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Effect of the solid state fermentation of cocoa shell on the secondary metabolites, antioxidant activity, and fatty acids

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Abstract During cocoa (*Theobroma cacao* L.) processing, the accumulated cocoa shell can be used for bioconversion to obtain valuable compounds. Here, we evaluate the effect of solid-state fermentation of cacao flour with *Penicillium roqueforti* on secondary metabolite composition, phenol, carotenoid, anthocyanin, flavonol, and fatty acids contents, and antioxidant activity. We found that the total concentrations of anthocyanins and flavonols did not change significantly after fermentation and the phenolic compound and total carotenoid concentrations were higher. The fermentation process produced an increase in saponin concentration and antioxidant activity, as well as significant changes in the levels of oleic, linoleic, gamma-linolenic, and saturated fatty acids. Based on our findings, we propose that the reuse of food residues through solid state fermentation is viable and useful.

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Introduction

Cocoa meal residue is generated during cocoa bean (*Theobroma cacao* L.) processing for chocolate production. Cocoa meal residue is mainly composed of the film/peel that surrounds the beans; which is morphologically equivalent to the mesocarp of the fruit. After the toasting step (at 150 °C until 2% moisture), the beans are mildly compressed and, thus, the beans are peeled; air blowers are used for the final separation between the nibs and the cocoa meal (Fig. 1). We believe that cocoa meal residue is underappreciated, typically being used in animal feed complementation without great success due to the presence of anti-nutritional components, such as theobromine [1–3].

Solid state fermentation (SSF) has been used to transform food waste into secondary metabolites of interest, including those with antioxidant action [4–7]. The need to identify compounds with biological functions (e.g., anti-tumor, anti-inflammatory, anti-aging, antioxidants, anti-mutagenicity, anti-carcinogenic) guide the search for phytochemicals compounds [8–10].

The selection of an appropriate microorganism is important for the success of bioprocessing. Fungi of the genus *Aspergillus, Trichoderma,* and *Penicillium* have been identified as good producers of enzymes [11, 12]. The first two genera have been extensively studied and reviewed, while research on *Penicillium* strains is relatively scarce [6]. *P. roqueforti* is a poorly investigated species; however, it has some characteristics favorable to fermentation, including good development in different pH conditions and the ability to use a variety of chemical

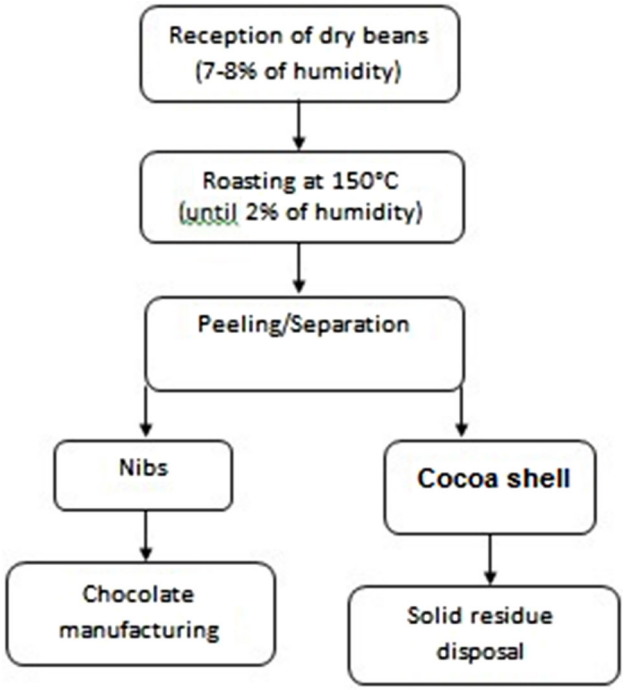

Fig. 1 Schematic representation of cocoa beans processing and cocoa shell generation

compounds as substrates, including pentoses and hexoses [13]. In addition, *P. roqueforti* is considered a GRAS (generally regarded as safe) fungus [14].

In our research group, several bioactive compounds have been identified in the cocoa shell (data not presented). In addition, the cultivation of fungi in waste food has been used of production of microbial enzymes [12, 15, 16]. Here we aimed to investigate the effect of solid state fermentation by *P. roqueforti* on the secondary metabolites, antioxidant activity, and fatty acids present in the cocoa meal.

Materials and methods

Solid state fermentation (FES)

The cocoa shell was donated by a cocoa processing company (Ilhéus, Bahia, Brazil) and was ground in a knife mill (Solab®). The granulometry was selected with a sieve with a diameter less than or equal to 200 mesh. *P. roqueforti* was used as the microorganism and was donated by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, RJ, Brazil), which was previously isolated, characterized, and deposited at the National Institute for Quality Control in Health (INCQS) 40075 with a lot number: 079840075. This fungus has been periodically preserved in silica and glycerol and kept at −80 °C. The inoculum was obtained by propagating the spores of *P. roqueforti* at 25 °C for 7 days in agar–agar and PDA (1:1) medium. The spores were collected, suspended in sodium phosphate buffer (50 mM, pH 7), and counted in a Neubauer's chamber, as described by Santos et al. [11]. The spore concentration used as the inoculum was 10^9 spores/g of a solid medium. To cultivate *P. roqueforti*, 20 g of the cocoa shell was autoclaved in a 250 mL Erlenmeyer flask, allowed to cool, and 1.0 mL of spore suspension plus 1.87 mL of distilled water were added. The humidity was adjusted to 50%, and FES was then conducted at 25 °C for 7 days.

Extracts

The aqueous and the hydroalcoholic extracts were obtained by shaking the fermented biomass (40 g) at room temperature for 1 h at a proportion of 1:7 g:mL (fermented biomass:solvent) with deionized sterile water and an alcoholic solution of hydroethanol (8:2 mL:mL) [17]. The obtained extracts were stored under refrigeration in an amber glass until analyses; which were all performed in triplicate.

Analysis of secondary metabolites

The assays for the in vitro identification of chemical constituents present in the aqueous and hydroalcoholic extracts were performed following the methodology described in the literature [18]. The results were evaluated visually and classified as positive or negative.

Total phenolic compounds

Folin-Ciocauteau reagent was applied to determine the total phenolic compounds [4]. The absorbance was read at 773 nm, and the results were expressed as mg equivalent of gallic acid (GAE) per 100 g of the cocoa meal.

Total anthocyanins and total flavonols

Anthocyanins and total flavonols were determined according to the procedure proposed in the literature [19]. The absorbance was measured at 535 and 374 nm for quantification of anthocyanins and flavonols, respectively. The results were expressed as mg of quercetin per 100 g of the cocoa meal.

Total carotenoids

For the determination of the total carotenoids, the applied procedure was proposed in the literature [20]. The

absorbance was obtained at 450 nm, and the results were expressed as mg β-carotene per 100 g of the cocoa meal.

Total flavonoids

The determination of flavonoids in the extracts was performed according to the literature [21], and the absorbance was measured at 415 nm. The results were expressed as mg of quercetin per 100 g of the cocoa meal.

Antioxidant activities: FRAP methodology

The ferric reducing antioxidant potential (FRAP) methodology was applied as described in the literature [22]. The absorbance was measured at 595 nm, and the results were expressed as μM ferrous sulfate per 1 g of the cocoa meal.

Antioxidant activities: DPPH methodology

The DPPH (2,2-diphenyl-1-picryl-hydroxyl) methodology followed the description in the literature [23]. Absorbance readings were taken at 515 nm. The results were expressed as percent inhibition of the DPPH radical.

Fatty acids profile

The methyl esters obtained in the transesterification of triacylglycerols [24] were separated on a gas chromatograph (Thermo-Finnigan), equipped with flame ionization detector and BPX-70 fused silica capillary column (120 m × 0.25 mm). The gas flow rate was 6.5 mL min^{-1} for N_2 trailing gas, 30 mL min^{-1} for N_2 auxiliary gas and 30 and 350 mL min^{-1} for flame H_2 and synthetic air gases, respectively. The sample split ratio was 90:10. The injector and detector temperatures were 250 and 280 °C, respectively. The total analysis time was 40 min, the initial temperature was 140 °C, and the final temperature was 238 °C. The injection volume was 1.5 μL. The areas of the peaks were determined using the Chromquest software. Identification of the fatty acids was performed by comparing the methyl esters retention times of Sigma (US) standards with those of the samples.

Statistical analysis

The results were analyzed with the System of Statistical Analysis and Genetics (SAEG) version 8.0, it was performed with the analysis of variance (ANOVA) and the Tukey test, at a significance level of 5%.

Results and discussion

Qualitative analysis of secondary metabolites

The compounds found after chemical prospection are listed in Table 1; according to these data, most of the metabolites that were detected in the cocoa shell before fermentation were also present after fermentation. Among the metabolites with confirmed antioxidant activity are the phenolic compounds, such as alkaloids and flavonoids (anticyclonic heterosides, tannins, catechins and free Anthraquinones) and triterpenoids, which belong to the carotenoids group. The compounds identified in the cocoa shell are also found in the cocoa bean and commercial chocolate [25, 26]. As shown in Table 1, saponins were only detected after FES, suggesting that these are a product of biotransformation by *P. roqueforti*. Saponins are natural surfactants produced by plants and also by some marine animals and bacteria.

Bioactive compounds

Comparing before and after fermentation, we detected no changes in the total anthocyanins and flavonols (Table 2). Unroasted cocoa beans are rich in flavonols and polyphenols, which explains the presence of these compounds in the residue even after processing [27].

For total phenolic compounds and total carotenoids (Table 2), a higher concentration was observed before fermentation, indicating a significant consumption of these metabolites by the fungus. Dulf et al. [5] observed a reduction of phenolic compounds after one week of fermentation by *Aspergillus niger* and *Rhizopus oligosporus*. Vattem et al. [28] attributed the reduction in the contents of the phenolic compounds to the polymerization of these

Table 1 Phytochemical prospection of cocoa shell extracts

Secondary metabolites	Extract	
	In natura	Fermented
Anthocyanic heterosides	+	+
Saponins	−	+
Gums and mucilage	+	+
Tannins	+	+
Catechins	+	+
Steroids and triterpenoids	+	+
Alkaloids	+	+
Cumarins	−	−
Phenolic compounds	+	+
Free antraquinones	−	−
Flavonoids	+	+

− Not detected; + detected

Table 2 Quantification of bioactive compounds in cocoa shell extracts

Total compounds	Extract	
	In natura	Fermented
Carotenoids (mg β-caroten/100 g cocoa shell)	0.8 ± 0.03^{a}	0.2 ± 0.03^{b}
Phenolics (mg galic acid/100 g cocoa shell)	2120 ± 20^{a}	926.6 ± 61^{b}
Anthocyanins (μg quercetin/100 g cocoa shell)	0.4 ± 0.03^{a}	0.5 ± 0.1^{a}
Flavonols (μg quercetin/100 g cocoa shell)	1.5 ± 0.2^{a}	1.5 ± 0.1^{a}

The average values (triplicates) followed by different letters in the same row present a statistical difference by the Tukey test ($p < 0.05$)

compounds by enzymatic oxidation, activated as part of a stress response associated with the reduction of available nutrients throughout the fermentation period. The total phenolic content of kojis prepared with *A. awanori*, *R. azygosporus* or *Rhizopus sp.* ranged from 16.6 to 27.2 mg GAE per g dry koji compared to unfermented control [19]; these values are well below that reported here (Table 2).

The total phenolic content found in the cocoa shell before fermentation was higher than that found in cocoa powder which ranged from 650 (Brazilian origin) to 2000 (US origin) mg GAE per 100 g. In our study, even after fermentation, the phenolic content of the cocoa shell was higher than that reported for Brazilian cocoa powder [29].

Ghana's cocoa beans, before and after fermentation of the seed, contain 6.30 and 65.24 mg GAE per 100 g of cocoa (611 mg per GAE for the cocoa powder) [19, 30]. These values are much lower than we found in the cocoa residue, before and after SSF.

Jonhson and Schroeder [31] have demonstrated that carotenoids can be biosynthesized by non-photosynthetic organisms such as bacteria, fungi, and yeasts. However, in view of the reduction of carotenoids observed in this work, we can consider *P. roqueforti* as a carotene non-biosynthesizing fungus, at least under the conditions and substrate evaluated in this work.

Flavonoid

We detected a higher concentration of total flavonoids in the hydroalcoholic extract (Table 3). After fermentation, there was a decrease in the amount of flavonoids in both the aqueous and hydroalcoholic extracts. Multiple studies have demonstrated the presence of a wide variety of flavonoid class compounds present in cocoa beans and derivatives and their relationship to antioxidant activity [19, 25, 27].

Antioxidant activity

The results of the antioxidant activities by the FRAP methodology are presented in Table 4. The highest value of reducing power was found in the inorganic hydroalcoholic extract (33.5 μM g^{-1}) followed by the pre-fermentation aqueous extract (28.6 μM g^{-1}); in the fermented extracts, the iron reducing power varied from 8 to 23.2 μM g^{-1}. Using methanol as the solvent extractor, reducing powers have been reported for white chocolate (2.3 μM g^{-1}) [32] and cocoa powder (18.0 μM g^{-1}), which are lower than the values for cocoa meal reported here.

The antioxidant activities determined by the DPPH methodology (2,2-diphenyl-1-picryl-hydrazyl) is also presented in Table 4 as a percent inhibition. From this data, it is observed that the fermented aqueous extract exhibited a moderate (50–70%) inhibition, whereas the hydroalcoholic extracts fermented by *P. roqueforti*, as well as two non-fermented extracts, exhibited a strong capacity (70%) to sequester DPPH. Xi et al. [33] state that some saponins may also exhibit antioxidant activity by capturing free radicals in both aqueous and hydrophobic phases. Chen et al. [34] reported 5 to 34% total antioxidant activity in the saponin rich extracts (concentration of 10 g/mL) of *Sapindus mukoross*.

Recent studies have shown that antioxidant activity is closely related to the molecular structure of polyphenols [29, 35]; thus, the presence of the various compounds

Table 3 Flavonoids quantification in cocoa shell extracts

Extracts	Flavonoids (mg quercetin/100 g cocoa meal)
Hydroalcoholic	37.5^{a}
Aqueous	31.5^{b}
Fermented/hydroalcoholic	23^{c}
Fermented/aqueous	21^{c}

Values followed by different letters in the same column present a statistical difference by the Tukey test ($p < 0.05$)

Table 4 Antioxidant activity of cocoa shell extracts by the methodologies: DPPH and FRAP

Extracts	DPPH (% of inhibition)	FRAP (μM ferrous sulfate/g cocoa meal)
Fermented/hydroalcoholic	81.3[a]	23.2[a]
In natura/hydroalcoholic	79.2[b]	33.5[b]
In natura/aqueous	78.5[b]	28.6[c]
Fermented/aqueous	57.7[c]	17.8[d]

Values followed by different letters in the same column present a statistical difference by the Tukey test ($p < 0.05$)

detected in the cocoa shell explains its high antioxidant capacity.

Zhang et al. [23] evaluating the effect of the fermentation by the fungus *Cordyceps militaris* on antioxidant activity observed in the aqueous extract 67.18% in control and 80.60% after the fermentation, on the activity of elimination of the radical DPPH, demonstrating that it seems wheat may be a raw material for the metabolization of bioactive compounds with antioxidant activity.

Fatty acids

Table 5 shows our fatty acids (FA) composition findings. Palmitic, margalic, stearic, oleic, linoleic, gamma-linolenic, arachidic, timnodonic, Alpha-linolenic, behenic, and lignoceric fatty acids were identified and quantified.

Table 5 Fatty acids composition in cocoa shell extracts

Fatty acids	Composition (%)	
	In natura	Fermented
Saturated (FAS)		
16:0	15.6 ± 2.1[a]	19.6 ± 1.9[b]
17:0	0.32 ± 0.04[a]	1.12 ± 0.06[b]
18:0	41.67 ± 3.7[a]	24.23 ± 3.5[b]
20:0	1.9 ± 0.4[a]	1.24 ± 0.35[a]
22:0	3.64 ± 0.5[a]	1.1 ± 0.3[b]
24:0	0.83 ± 0.2[a]	0.6 ± 0.25[a]
Mono-unsaturated (FAMU)		
18:1n9c	28.7 ± 3.8[a]	35.8 ± 3.3[b]
Poly-unsaturated (FAPU)		
18:2n6	2.6 ± 0.5[a]	5.4 ± 0.6[b]
18:3n6	2.3 ± 0.4[a]	8.26 ± 0.5[b]
20:5n-3	1.13 ± 0.3[a]	1.73 ± 0.4[a]
Total FAPU	34.7	51.2
Total FAS	64	48
Ratio FAPU/FAS	0.09	0.32

The average values (duplicates) followed by different letters in the same row present a statistical difference by the Tukey test ($p < 0.05$)

FAS saturated fatty acids, *FAPU* polyunsaturated fatty acids

We found that the FA concentration was modified after fermentation, as expected. The non-fermented cocoa shell had higher levels of the saturated FA and stearic acid (18:0), and, after fermentation, it was observed a reduction of around 42% in this concentration and an increase of around 25% on the monounsaturated FA, oleic acid (18:1n9c), which was the predominant FA after fermentation. Oleic acid can supposedly lower LDL-cholesterol levels. Moreover, oleic acid in food likely has a preventive effect on several chronic diseases (cardiovascular diseases, cancer or age-related cognitive decline) and, therefore, may increase human longevity [36].

Unsaturated fatty acids might arise in a biosynthetic route by a mechanism called desaturation, where the fungus can desaturate or catalyze the formation of a double bond between the consecutive carbons of one or more fatty acid to produce a fatty acid mono- or polyunsaturated, or precursor thereof [37]. Here we demonstrate the conversion of stearic acid to linoleic acid and gamma-linolenic acid by *P. roqueforti*; a change of more than 60% in the unsaturated fatty acids profile.

Cacao seeds from Ghana and Ecuador highlight fatty acids C16:0 (25%), C18:0 (33%) and C18:1 (34%) the most important for both origins [38]. Another important finding of this study was the significant increase in linoleic (18:2n6) and γ-linolenic (18:3n6) acids following fermentation since these FAs are considered an essential part of the animal diet (they are not biosynthesized by animals, including humans).

According to the UK Department of Health, the optimal FAPU ratio for FAS for food is 0.40, and values below 0.45 are unhealthy [39]. In Ecuadorian and Ghanaian cacao, the FAPU ratios are 1.65 and 1.72 respectively [38], clearly indicating a high proportion of FAPU per FAS. Here, we detected an FAPU ratio of 0.09 to 0.32, making the cocoa shell healthier than the cocoa bean.

The cocoa shell is a residue containing several bioactive compounds with antioxidant activity, such as phenolic compounds and flavonoids. Also, the cocoa shell extract can promote radical sequestration (DPPH) and reduce iron (FRAP). SSF with *P. roqueforti* increased the antioxidant capacity (DPPH), produced saponins, reduced the content of saturated fatty acids, and increased the content of oleic,

linoleic, and gamma-linolenic acids (which are important for good health). Based on our findings, we propose that SSF of the cocoa meal residue byproduct of the cocoa industry by *P. roqueforti* can be applied in the food industry as a replacement for synthetic antioxidants.

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