

# MAPPING THE EFFECTS OF STARTER CULTURE ADDITION ON COCOA BEAN FERMENTATION

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## Abstract

Fermentation is an essential step in the formation of aroma and flavour of cocoa beans. During the fermentation, cocoa bean pulp is degraded to form metabolic products, one of which functions as the precursor for the aroma and flavour of cocoa beans. Previous research suggested that the addition of starter culture on the cocoa bean fermentation process can improve the fermentation performance. In this research the effects of various starters culture combinations, including *Saccharomyces cerevisiae* var. Chevalieri, *Lactobacillus plantarum*, and *Acetobacter aceti* were studied. The addition of starter culture was confirmed by analysis of microbial population during the fermentation. Fermentation performances were measured as fermentation index and cocoa bean pH, as well as sugar and metabolic product concentration. The obtained results showed that in general the addition of starter culture accelerated sugar consumption and increased metabolites production. The highest fermentation index was  $1.38 \pm 0.07$ , obtained from fermentation added with starter culture combination containing *Saccharomyces cerevisiae* var. Chevalieri and *Lactobacillus plantarum*. This fermentation advanced faster and was completed at 4 days, or shortened the overall fermentation time. On the other hand fermentation added with starter culture containing only *Acetobacter aceti* gave the lowest fermentation index,  $0.96 \pm 0.04$ .

**Keywords:** Cocoa bean pulp, Fermentation, Metabolite, Microbial population, Starter culture

## Introduction

Cocoa (*Theobroma cacao*, L.) is an important agricultural commodity for Indonesian national economy. Cocoa becomes a source of foreign exchange, regional development, driving the development of agribusiness and agro-industry. As a high commercial commodity, quality is a very important factor in world cocoa market competition. Therefore, processing facilities, quality control and the application of technology in all phases of the processing cocoa beans must be considered. However, post-harvest processing of cocoa in particular at the farm level has not been properly done: cocoa beans are often found mixed with foreign substances, are not properly dried, and mostly unfermented. Thereby in the international market, Indonesian cocoa beans are often priced low and even imposed discounts.

Fermentation is such an essential step in the cocoa bean processing. During the fermentation, the mucilaginous pulp surrounding the cocoa bean is removed. Microbial activities degrade the the sugar-rich cocoa bean pulp into metabolic products, such as ethanol, lactic acid, and acetic acid. The heat released from this process and the diffusion of metabolic products into the beans put the bean germination into halt. Further the metabolic products also serve as precursors for the aroma and flavour of cocoa beans. The overall process takes around 5 – 7 days to complete and it is considered too long. Regardless of the quality, farmers tend to sell their cocoa beans unfermented. In order to remove the pulp, the beans are soaked in the water before being dried. To improve the

quality of Indonesian cocoa beans, it is necessary to develop a simple, rapid but also of high quality, cocoa bean fermentation method that can be implemented by farmers.

Cocoa bean fermentation is usually initiated by indigenous microorganisms that naturally present in the cocoa bean pulp including yeast, lactic acid bacteria and acetic acid, bacilli, and fungi [1]. Schwan *et al.* found more than 40 species of microbes that grow during fermentation of cocoa [2]. The dominant yeast species is *Saccharomyces cerevisiae* and *Candida tropicalis* with a high survival rate,  $10^7$  cfu/g during 36 hours [3]. Typical lactic acid bacteria significantly present during the cocoa bean fermentation process is *Lactobacillus plantarum*. While typical acetic acid bacteria actively presents in the first 24 hours of fermentation is *Acetobacter aceti*. But not all of these microbes play an important role in the fermentation. It needs to be selected against microbes that have a major role in the formation of aroma, colour, flavour and chemical components of cocoa beans.

The addition of starter culture to cocoa bean fermentation may modified microbial composition and thus improving the quality of fermentation [4]. Cempaka *et al.* [5] and Kresnowati *et al.* [6] suggested the addition of a starter *S.cerevisiae* var. Chevalieri and *Lactobacillus plantarum*, separately, can improve the fermentation index or in other words, can shorten the fermentation time. *S.cerevisiae* var. Chevalieri was chosen in particular, considering its pectinolytic activity that may improve pulp degradation [2]. Leal *et al.* evaluated *Kluyveromyces marxianus*, another pectinolytic yeast, as starter culture and showed that resulted cocoa bean had better sensorial evaluation [7]. The use of mixed starter culture containing yeast, LAB, and AAB was suggested to obtained high quality cocoa bean fermentation [8]. Indeed Kustyawati and Setyani showed that the addition of starter mixture consisting of dominant microorganisms comprised of *Saccharomyces cerevisiae*, *Acetobacter aceti* and *Lactobacillus lactis* in the early stages of fermentation (day 0) can optimize the fermentation process [9]. Lefeber showed that the addition of *S.cerevisiae*, *L.fermentum* and *A.pasteurianus* as mix starter culture gave better and reliable flavour [4].

It is the aim of this research to map the effectiveness of various combination of mixed starter culture addition containing *S.cerevisiae* var. *Chevalieri*, *L.plantarum* and *A.aceti* to the cocoa bean fermentation quality. Interaction between the added species would be tested and further their impact on the dynamic profiles of sugary pulp compositions, and primary metabolic products, pH and the fermentation index were studied.

## Materials and Methods

### Cocoa Bean Fermentation

Cocoa beans were obtained from PT Bajabang, West Java, Indonesia. Lab scale cocoa bean fermentations were performed as such to mimic the real cocoa bean fermentation in the farm, following [6] and [10]. The fermentation was conducted in 250 mL erlenmeyer, each contained 20 gram of cocoa beans. The fermentation temperature was controlled by as such to follow the increasing temperature profile of the cocoa bean fermentation: at fermentation time of 0-24 hour at 30°C, 24-48 hour at 35°C, 48-72 hour at 40°C, and 72-120 hour at 50°C. Samples were taken every 24 hour during the 5 day fermentation and processed for the analysis of microbial composition, concentration of sugary compounds and primary metabolic products, pH as well as fermentation index. Each fermentor provided for 1 sample-time.

### Starter Culture Preparation

Microbial strains used in the starter were combinations of *Saccharomyces cerevisiae* var. Chevalieri ITBCC R63, *Lactobacillus plantarum* ITBCC 188 and *Acetobacter aceti*

ITBCC B166. Each strain was cultivated in different media. *Saccharomyces cerevisiae* var. Chevalieri was cultivated in Potato Dextrose Agar (PDA) media, *Lactobacillus plantarum* were cultivated in MRS-A media, whereas *Acetobacter aceti* was cultivated in Acetobacter Agar media. Each was incubated for 2 days before being inoculated at the beginning of the cocoa bean fermentation.  $10^3$ - $10^4$  CFU of each microbial strain, as was indicated in the experimental design, per gram cocoa beans were used for each fermentation.

### Experimental Design

A single factor experiment was conducted with the addition of starter culture as the experimental factor (6 levels of treatment and 2 replications). Experiments were performed in two batches. To take into accounts possible variations in cocoa beans used in the each batch of experiments, control experiments (K) were performed in each batch. The order in which the experiments were carried out is presented in Table 1.

**Table 1. Starter Culture Combination Used in the Experiments**

Code	Specific Treatment			Batch 1	Batch 2
	<i>S.cerevisiae</i>	<i>L.plantarum</i>	<i>A.aceti</i>		
K	-	-	-	✓	✓
R	✓			✓	
RA	✓		✓	✓	
RL	✓	✓			✓
RAL	✓	✓	✓		✓
A			✓		✓

### Data Analysis

The dynamics in microbial population was measured as the concentration of yeast, lactic acid bacteria, and acetic acid bacteria by Total Plate Count (TPC) method. The samples were spread on specific enrichment agar as indicated previously and were incubated for 24 hours at 30°C for yeast and acetic acid bacteria, and at 37°C for lactic acid bacteria, before the measurement of total colony formed.

The remaining sugars and primary metabolic products were extracted from the cocoa bean pulp and measured by HPLC following the method described in [5]. The quality of fermentation was presented as fermentation index that was measured by spectrophotometry following [11].

### Data Processing

In order to obtain a comprehensive review of the fermentation dynamics of microbial population, sugars concentration and the primary metabolic products concentration, as well as the fermentation index (IF) and bean pH were expressed as the area of the dynamic curve that was calculated as the area between the measured concentration and x-axis, within the time of observation. Pivot points of the dynamic profile of each parameter were also calculated to give an indication the time of maximum concentration.

Data of curve area and pivot points were statistically analysed by Analysis of Variance (ANOVA) followed by Tukey's test of multivariate comparison method at 95% confidence level using Minitab 16.0.

## Result and Discussion

### Batch to Batch Variations

Figure 1 presents variations in the initial concentration of sugary compounds in the cocoa bean pulp, measured as sucrose, glucose, and fructose, as well as variations in the initial microbial population in the control experiments, measured as yeast, acetic acid bacteria, and lactic acid bacteria. The initial concentration of sugary compounds in the cocoa bean pulp measured in both control experiments were significantly different (Figure 1a). Sugar concentrations in the second control experiments (K2) were significantly higher than those in the first control experiments (K1). The initial concentration of citric acid, which was reported to be significantly present in the cocoa bean pulp [3], was also measured in this experiment. However, in agreement with our previous observation [5], its level was measured to be very low and thereby its dynamics will not be discussed further. Comparable initial microbial concentrations were measured in both control experiments (Figure 1b). The observed differences in the initial pulp content may lead to different fermentation pattern.

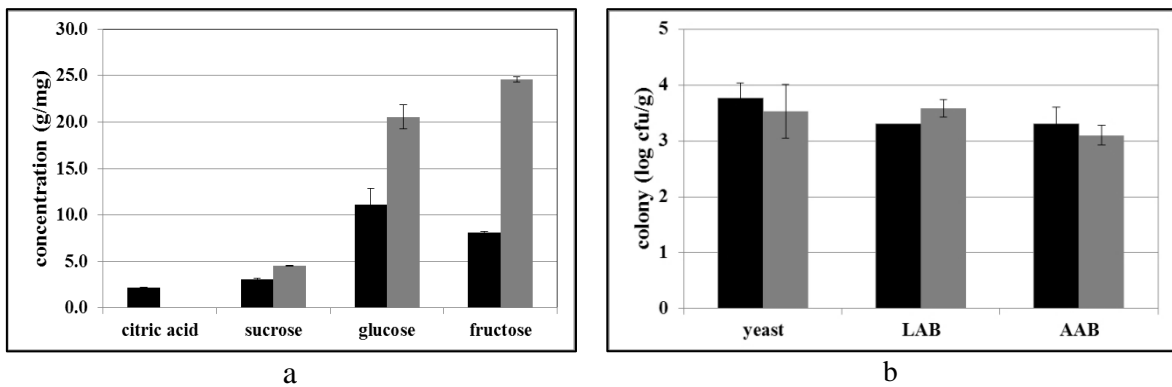


Figure 1. a. Initial concentration of sugary compounds in pulp, b. Initial concentration of microbial population (black columns represent control experiment 1 (K1) whereas grey columns represent control experiment 2 (K2))

Dynamics in microbial population during the fermentations are presented in Figure 2, whereas the associated dynamics in sugar concentrations and primary metabolic products are presented in Figure 3 and 4. The different colour used in the figures represents different batches of experiments. Indeed, statistical analysis showed that some variables i.e. lactic acid bacteria concentration, sucrose, fructose, ethanol, and acetic acid, at the two control experiments were significantly different at confidence level 90% (Table 2). Thereby it is necessary to compare the experimental results to its related control experiments.

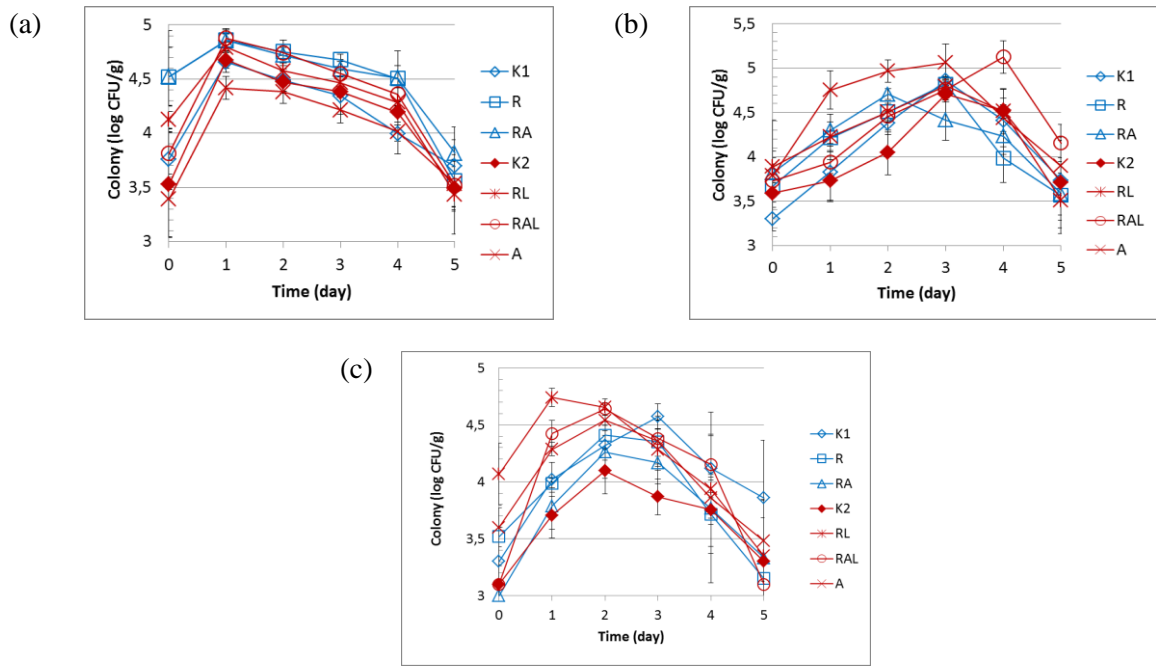


Figure 2. Dynamics in microbial population during the fermentation: a. yeast, b. LAB, c. AAB

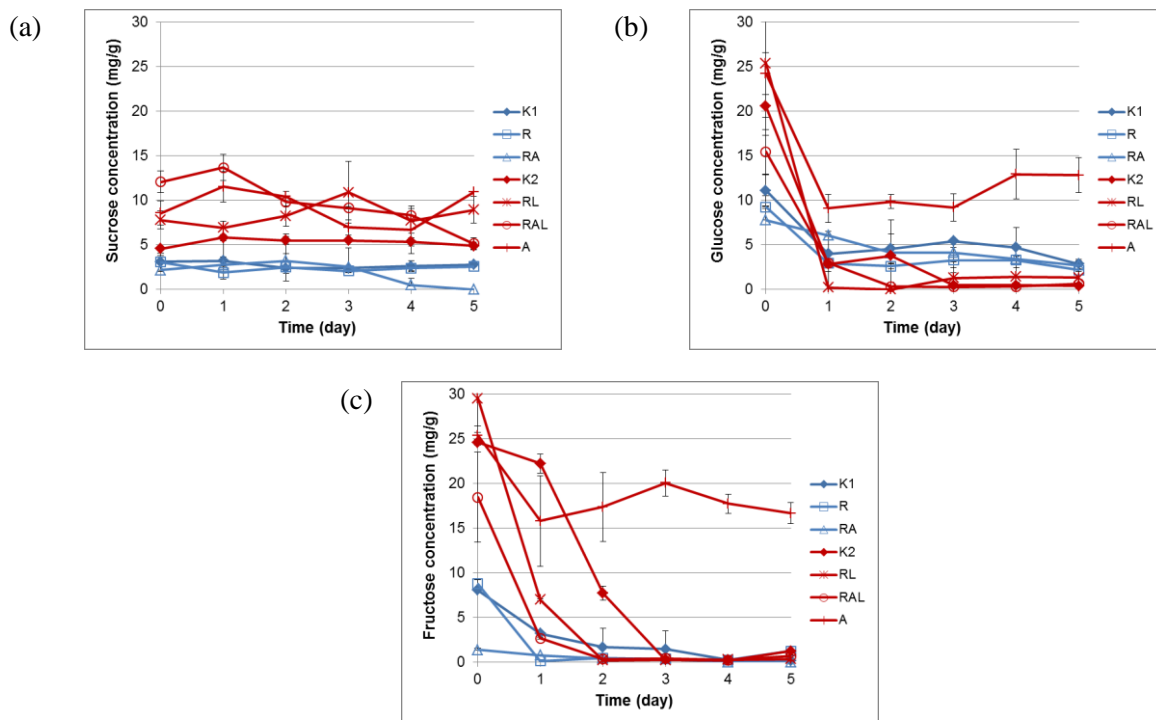


Figure 3. Dynamics in sugar concentration in cocoa pulp during the fermentation: a. Sucrose, b. Glucose, c. Fructose

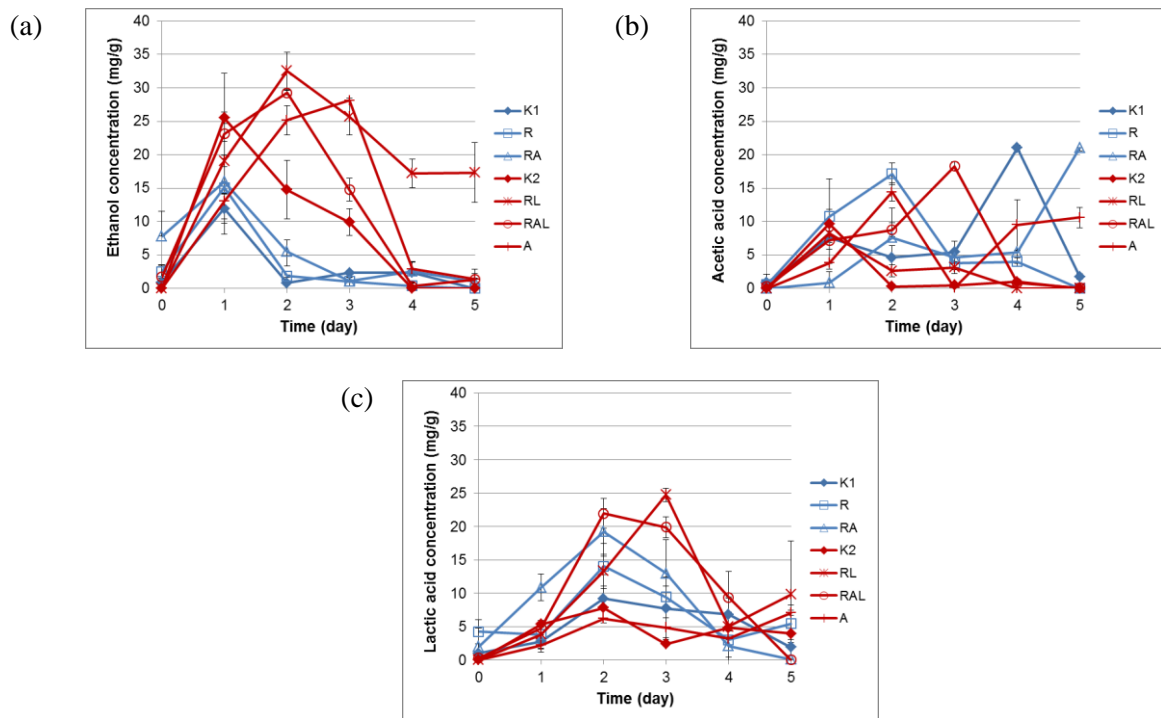


Figure 4. Dynamics in primary metabolic product concentrations during the fermentation:  
a. Ethanol, b. Acetic acid, c. Lactic acid

**Table 2. Comparison of the Two Control Experiments, Each Represents Different Batches**

Variable	Curve Area		p-value*
	Control Batch 1 (K1)	Control Batch 2 (K2)	
Yeast population (log cfu.g <sup>-1</sup> .day)	21.2±0.1	21.2±0.0	0.428
AAB population (log cfu.g <sup>-1</sup> .day)	20.1±0.2	20.7±0.5	0.703
LAB population (log cfu.g <sup>-1</sup> .day)	20.5±0.9	17.8±1.7	0.029**
Sucrose (mg.g <sup>-1</sup> .day)	13.6±0.9	26.9±1.4	0.015**
Glucose (mg.g <sup>-1</sup> .day)	25.6±2.6	18.0±1.2	0.218
Fructose (mg.g <sup>-1</sup> .day)	10.7±4.7	43.4±1.6	0.086**
Ethanol (mg.g <sup>-1</sup> .day)	17.9±2.7	50.2±5.5	0.040**
Acetic acid (mg.g <sup>-1</sup> .day)	39.9±0.4	11.4±7.1	0.100**
Lactic acid (mg.g <sup>-1</sup> .day)	27.9±16.5	22.3±0.1	0.710
Fermentation index / IF (unit IF.day)	3.9±0.0	3.9±0.1	0.560
pH of bean (unit pH. day)	33.2±0.1	33.7±0.1	0.930

\* the probability associated with a Student paired t-Test, with a two-tailed distribution.

\*\*significantly different at confident level 90%

### Dynamics of Microbial Population

Dynamics in microbial population were followed to confirm whether the addition of a starter culture increase the concentration of that particular microbial species during the fermentation and to evaluate their corresponding interaction. The addition of yeast in the starter culture increased the initial yeast concentration in the fermentation (Figure 2a) and increased the overall yeast concentration in the microbial population during the cocoa bean

fermentation (Figure 5a). Similar trends were observed for lactic acid bacteria (Figure 2b and Figure 5b) and acetic acid bacteria (Figure 2c and Figure 5c).

Yeast plays an important role in the cocoa fermentation. In particular *S. cerevisiae* var. Chevalieri has the capability of producing pectinase, the enzyme for catalysing the hydrolysis of pectin into simple sugars. Similar trends of yeast concentration were observed in all run. Yeast concentration increased from start of fermentation until day 1 and then continuously decreased until the end of fermentation (Figure 2a). However, significant difference with confidence level of 95% was measured for the overall yeast concentration between most treatments: R, RA, and RAL (Figure 5a).

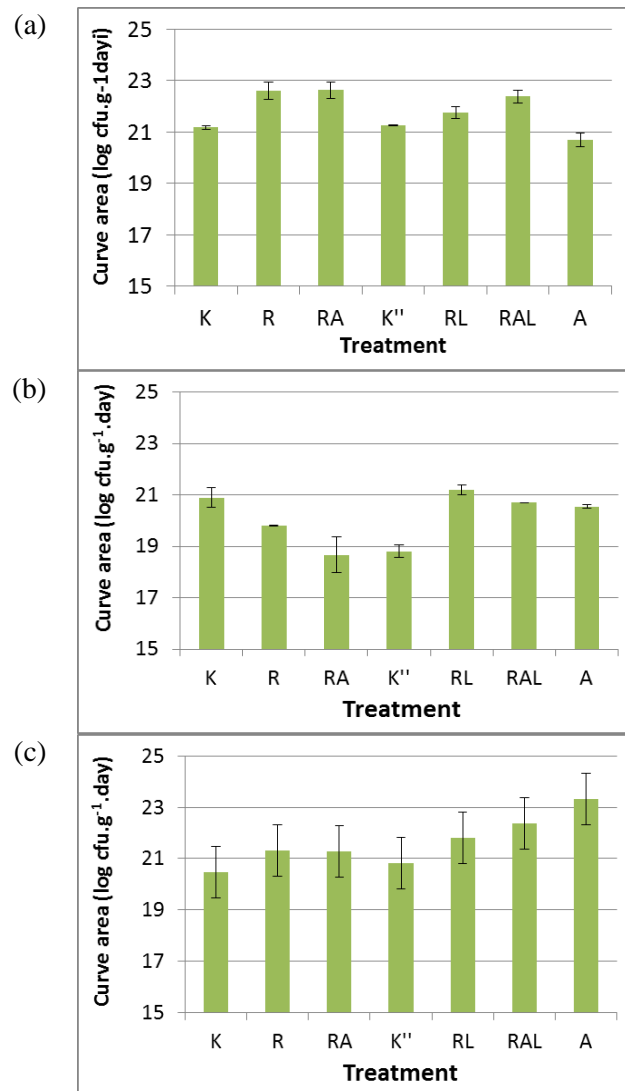


Figure 5. Effects of starter culture addition on microbial population : a. Yeast, b. LAB, c. AAB

Different trends of lactic acid bacteria concentration during the cocoa bean fermentations were also observed due to the addition of starter culture (Figure 2b). Lactic acid bacteria in the starter culture (run RL and RAL) increased the overall population of lactic acid bacteria during fermentation (Figure 5b). On the other hand, starter cultures containing yeast without lactic acid bacteria (R and RA) has negative effect on the overall population of lactic acid bacteria during fermentation (Figure 5b). Both yeast and lactic acid bacteria competed for the same substrate and the dominant yeast population in the

beginning of fermentation decreased the overall lactic acid bacteria population during the fermentation.

Different trends of acetic acid bacteria concentration during the cocoa bean fermentations were observed due to the addition of acetic acid bacteria in the starter cultures. The addition of acetic acid in the starter culture was observed to shift the maximum acetic acid concentration earlier (Figure 2c). Figure 5c shows that the addition of a monoculture starter *Acetobacter aceti* (A) resulted in the largest overall acetic acid bacteria concentration during the fermentation,  $23.3 \pm 0.4 \log \text{cfu g}^{-1} \text{day}$ .

### Sugar Reduction in Pulp

Consistent with literatures [3], fresh cacao pulp contains high concentration of sugar. During the fermentation, metabolites that are already presents in the pulp such as citric acid, sucrose, glucose, and fructose are consumed by microorganisms to grow. At the same time metabolites are produced, some of which act as the precursors for cocoa beans aroma and flavours. Various sugars metabolized by yeast into ethanol, in addition to the lactic acid bacteria also convert most of the sugar and citric acid to lactic acid and acetic acid. The performance of cocoa bean fermentation will affect the quality of fermented cocoa beans.

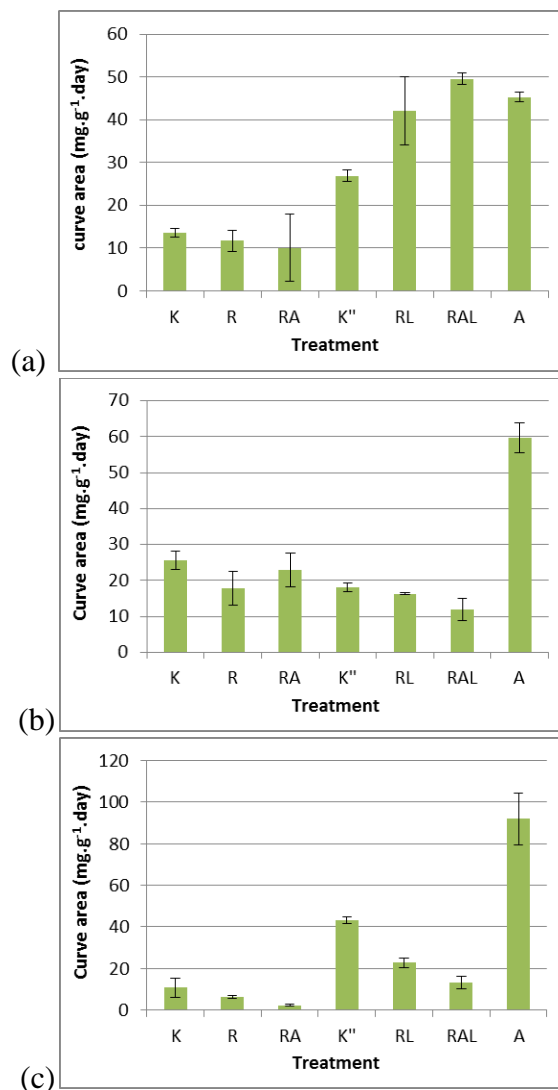


Figure 6. Effects of starter culture addition on overall sugar degradation during the cocoa bean fermentation : a. Sucrose, b. Glucose, c. Fructose



Dynamic profiles of sugar concentrations during the fermentation are shown in Figure 3. Similar trends were observed from all run, the concentration of glucose and fructose rapidly decrease from the beginning of fermentation (Figure 3b – c), whereas sucrose concentration was relatively constant during the fermentation (Figure 3a).

Figure 6 shows the overall sugar consumption/degradation during the fermentation. The lowest area indicated rapid sugar consumption, whereas high area indicated that sugar concentration was relatively constant during the fermentation. However, it is important to note that the initial sugar concentration in the first batch of experiments were significantly lower than those in the second batch of experiments (Figure 1a). Thereby, the associated area was lower than those of the second batch of experiments.

Different microbial compositions are expected to affect the cocoa bean fermentation. No significant different was observed in the sugar area of all treatments in the first batch of experiment (K1, R, and RA). Meanwhile, in the second batch of experiments (K2, RL, RAL, and A), the sugar area of treatment A, in particular glucose and fructose (Figure 6b – c), was observed to be significantly higher than others. This shows that the pulp was less degraded during the fermentation with the addition of only acetic acid bacteria in the starter culture. The acetic acid bacteria prefer to consume ethanol that is produced by yeast fermentation. On the other hand, the sugar area of treatment RL and RAL was observed to be significantly lower than others. This shows that the presence of mixed microorganisms: yeast, lactic acid bacteria, and acetic acid bacteria, in the starter culture to the fermentation, synergically improve the pulp degradation process.

### **Metabolic Formation**

During the cocoa bean fermentation various sugars are metabolized by yeast into ethanol and by lactic acid bacteria mostly into lactic acid. The produced ethanol is later consumed by acetic acid bacteria and converted into acetic acid. These metabolites will also diffuse into the bean, leading the cell death as well as the formation of various specific cocoa aroma and flavour compounds [2, 12]. The time profiles of primary metabolites observed in the experiments are shown in Figure 4.

Figure 7 shows the overall production of primary metabolites during the cocoa bean fermentation. The high area indicates that high production of those particular metabolites, despite their further consumption or diffusion into the bean. No significant difference was observed between the overall primary metabolites profile during the first batch of experiments, that are K1, R, RA (Figure 7). Unlike the results of first batch experiments, the second batch experiments, that are K2, RL, RAL, and A, gave more diverse results. Obviously the addition of lactic acid bacteria in the starter culture, treatment RL and RAL, significantly increased the overall lactic acid production (Figure 7b), whereas the addition of acetic acid bacteria in the starter culture, treatment RAL and A, significantly increased the overall acetic acid production (Figure 7c). The observed overall trend in ethanol production was less clear. The addition of yeast in the starter culture did not consequentially increase the overall production of ethanol significantly. Only at RL treatment was a significant increase in the ethanol production observed (Figure 7a). In treatment RAL, the increase in ethanol production would be directly followed by its consumption by acetic acid bacteria for acetic acid production. An increase in overall acetic acid production was observed, instead (Figure 7c).

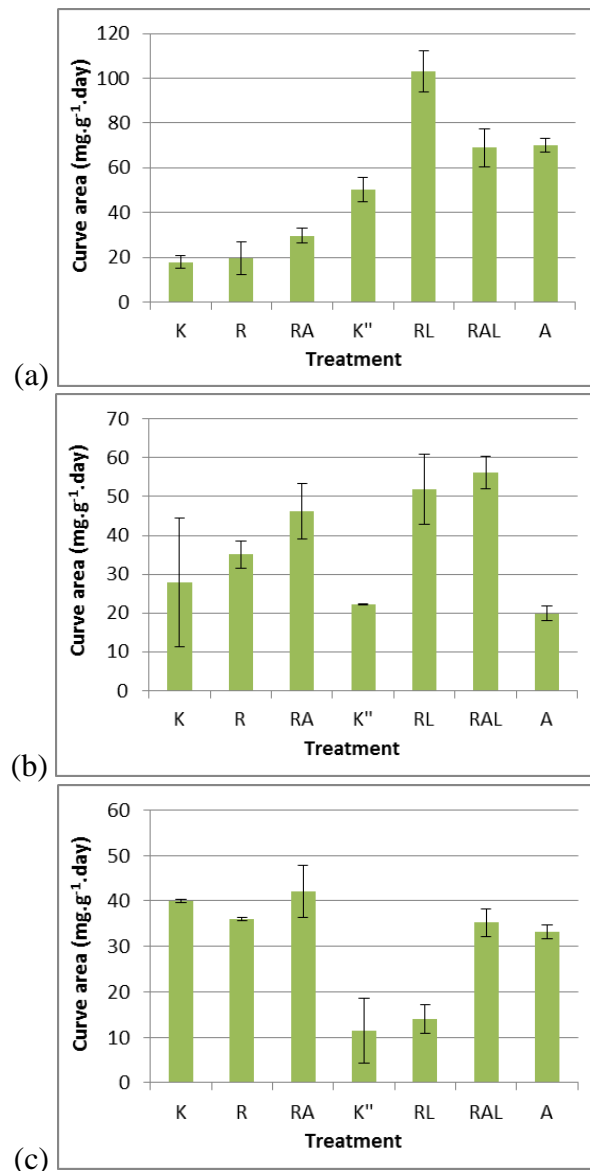


Figure 7. Effects of starter culture addition on overall primary metabolites production during cocoa bean fermentation : a. Ethanol, b. Lactic acid, c. Acetic acid

### pH of Bean and Fermentation Index

Acidity (pH) may indicate the death of cocoa beans. Figure 8a shows the initial pH of the cocoa beans used in the experiments were in the range of 7.05 to 7.22. These numbers decrease as the fermentation proceed along with the diffusion of the produced organic acid into beans. According to Camu *et al.* [13] a decrease in the internal pH on beans was accompanied by an increase in temperature up to 50°C and damage bean grain structure will facilitate the development of flavour precursors and pigment degradation by endogenous enzymes, such as invertase, glycosidases, proteases and polyphenol oxidase.

Ardhana and Fleet reported the pH of fresh pulp pH to be in the range of 3.6 - 3.9 [3]. In agreement with their observation, the initial pH of the fresh pulp was measured to be relatively low, ranging from 3.59 to 3.95. This implies the presence of citric acid in cocoa pulp [3], despite very low level of citric acid was measured in this experiment (Figure 1). Correspond to the decrease in beans pH as the fermentation proceeded, an increase in pH of pulp was observed (results not shown).

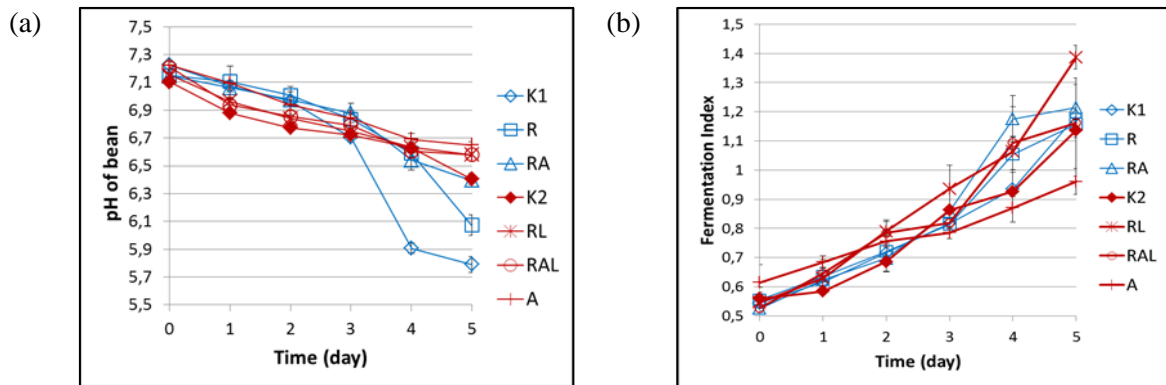


Figure 8. a. Dynamics in pH of bean during the fermentation, b. Dynamics in fermentation index during the fermentation

Fermentation index measures the colour change of cocoa that can be used as the indicator of completeness of the fermentation [11]. Cocoa bean polyphenols which impart the bitter and astringency of cocoa are stored in the whitish purple pigment in the cotyledon. During the fermentation the polyphenols diffuse out, degrade, and react with other cellular compounds, decreasing the astringency as well as turning the colour into brown. The completion of the fermentation is marked by the fermentation index being equal to or higher than 1.0.

Figure 8b shows an increasing profile of the fermentation index for all treatment. By the end of fermentation, at day 5, the measured fermentation indices were mostly higher than 1 or in another word the fermentation was complete. Only at treatment A, in which only acetic acid bacteria was added as the starter culture, was the fermentation index measured to be less than 1. This result supports other measurements that at treatment A less sugar was degraded, less primary metabolites were produced, and less decrease in the beans' pH. On the other hand, treatment RL gave significantly higher overall fermentation index than other treatment. By day 5 the fermentation index was measured to be  $1.38 \pm 0.07$ . This result also supports other measurements that at treatment more sugar was degraded and more primary metabolites were produced.

Figure 8b also shows that by day 4, the fermentation indices of fermentations with added starter cultures, except for treatment A, have exceeded 1. Thereby the fermentation can actually be ended one day shorter. Overall, the obtained results show that the addition of starter can accelerate the fermentation process.

## Conclusions

Most combinations of *S.cerevisiae* var. Chevalieri, *A.aceti*, and *L.plantarum* as starter culture for cocoa bean fermentation improved the fermentation performance through modification of microbial population, acceleration of sugar reduction and metabolites production and improvement of fermentation index. These indicated that with addition of mix starter culture in the beginning of fermentation, the execution of cocoa bean fermentation can be shortened. Further, the addition of starter culture modified microbial composition during the fermentation and thus directed the fermentation towards the targetted fermentation mechanism, regardless the initial cocoa bean conditions.

The best result was obtained from the addition combination of yeast and lactic acid bacteria in the starter culture, giving more sugar degradation and more primary metabolites production which leading to the fermentation index of  $1.38 \pm 0.07$  by the end of

fermentation. Further study should be done to verify the effect of this particular starter culture combination into the formation of cocoa bean flavour, for example by organoleptic.

On the other hand, the addition of a single culture acetic acid bacteria in the starter culture resulted in less sugar degradation and less primary metabolite production leading to the fermentation index lower than 1 by the end of fermentation. This particular combination was not suggested for further used in the application.

## Acknowledgment

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