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Carbohydrate Enrichment in In-Vitro Civet Coffee Fermentation: Impact on Arabica Coffee Beans

Darwin*, Ramayanty Bulan, Hapirayani, Ami Muliawati, Faradilla, Ade' Ikhtiara Irnanda, Marcella Br Kaban

Department of Agricultural Engineering, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia

Corresponding Author Email: darwin_ae@usk.ac.id

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ABSTRACT

This study aimed to evaluate the performance of in-vitro fermentation processes on civet coffee, employing a mixed-microbial enrichment culture, and to assess its impact on the physicochemical properties of the fermented product. Arabica Coffee cherries were utilized as the subject in this investigation. The inoculum, derived from civet fecal suspensions, was enriched through the augmentation of various carbohydrates, including soluble (e.g., glucose) and insoluble (e.g., starch) carbohydrates, mirroring the primary components present in civet food. Over an incubation period of four hours, a drop in pH from 7.0 to 5.6 was observed in the cultures, and volatile fatty acid (VFA) was detected at 2.8 mmol/L. It was found that the sugar conversion rate during in-vitro coffee fermentation by the starch-consuming microbial culture (2.1 mM/hour) significantly outperformed that of the glucose-consuming culture (0.9 mM/hour). The caffeine content in in-vitro fermented coffee (0.27-0.28 mg/100g) was found to closely approximate that of in-vivo fermented coffee via civet digestion (0.254 mg/100g). Notably, both in-vitro and in-vivo fermentation yielded caffeine content substantially lower than that of conventionally fermented coffee, which ranged between 1.5 and 2.7 g/100 g. In summary, the fermentation of Arabica coffee cherries using a mixed microbial culture, enriched with starch supplementation (SCM), demonstrated a faster rate compared to a culture enriched with glucose (GCM). The findings provide important insights for optimizing in-vitro civet coffee fermentation processes.

1. INTRODUCTION

Coffee, a universally consumed beverage, ranks as one of the most traded commodities in the world, with its global consumption witnessing a steady annual increment of approximately 1.9% over the past five decades [1, 2]. Currently, Brazil and Indonesia hold the leading positions in the production and export of Arabica and Robusta coffee respectively, collectively accounting for 70% of Arabica and 30% of Robusta in the global coffee market [3, 4]. Major consumer markets for coffee are primarily located in the United States and European countries [5].

The appeal of coffee lies largely in its distinctive flavor and inviting aroma [2], which are influenced by a myriad of factors, including agricultural practices, cultural preferences, microbiological interactions, physicochemical properties, and processing and storage conditions [6, 7]. From plantation to cup, various stages, particularly the bio-processing or post-harvesting processes, play pivotal roles in determining the taste and aroma of coffee [8, 9].

Bio-processing, despite its significant impact on coffee aroma, remains insufficiently explored. Sensory analysis suggests that coffee subjected to biological processing via wet fermentation exhibits a more favorable aroma and taste [2, 10]. Controlled coffee fermentation processes have been shown to enhance desirable traits while preventing off-flavors caused by undesired fermentation [2, 11]. However, the effects of fermentation on coffee's aroma and flavor are often overlooked, with the primary focus being on the removal of pulp and mucilage layer and the reduction of water content [12].

Coffee pulp and mucilage contain substantial nutrients, with the mucilage comprising starch, cellulose, and polysaccharides, and the pulp primarily consisting of minerals, free amino acids, fatty acids, crude protein, and carbohydrates [13-15]. These components serve as vital substrates for microbes during coffee fermentation, potentially enhancing the quality of coffee beans [15, 16]. The fermentation of carbohydrates, both soluble (e.g., sugar) and insoluble (e.g., starch), typically results in the production of fermentation end-products such as alcohols, volatile fatty acids (VFA), and lactic acid, which could considerably enhance the quality, aroma, and taste of coffee beans [2, 16]. Microbial activities during coffee fermentation transform components of pulp and mucilage layer, like free amino acids and sugar, into fermentation end-products, which subsequently influence the roasting process by releasing volatile and Maillard compounds that modulate flavors and aroma [2, 17, 18].

Currently, natural fermentation, aimed at removing mucilage, is widely practiced in coffee processing [3]. This method, reliant on the endemic microbial community associated with the coffee plantation microbiota [3, 19], produces limited effects on the quality of coffee beans [2]. A significant drawback of this conventional practice is the potential for irreproducible results due to contamination since the fermentation process occurs in an open environment. Coffee fermentation using a defined microbial culture could potentially offer a controlled process and more reproducible results compared to the traditional practice [20, 21].

Several studies have reported coffee fermentation employing specific microbes, such as yeasts (e.g., *Saccharomyces cerevisiae*), acetic acid bacteria (e.g., *Acetobacter xylinum*), and lactic acid bacteria (LAB) [22-24]. However, the use of a pure microbial culture may not significantly enhance the physicochemical properties of coffee, as it tends to alter a specific component to produce a particular fermentation end-product [25]. In contrast, mixed microbial cultures could potentially enhance the physicochemical characteristics of coffee [20] and promote the desired tastes and aroma [17]. This is attributable to the diverse types of microbes and/or microbial consortium present in mixed cultures [16, 26, 27].

The introduction of a mixed microbial culture, derived from civet fecal suspension, to coffee fermentation could aid in the oxidation and conversion processes of the compounds in coffee pulp and mucilage, forming various metabolites [20]. This is attributed to the inoculum from civet fecal suspension, comprising a diverse array of digestive microorganisms adapted to the fermentation of coffee cherries [20]. This process could not only reduce undesirable coffee components, such as caffeine, but also generate fermentation end-products, contributing to the modulation of coffee flavor and aroma [17, 28]. This study seeks to evaluate the in-vitro civet coffee fermentation processes using a mixed-microbial enrichment culture supplemented with carbohydrates, and assess their impact on the physicochemical properties of the fermented coffee, including sugar and caffeine content. The carbohydrates supplemented in the cultivation process comprise both soluble and insoluble types.

2. MATERIALS AND METHODS

2.1 Coffee bean collection

Coffee cherries used for the experiment of in-vitro luwak fermentation were Arabica Coffee (*Coffea Arabica*) collected from the coffee plantation located in the Jeget Ayu Village, Jagong Jeget subdistrict, Central Aceh Regency, Takengon, Aceh Province, Indonesia. The coffee cherries picked was the ripe coffee in which the coffee was ready to harvest and its skin had turned from green to red color. After collecting, the coffee cherries were cleaned and sorted shortly after collection to get rid of unnecessary materials including sands, stalks and leaves, which may obstruct fermentation processes.

In this study, original luwak coffee beans used as comparing data of in-vivo fermentation were also collected from the field of the coffee plantation.

2.2 Inoculum preparation and cultivation processes

The coffee cherries were fermented by using a starter culture derived from selectively enriched civet fecal suspension. The civet poop used for the selective enrichment process was collected from the coffee plantation in the Jagong Jeget subdistrict, the district of Central Aceh. Prior to starting the cultivation process, the poop was kept under the temperature 37 ± 0.5℃ to activate microbes.

The cultivation processes were performed by feeding different substrates of soluble and insoluble carbohydrates. The culture media was prepared by dissolving 1 g of ammonium chloride (Merck), 1 g of Sodium Hydrogen Carbonate/Sodium Bicarbonate (Merck), 1 g of Bacto Peptone (Merck) and 1 g of substrates (i.e. glucose anhydrous (Merck) and/or starch powder) to 100 ml of DI water. The prepared culture media was then mixed with 100 g of the civet poop. The selective enrichment process was performed by daily topping up 100 ml of culture media with a particular substrate (glucose or starch) into 0.5 L fed-batch reactor. Glucose and starch were used in this experiment as these types of carbohydrate would generate different fermentation end-products and may induce different types of fermentative microbes [16]. Hence, by supplementing these carbohydrates potentially could affect coffee fermentation and improve its quality [20]. This process was performed until the volume culture reached 500 ml. The cultivation process was conducted under the mesophilic temperature of 37 ± 0.5°C [29, 30]. The physicochemical characteristics of civet fecal suspension utilized as starter culture for in-vitro luwak fermentation of Arabica Coffee cherries were shown at Table 1.

Table 1. Physical-chemical properties of civet fecal suspension

Parameters	Unit	Value
pH	-	7.58
Total dissolved solids	mg/l	91
Optical Density	OD_{600}	0.539
Electrical Conductivity	µS/cm	181
Salinity	mg/l	89

2.3 Experimental design and procedures

The experiments were set up to compare effective starter cultures between glucose consuming microbes and starch consuming microbes used as inoculum for performing in-vitro fermentation of Arabica coffee cherries. The fermented coffee beans would be compared with original luwak coffee beans or coffee beans fermented in-vivo via civet digestion. A series of batch reactor with the working volume of 80 ml was utilized to perform an in-vitro luwak fermentation of Arabic coffee cherries. Inoculum added to each reactor was 40% of the total volume. The concentration of inoculums used in this experiment was based on the previous study revealing that 40% of inoculums were a feasible concentration that could enhance conversion of sugar and caffeine in the fermentation of Arabica coffee [20].

To ensure all coffee cherries completely digested, the fermentation process was performed for about 48 hours. Typically, fermentation in-vivo by civet lasts for less than 24 hours. As the current study is performed in-vitro, 48 hour of incubation was set up to provide sufficient time for microbes to ferment the coffee [20]. In order to have in-vitro fermentation closely similar to the in-vivo process, the working temperature established was around 37 ± 0.5℃, which was relatively close to the body temperature of civet

[31]. After fermentation, each trial including coffee beans fermented using inoculum enriched with starch consumption and coffee beans fermented using inoculum supplemented with glucose was evaluated as well as compared with luwak coffee beans or original coffee beans fermented in-vivo by civet. The data of physicochemical characteristics (i.e. glucose, caffeine and solid contents) of fermented coffee obtained was desriptively presented and compared.

2.4 Analytical methods

Analysis of all samples was carried out periodically to evaluate the impact of fermentation using different conditions of inoculums towards the physicochemical characteristics of coffee. Samples of coffee cherries and the types of inoculums were analyzed to assess their characteristics prior to using them in in-vitro fermentation. During the processes of selective enrichment and fermentation, pH of the culture was regularly recorded utilizing a Lab Benchtop pH meter multifunction with complete probe. The pH meter was calibrated and validated with buffer solution prior to the measurements [32, 33].

Optical density (OD) analysis was carried out to investigate the growth of microbes during the selective enrichment processes or microbial culture cultivation utilizing a Lab UV-VIS Spectrophotometer, 325-1000nm 4nm Ultraviolet. The OD measurement was performed at the wavelength of 600. A blank of DI water was used in a spectrophotometer prior to OD analysis of test samples.

Moisture content (MC) and total solid (TS) were analyzed using a laboratory oven while ash content was measured using furnace. The temperature for total solid and moisture content analysis was set up at around 105ºC. Samples of total solid and moisture content analysis were then placed in the furnace for measuring ash content at the temperature of 500ºC. The analytical procedures were carried out based on the Standard Methods [34, 35].

Volatile fatty acid (VFA) analysis was carried out to measure the total amount of VFA produced during the fermentation of coffee cherries. The determination of VFA on the fermented coffee was performed via titration method. The VFA measurement was conducted using a Lab Standard pH meter. Prior to start of analysis, samples were firstly acidified with HCl to pH 3 to change all bicarbonates to the form H_2CO_3 $\leftrightarrow$ CO_2, and aerated the samples to get rid of CO_2. The samples were subsequently back titrated with sodium hydroxide (NaOH) from pH 3.9 to 5.6. Each procedure adopted for VFA determination was carried out based on the study by Lützhøft et al. [36].

Caffeine analysis was carried out to assess caffeine content on coffee cherries and the fermented coffee. The analysis was performed by a Lab. UV-Visible Spectrophotometer at the wave length of 275 nm [37, 38]. A gram of ground fined coffee beans was dissolved and mixed into 150 ml hot DI water. The mixture was subsequently screened utilizing a filter paper and poured it into a beaker. The solution was then mixed with 1.5 g $CaCO_3$, and extracted 4 times with 25 ml chloroform. The extract was heated to get rid of the chloroform residue using oven. The caffeine extract was then diluted ten times with DI water prior to the measurement using Spectrophotometer [20, 39].

The determination of carbohydrate or sugar content of the fermented coffee as well as raw coffee cherries was carried out utilizing the Glucometer BioSensor AGM-2100 with an assay method of electrochemical method (Gold electrode). The sugar content analysis was conducted under the ambient temperature. The samples were diluted 10 times to avoid an overestimate value of sugar content of coffee. Each procedure adopted for the measurement of soluble carbohydrates concentration of coffee was carried out based on the method developed by Darwin [40].

3. RESULTS AND DISCUSSION

Results revealed that microbial growth represented in OD value of the mixed microbial culture supplemented with both soluble and insoluble carbohydrates gradually increased from day 1 to day 5 of incubation period (Figure 1). The culture enriched with the supplementation of glucose had higher of OD value (0.0250) in comparison to that of the culture fed with starch (0.022). This would be attributed to that glucose is a soluble carbohydrate classified as single sugar, which was easily utilized and converted by microbes rather than starch considered as non-soluble carbohydrate of polysaccharide [26, 41]. In addition, the results of the current study showed that pH of the culture supplemented with glucose was lower (pH 6.7) than that of the culture fed with starch (7.20). This indicated that microorganisms of various species live and grow in different levels of pH [42]. Typically, this would specify the end-products generated during the processes of coffee fermentation [43, 44], and subsequently would influence final product of coffee including taste and flavor modulation [45].

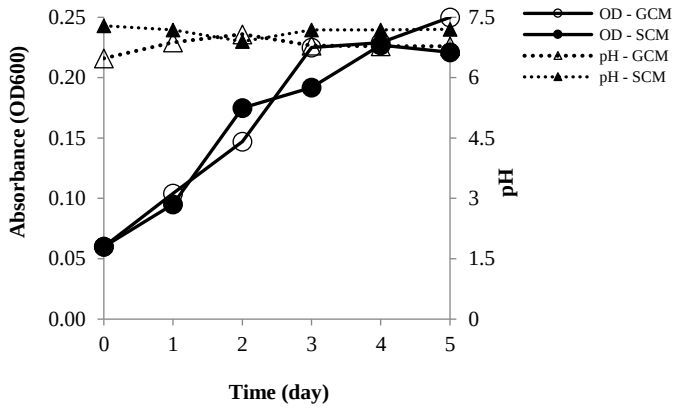

Figure 1. Microbial growth as measured by absorbance (OD600) and pH of the inoculum fed with different carbohydrates during the cultivation process of civet fecal suspension
GCM - glucose consuming microbes; SCM - starch consuming microbes

The type of coffee used in this study is *Arabica* coffee cherries, and the characteristics of coffee used for in-vitro civet fermentation process was presented in Table 2. Results showed that pH of the coffee cherries utilized were quite acidic that was around 5.53. The alkalinity of the coffee cherries was measured to evaluate its ability to neutralize acids and/or bases generated during the fermentation including oxidation reactions involved [46], and the alkalinity of Arabica coffee cherries used in this study was found at around 1.2 mmol/L. The results of the current study showed that coffee cherries used in in-vitro civet fermentation were rich in nutrients and minerals comprising of ash (8.01%), caffeine (2.81 mg/100g) and total solid (60%), which may be required

for fermentative microorganisms. The coffee also contained soluble carbohydrate represented in glucose content (21 mmol/L). This could be utilized as source of energy and oxidized into metabolites including volatile and non-volatile compounds (i.e. alcohols and organic acids), which necessarily affect the quality of coffee [47, 48].

Table 2. Physico-chemical properties of *Arabica* coffee cherries prior to fermentation

Parameters	Unit	Value
Moisture content	%	40.3
Ash content	%	8.01
Solid content	%	59.7
Glucose	mmol/L	20.85
Alkalinity	mmol/L	1.2
Caffeine	mg/100g	2.81
pH	-	5.53

The results of the present study showed that within 2 hours of incubation, pH culture of coffee fermentation inoculated with glucose consuming microbes decreased from 6.8 to 6.0 while the pH culture of the coffee fermented using starch consuming microbe (SCM) dropped significantly from 7.2 to around 5.8 (Figure 2). Within 4 hours of incubation, pH of both cultures dropped into 5.70, and within this period the cultures were in acidic condition [49] in which volatile fatty acid (VFA) was build-up and gradually increased from 0 to 2.75 mmol/L. The formation of VFA in the coffee fermentation inoculated with SCM continuously increased and reached a peak at 8 hours of incubation (6 mmol/L) while the culture inoculated with SCM the VFA dropped from 2.8 to 1.4 mmol/L. The formation of VFA during the processes of fermentation may improve the quality of the produced coffee beans. This is because as a volatile compound, VFA generated during the coffee fermentation may likely influence the modulation of coffee flavor and aroma [50].

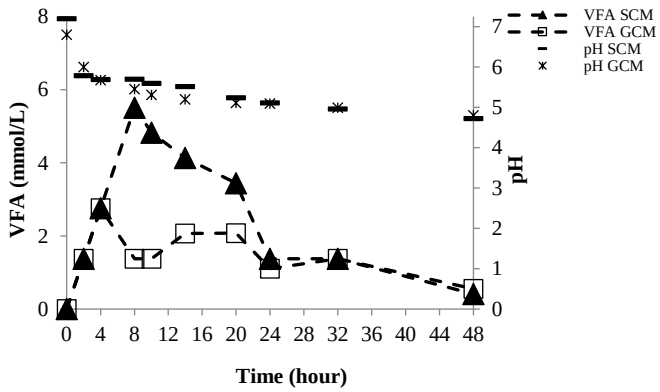

Figure 2. PH and VFA of the coffee beans during fermentation
GCM - glucose consuming microbes; SCM - starch consuming microbes

As depicted in Figure 2, from 24 hours of incubation to the rest of the process, coffee fermentation process was under the acidic condition in which pH was around 4.7, and VFA concentration tended to decrease. Study mentioned that the relative concentrations in which VFA are generated, would be affected by some factors including substrate availability and rate of depolymerization, substrate composition, and microbial species present [51]. Besides, typically during VFA formation via acid stage fermentation, the formation of VFA tend to decrease once pH of the culture drop under the pKa value of VFAs (pH < 5.0). This is due to the fact that some VFA producers cannot survive under the extremely acidic pH [52]. Under the extreme low pH, there would be metabolism shifted in which the formation of VFA may be shifted to other fermentation end-products such as lactic acid ethanol. This is because lactic acid and ethanol production may occur under the extreme low pH (pH<5.0), and thereby lactic acid and alcohol producers would be the dominant microbes at this condition [27, 49].

Results of the current study showed that within 4 hours of incubation glucose concentration of the coffee fermented using SCM decreased sharply from 19 to 10.4 mmol/L while the coffee fermentation inoculated with GCM slightly decrease from 19 to 15.4 mmol/L (Figure 3). This indicated that the sugar conversion rate of coffee fermented by SCM was greater (2.1 mM/hour) than that of GCM (0.9 mM/hour). As shown in Figure 3, the fermentation of Arabica coffee cherries using SCM was faster than that of GCM. This is because SCM was able to convert soluble carbohydrate merely 14 hours while the coffee fermentation using GCM may convert the sugar above 32 hours of incubation. This could be attributed that the starter culture of SCM used in the fermentation of coffee is more adaptable towards the components of Arabica coffee cherries specifically carbohydrate (i.e. polysaccharides and monosaccharide) since it can easily degrade and convert soluble sugar of the coffee. The culture of starch consuming microbes is typically dominated by lactic acid bacteria producers, and they are able to live and grow under the acidic condition or low pH [53]. Hence, the conversion of carbohydrates into metabolites during the coffee fermentation would be completed faster by using the culture containing starch consuming microbes.

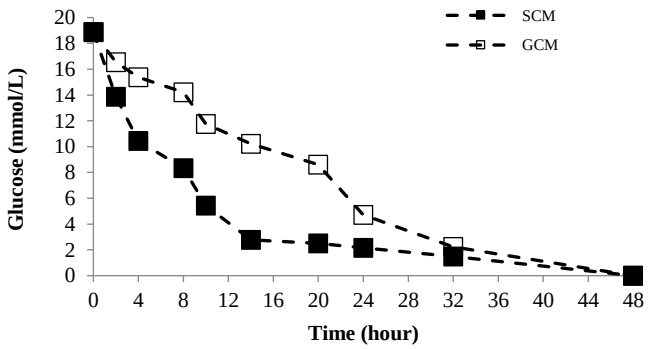

Figure 3. Glucose concentration of the fermented coffee beans using different treatments of starter
GCM - glucose consuming microbes; SCM - starch consuming microbes

The results revealed that coffee fermentation using SCM was able to degrade caffeine faster than that of the GCM. As depicted in Figure 4, coffee fermentation using SCM could reduce caffeine content of the coffee from 3.4 to 2.5 mg/100g solely within 2 hours of incubation while the coffee inoculated with GCM merely reduced the caffeine from 3.4 to 3.3 mg/100g. This indicated that the caffeine degradation rate of the coffee fermented using SCM was about ten times higher (0.06 mg/100g per hour) than that of GCM (0.005 mg/100g). Overall, with 48 hours of duration of fermentation both starter cultures could reach around 80% of caffeine degradation. Besides, after 48 hours of incubation, the caffeine concentration of the fermented coffee was quite low that was around 1.00 mg/100g. The caffeine content of the fermented coffee is quite lower than that of other common coffee beans,

which was between 1.5 and 2.7 g/100 g [54, 55]. The results suggested that the coffee fermentation using enriched microbes consuming carbohydrates may effectively reduce caffeine content of the fermented coffee.

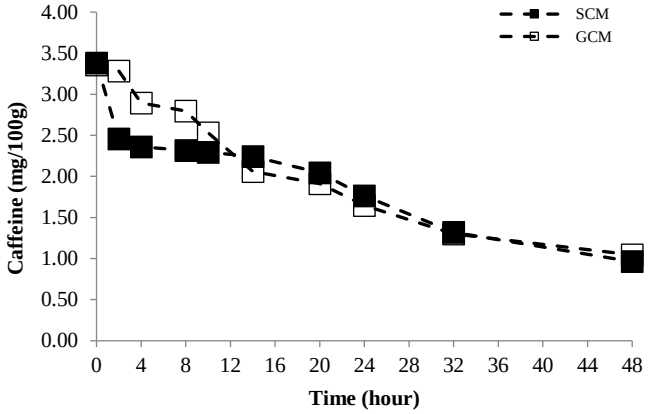

Figure 4. Caffeine of the fermented coffee beans using different treatments of starter
GCM - glucose consuming microbes; SCM - starch consuming microbes

The current study showed that the caffeine content of the coffee fermented in-vitro using mixed microbial cultures enriched with carbohydrate supplementation (1.65-1.76 mg/100g) were higher than that of the original luwak coffee bean (1.34 mg/100g) (Table 3). This result was obtained from the coffee beans analyzed after 24 hours of digestion of Arabica coffee cherries. This indicated that in-vivo coffee fermentation by civet or luwak is still more effective and faster for decaffeination process [56] than that of in-vitro fermentation by mixed microbial culture from civet fecal suspension enriched with carbohydrate supplementation. Further, sugar concentration represented in glucose content of the fermented coffee beans revealed that in-vivo civet digestion was faster for degrading and converting sugar (0 mmol/L) of the coffee compared to that of the in-vitro process (2.17-4.72 mmol/L) after 24 hours of incubation period (Table 3). This suggested that in-vivo civet digestion could completely degrade caffeine and convert soluble carbohydrate into metabolites faster, and thereby may significantly contribute to the modulation of flavors and aroma of the coffee [57, 58]. Besides, to generate similar characteristics between the original luwak coffee and the coffee fermented in-vitro, the process of coffee fermentation may take longer to complete the processes.

Table 3. Physical-chemical properties of fermented coffee beans

Parameters	Unit	CFIS	CFIG	LC
Caffeine	mg/100g	1.76	1.65	1.34
Glucose	mmol/L	2.17	4.72	0
pH	-	5.11	5.1	5.34
VFA	mmol/L	1.38	1.10	1.00
Moisture content	%	52.2	54	32
Ash content	%	5.51	4.75	5.2
Solid content	%	47.8	46	68

Note: data taken after 24 h incubation (as comparing data towards in-vivo civet fermentation). CFIS – coffee fermented using inoculum fed with starch; CFIG - coffee fermented using inoculum fed with glucose; LC – luwak coffee or coffee fermented in-vivo by civet

The current study showed that the physical chemical properties of the dried fermented coffee beans generated from in-vitro fermentation were somewhat close to that of the in-vivo digestion or luwak coffee bean (Table 4). The results revealed that moisture content (MC) and solid content (SC) of the coffee fermented in both in-vitro and in-vivo was quite similar in which MC was about 12%, and SC was around 88%. The results of the study showed that the caffeine content of the coffee fermented in-vitro was slightly higher (0.27-0.28 mg/100g) than that of in-vivo (0.254 mg/100g). The result also revealed that there is no a big difference in terms of caffeine content of the fermented coffee among the cultures used in in-vitro fermentation whether enriched with glucose supplementation or starch addition.

Overall, the caffeine content of the coffee processed via fermentation using mixed microbial cultures from civet fecal suspension enriched by carbohydrate supplementation was still significantly lower than that of the coffee processed conventionally. This could be attributed to the study revealing that on average the caffeine content of a single Arabica coffee bean ranges from 1.2 to 1.5 g/100 g while a single Robusta coffee bean may contain from 2.2 to 2.7 g of caffeine per 100g [55, 59, 60].

Table 4. Physicochemical characteristics of dried fermented coffee beans

Parameters	Unit	CFIS (in-vitro)	CFIG (in-vitro)	LC (in-vivo)
Moisture content	%	11.60	12.02	12
Solid content	%	88.44	87.98	88
Caffeine	mg/100g	0.27	0.28	0.254
Glucose	mmol/L	0	0	0

CFIS – coffee fermented using inoculum fed with starch; CFIG - coffee fermented using inoculum fed with glucose; LC – luwak coffee or coffee fermented in-vivo by civet

4. CONCLUSIONS

The fermentation process of Arabica coffee cherries using mixed microbial culture enriched with starch supplementation (SCM) was faster than that of the culture enriched with glucose addition (GCM). This may be attributed that SCM could reduce glucose content of the fermented Arabica coffee cherries faster merely less than 12 hours of incubation than that of GCM in which it took longer above 24 hours to complete the fermentation process. Both SCM and GCM cultures are able to degrade caffeine content of the fermented coffee that was around 80% within 48 hours of incubation. The caffeine concentration of the fermented coffee was quite lower (1.00 mg/100g) than that of the conventional processing coffee (>2.5 mg/100g). Sensory analysis of the fermented coffee would be significant to assess the taste and/or aroma of both coffee fermented in-vitro and in-vivo.

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