation of sugars to ethanol, production of organic acids and some volatile organic compounds. Then, yeasts are briefly eclipsed by lactic acid bacteria

(LAB), which ferment sugars and citric acid to lactic acid, acetic acid, glycerol and mannitol. As the pulp disappears, with the penetration of oxygen into the bean mass acetic acid bacteria (AAB) will dominate and be responsible for the aerobic exothermic conversion of ethanol into acetic acid (Camu et al., 2008a). These microbial activities lead to the death of the bean since metabolites, mainly ethanol and acetic acid, penetrate through the husk into the cotyledons; internal pH drops from 6.5 to 4.8; bean temperature rises up to 50 °C; and internal cocoa bean structure is damaged, resulting in cocoa flavour precursor development and pigment degradation by endogenous enzymes (Camu et al.,

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2008a). At the end of the process, aerobic spore-forming bacteria (e.g. *Bacillus* strains) and filamentous fungi may appear, producing a variety of chemical and lactic acid, which may contribute to the acidity and off-flavors of fermented cocoa beans (Camu et al., 2008a; Schwan, 1998).

Fermentations are normally carried out at farms where great variation in fermentation techniques among farmers leads to the lack of uniformity of fermented bean quality, e.g. problems of acidity or lack of cocoa flavor due to underfermentation or off-flavors due to overfermentation and spoilage of beans (Schwan, 1998). The similar problems are faced in Vietnam (Goletti, 2008). A large number of microorganism species has been isolated from natural cocoa fermentation process, however, not all of them are likely to be really essential (Schwan, 1998). There have been several studies to use microbial starter to better control the cocoa fermentation process to stabilize or improve the fermented bean quality. Samah et al. (1992) successfully used an inoculate of wild strain *Saccharomyces cerevisiae* to yield good cocoa fermentation (Samah et al., 1992). Schwan (1998) showed that a mix culture of predominating microorganisms of yeasts (*Saccharomyces cerevisiae* var. *chevalieri*), LAB (*Lactobacillus lactis* and *Lactobacillus plantarum*) and AAB (*Acetobacter aceti* and *Gluconobacter oxydans* subsp. *suboxydans*) isolated from natural fermentation of cocoa mass could be used at the beginning of fermentation process to replace wild microflora in order to stabilize and improve the quality of fermented beans (Schwan, 1998). Leal et al. (2008) successfully used a hybrid *Kluyveromyces*

marxianus yeast strain with high exogenous pectinolytic activity to improve product quality due to increase of liquid draining, improved seed protein degradation, and reduction of titrable acidity (Leal et al., 2008). Lefeber et al. (2011) tested cocoa-specific strains of *Lactobacillus fermentum* in simulated cocoa pulp fermentation and indicated that LAB strains were important compositions of starter cultures for controlling fermentation processes to obtain good quality of fermented cocoa beans (Lefeber et al., 2011). This work was to study the application of a LAB dried product of *Lactobacillus fermentum*, isolated from natural cocoa fermentation, to ferment the cocoa mass in small boxes.

Materials and methods

Materials

Cocoa pods were purchased from Ben Tre province (Vietnam), only matured yellow or red pods of different clones (most popular TD3 and TD5) were selected and all damaged and diseased individuals were eliminated. Each pod was about 0.5-0.8 kg. Pods were then transported to the laboratories of Nha Trang University in Khanh Hoa province by car and used for fermentation within 7 days of harvest. The pods were broken open manually using stainless steel knives, the cocoa mass was placed in 10-kg wooden boxes with holes at the bottom to allow drainage of sweatings from the fermentation process.

A LAB product containing at least 2.3×10^7 CFU of *Lactobacillus fermentum* per g, previously isolated from natural fermentation (Loc et al., 2013), was used for this study.

Fermentation of cocoa mass

Fermentation was carried out in above described wooden boxes. A LAB product was added at different ratios to the bean mass, namely 0% (for comparison), 2%, 4% and 6%

(w/w) from start; and/or 2% after 24-hour intervals of fermentation, which made 16 different regimes all together (Table 1). Fermentation was last up to 7 days and mixing of the beans was done after 48 and 96 hours of fermentation.

Table 1 Ratio of LAB product (%) added at different time intervals during fermentation

Regime	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Day 1	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6	0
Day 2	0	0	0	2	2	2	2	2	2	2	2	2	2	2	2	0
Day 3	0	0	0	0	0	0	2	2	2	2	2	2	2	2	2	0
Day 4	0	0	0	0	0	0	0	0	0	2	2	2	2	2	2	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Enumeration of microflora and cut test

Samples of 250 g of beans were aseptically scooped from the center of the cocoa mass every 24 hours. They were then mixed with 250 ml of 0.1% peptone water (Oxoid) in a Stomacher bag and vigorously shaken to have a uniform homogenate. Samples of 1.0 ml of the homogenate were serially diluted in 0.1% peptone water. Their aliquots of 0.1 ml were spread in duplicate over the surface of plates with specific media for the isolation and enumeration of target organisms.

Medium for yeast isolations was MEA (Malt Extract Agar, Half Strength, ATCC Medium 2418). Compositions of 1 litre were Agar 15 g, Malt Extract 10 g, pepton 2.5 g, chloramphenicol 100 mg/l, chlortetracycline 50 mg/l (Loc et al., 2013; Loc, 2012). Yeasts were identified based on the morphology, physiology, fermentation and assimilation properties (Ardhana and Fleet, 2003).

LAB were isolated using MRS agar (Merck), incubated at 30 °C for 3-4 days. LAB were

identified according to cell and colony morphology, Gram, catalase and oxidase reactions, gas production from glucose, growth at pH 3.0–5.5, growth at 15–47 °C (Ardhana and Fleet, 2003).

AAB were isolated by Glucose Yeast Extract Calcium Carbonate Agar (GYC Agar) containing glucose 50g/l, yeast extract 10g/l, calcium carbonate 30 g/l, agar 20 g/l, with 100 mg/l of cycloheximide at 25 °C for 5-8 days [11]. AAB were identified by standard methods (Schwan, 1998).

Cut test: Samples of 300 fermented beans (after being sun dried) were cut lengthwise through the middle. The cotyledon surface was examined in full daylight and beans were categorised into the following groups: fully brown (fermented); partly brown; partly purple (partly fermented); purple (underfermented); slaty (not fermented); insect damaged; moldy; or germinated (Schwan, 1998; Camu et al., 2008b).

Results and discussion

Quality of fermentented beans evaluated by cut test

It can be seen from **Figure 1**, showing the results for fermented beans, that adding of LAB product at different ratios and different time intervals to the bean mass has affected the fermented

product quality. Control sample (with no LAB addition) and the sample with only 2% LAB added at the beginning yield the worst cut test quality compared to other samples. Sample 12, i.e. the one added with 6% LAB product at start and 2% bacteria at day 2, day 3 and day 4 of fermentation gave the highest quantity of fully fermented beans (98.43%).

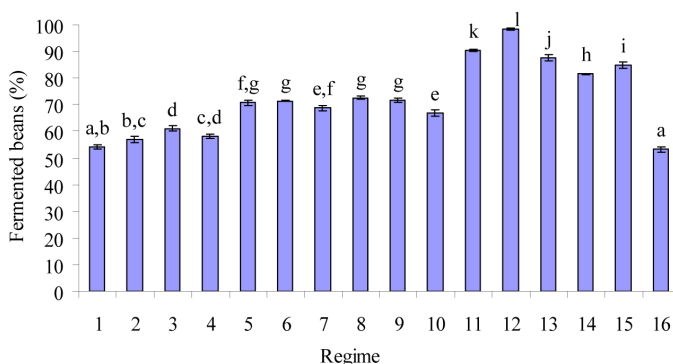
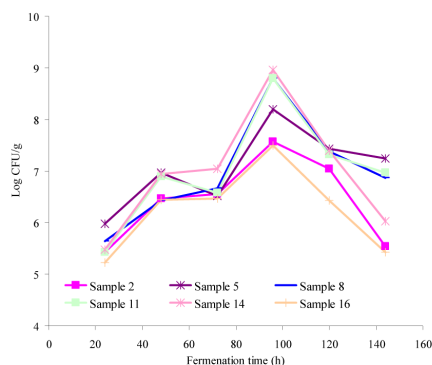
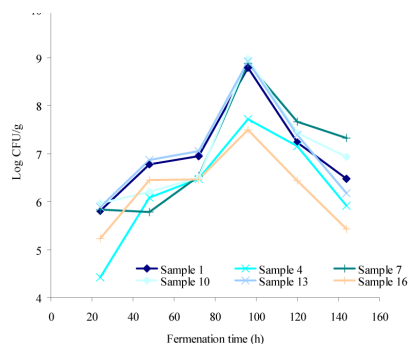


Figure 1 Quantity of fermented beans (%) at the 16 studied regimes. Different letters indicate significant differences ($p < 0.05$) of the samples

Enumeration of yeasts during fermentation

The changes of yeast counts during 144 hours of fermentation are illustrated in **Figure 2**. It was observed that the counts in all the samples raised from start and reached their maximal values after 96 hours (up to 8.97 log CFU/g), and then dropped to below 7.49 log CFU/g. Interestingly, sample 16 with no LAB addition had the lowest yeast counts at the end of the fermentation process. Generally, addition of LAB did not affect the course of the yeast evolution in the bean mass. This result is in good agreement with other studies that yeasts are dominating at the beginning of the fermentation and their number decreases at the end of the process (Camu, et al., 2008b).



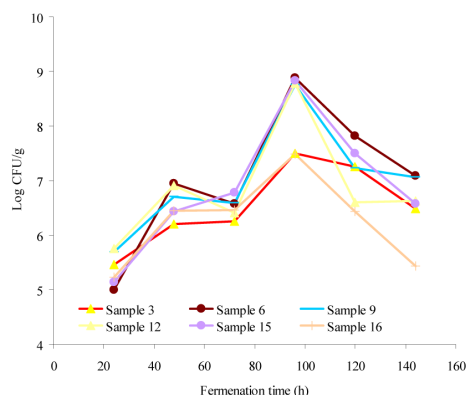


Figure 2 Evolution of yeasts in the cocoa mass during fermentation of the 16 studied regimes

Enumeration of LAB during fermentation

The changes of LAB density during 144 hours of fermentation are illustrated in **Figure 3**. It can be seen that the LAB counts in all the samples, except for sample 1, reached their peaks after 96 hours (up to 8.70 log CFU/g), and then decreased to below 7.12 log CFU/g at the end of the fermentation process. The control sample (sample 16) with no LAB addition had the lowest yeast counts after 7 days (4.12 log CFU/g). Samples 12, 11, and 13 had the highest end counts (in descending order). When comparing this result with the quality from the cut test, it revealed that those samples (samples 12, 11, and 13) with the highest LAB end counts also had the highest quality at the same time (**Figure 1**). This indicates that LAB supplement to the cocoa mass at different time intervals of the fermentation affected the quality of the fermented beans. It is understood because LAB play an important role in fermentation of sugars and citric acid to lactic acid, acetic acid and mannitol (Camu, et al., 2008a; Schwan, 1998).

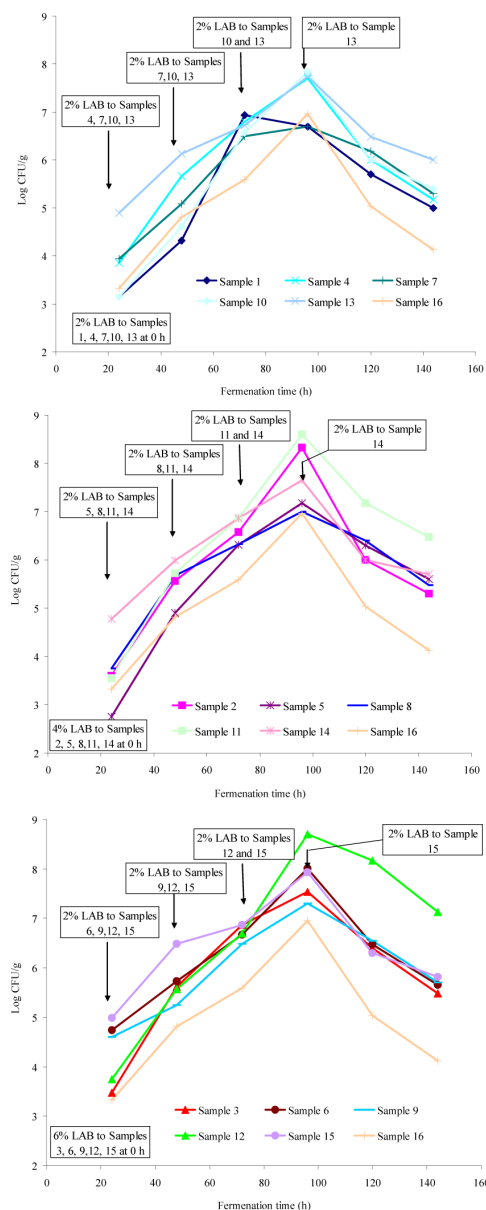


Figure 3 Evolution of LAB in the cocoa mass during fermentation of the 16 studied regimes

Enumeration of AAB during fermentation

Figure 4 shows the evolution of AAB in the bean mass by fermentation time. Similar to yeasts and LAB, AAB in all 16 samples increased with

time and reached maximum after 96 hours, then the AAB counts decreased till the end of the fermentation. However, in the samples with no addition or with one-time addition of LAB at the fermentation start, AAB counts were the lowest at the end of the process, e.g. 4.46 log CFU/g, 5.30 log CFU/g, 5.00 log CFU/g, and 5.54 log CFU/g in samples 16, 1, 2, and 3, respectively, corresponding to the poorest quality of the fermented products (Figure 1). The samples with the highest

end AAB counts, namely samples 12, 11 and 13, were also those with the best fermented bean quality (Figure 1). All these indicate that addition of LAB influenced on the AAB population, which were responsible for producing acetic acid from ethanol (Camu, et al., 2008a; Schwan, 1998). Consequently, supplementation of LAB at different ratios and at different time points affected the quality of fermented beans.

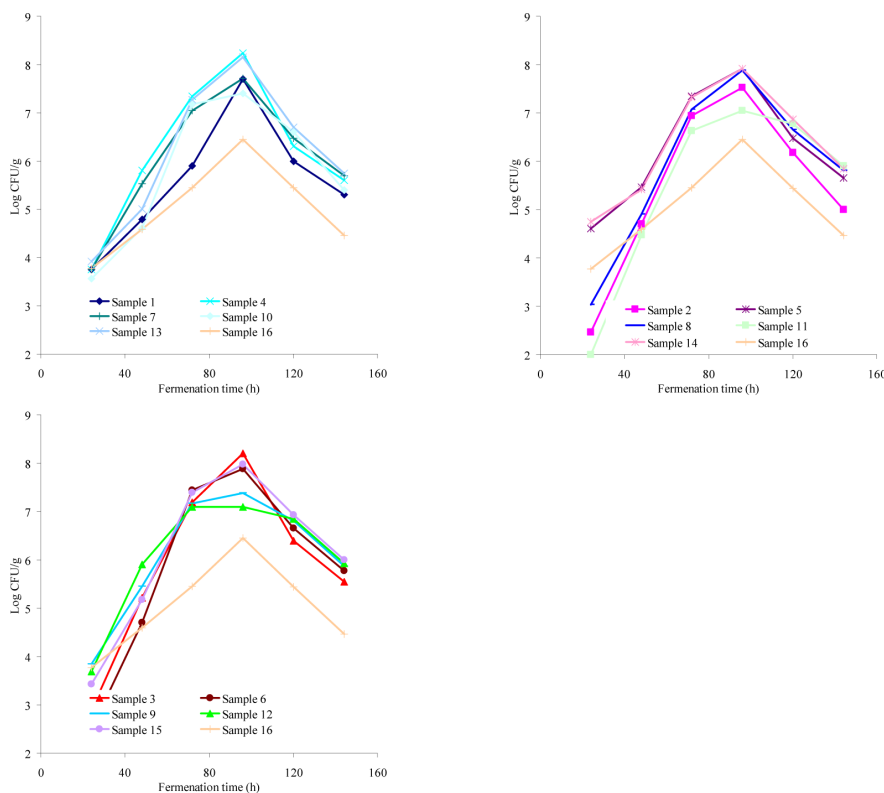


Figure 4 Evolution of AAB in the cocoa mass during fermentation of the 16 studied regimes

To summarise, the ratios of LAB used and adding times significantly influenced the quality of fermented beans. The samples added 6% LAB product at start and 2% at day 2, day 3 and day 4 of fermentation yielded the best results. In these

samples, the counts of LAB and AAB at the end of fermentation were also the highest in comparison with other samples. Addition of LAB at certain concentrations and time intervals gave positive effect on the bean quality

References

- Ardhana, M. M., and G. H. Fleet. 2003. The microbial ecology of cocoa bean fermentations in Indonesia. *Int. J. Food Microbiol.* 86: 87-99.
- Camu, N., T. D. Winter, S. K. Addo, J. S. Takrama, H. Bernaert, L. D. Vuyst. 2008a. Fermentation of cocoa beans: influence of microbial activities and polyphenol concentrations on the flavour of chocolate. *J. Sci. Food Agr.* 88: 2288–2297.
- Camu, N., A. González, T. D. Winter, A. V. Schoor, K. D. Bruyne, P. Vandamme, J. S. Takrama, S. K. Addo, L. D. Vuyst. 2008b. Influence of Turning and Environmental Contamination on the Dynamics of Populations of Lactic Acid and Acetic Acid Bacteria Involved in Spontaneous Cocoa Bean Heap Fermentation in Ghana. *Appl Environ Microbiol.* 74(1): 86–98.
- Goletti, F. 2008. Study on suitability, feasibility, and socioeconomic benefits of cocoa production in Vietnam. Final report of Agrifood Consulting International for Ministry of agriculture and Rural Development of Vietnam; 2008. 286 p.
- Leal, J. G. A., L. H. Gomes, P. Efraim, C. A. F. Tavares, and A. Figueira. 2008. Fermentation of cacao (*Theobroma cacao* L.) seeds with a hybrid *Kluyveromyces marxianus* strain improved product quality attributes. *FEMS Yeast Res.* 8: 788–798.
- Lefeber, T., M. Janssens, F. Moens, W. Gobert, and L. D. Vuys. 2011. Interesting Starter Culture Strains for Controlled Cocoa Bean Fermentation Revealed by Simulated Cocoa Pulp Fermentations of Cocoa-Specific Lactic Acid Bacteria. *Appl. Environ. Microbiol.* 77: 6694-6698.
- Loc, V. T. M. 2012. Isolation main microflora species from natural cocoa fermentation in Vietnam. Master thesis in Post-harvest Technology, Nha Trang University, Vietnam, 2012.
- Loc, V. T. M., M. T. T. Nga, and T. M. Nguyen. 2013. Changes in the number of lactic bacteria in natural fermentation of cocoa and selection of lactic bacterial strain suitable for cocoa fermentation. *J. Fish. Sci. Technol.* 2: 118-122.
- Samah, O. A., M. F. Ptih, and J. Selamat. 1992. Biochemical changes during fermentation of cocoa beans inoculated with *Saccharomyces cerevisiae* (wild strain). *J. Food Sci. Technol.* 29: 341–343.
- Schwan, R. F. 1998. Cocoa Fermentations Conducted with a Defined Microbial Cocktail Inoculum. *Appl. Environ. Microbiol.* 64(4): 1477–1483.
- Schwan, R. F., and A. E. Wheals. 2004. The microbiology of cocoa fermentation and its role in chocolate quality. *Crit. Rev. Food Sci. Nutr.* 44: 205–221.
- Schwan, R.F., R. F. A. H. Rose, and R. G. Board. 1995. Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. *J. Appl. Bacteriol. Symp. Suppl.* 79: 96S–107S.