



Lactobacilli Cultures against Ochratoxin A Producing Moulds Isolated from Cocoa in the South West Region of Cameroon

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Authors' contributions

This work was carried out in collaboration between all authors. Author BTF designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BGN and GTN managed the experiments. Authors SW and RN managed the analysis of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The presence of ochratoxin A (OTA) in cocoa beans is a major health concern, due to its deleterious effects on humans and animals. During the traditional processing and storage of cocoa, fungi contamination occurs. Many of these fungi, produce mycotoxins that can cause acute or chronic intoxication and damage to human and animals after ingestion of the contaminated food and feed. The bio-control of ochratoxigenic moulds by lactic acid bacteria (LAB) could provide a safer alternative compared to the use of fungicides. The present study aims at investigating the potential of selected lactobacilli strains to inhibit the growth of OTA producing moulds. Cocoa samples were collected in the South West region of Cameroon. The mycoflora of cocoa beans were isolated and identified using phenotypic characteristics on Potato Dextrose Agar (PDA) and

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microscopy after application of lactophenol cotton blue stain. LAB used were isolated from fermented palm and raffia wines and fermented pineapple juice by pour plate method on De Man Rogosa and Sharpe Agar. Phenotypic identification of LAB were identified using API 50 CHL kit. Colony PCR was used to confirm whether the isolates belong to LAB group. The inhibitory activity of *Lactobacillus sp* against ochratoxigenic moulds was carried out on agar using the overlay method and in broth by turbidimetric analysis. *Aspergillus sp* were the most prevalent fungi in cocoa samples. *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus niger* were isolated in all samples. *Lactobacillus plantarum* exhibited the highest inhibitory activity against OTA producing moulds. The percentage of inhibition was ranged from 10 to 46.5%. *A. ochraceus* was the most susceptible mould with the various LAB isolates. The quantification of OTA using ELISA kit showed significant reduction in OTA production ($p < 0.05$) during mixed culture of moulds with lactobacilli. Lactobacilli from fermented drinks could be used for biological control of OTA producing fungi.

Keywords: Cocoa; ochratoxin A; *Aspergillus carbonarius*; *Aspergillus ochraceus*; biocontrol.

1. INTRODUCTION

In Africa, Cameroon is the fourth leading producer of cocoa and currently the world's fifth leading cocoa producer after Ivory Coast, Ghana, Indonesia and Nigeria. Cocoa production in Cameroon was estimated at 256,000 tons in 2012 harvest. The South-Western region adjacent to the Nigerian border has dominated Cameroonian production accounting for close to 60% of total production (Kumba being the main area of production) [1]. Production in recent decades in other Cameroonian regions has increased rapidly, most notably in the Centre Region which now produces an almost similar volume to the South West Region [2].

Processing of cocoa entails harvesting of ripe fruit, manually from trees. The pods are then opened to remove the cocoa beans and pulp as soon as possible. Depending on fermentation practice; heaped, bags or boxes, the cocoa beans are allowed to ferment. Fermented cocoa beans are usually sun dried in an open dry yard or suspended on tables. Sun and mechanical drying can be combined and used together. After adequate drying, the cocoa beans are sorted to remove flat beans, shrivelled beans, black beans and other damaged beans. The beans are then put into appropriate bags and stored [3]. Contamination by OTA-producing fungal spores set in during the opening process of cocoa pod and subsequent processes involved in conversion of the cocoa beans to other cocoa derivatives [3].

Ochratoxins are a group of mycotoxins produced as secondary metabolites by several fungi of the *Aspergillus* or *Penicillium* families and are weak organic acids. The family of ochratoxins consists of three main members, A, B, and C which differ slightly from each other in chemical structure.

These differences, however, have marked effects on their respective toxic potency. Ochratoxin A (OTA) is the most abundant and hence the most commonly detected member; it is also the most toxic of the three. It is a potent toxin affecting mainly the kidney [4,5]. It was concluded by the Committee on Toxicity of Chemicals in Food, Consumer Products and Environment (COT) that OTA is a genotoxic carcinogen, and proposed that levels in foods be reduced to the lowest level that can be technologically attained [6]. In 1993, the International Agency for Research on Cancer (IARC) classified ochratoxin A as a possible human carcinogen (Group 2B) and concluded that there was sufficient evidence in experimental animals for the carcinogenicity of ochratoxin A and inadequate evidence in humans for the carcinogenicity of ochratoxin A [7]. The joint expert Committee on Food Additives of the WHO and FAO set a provisional maximum intake of 100 ng/kg body weight (bw), while the Scientific Committee on Food of the European Union proposed that the maximum daily intake of OTA should not exceed 5 ng/kg bw [8].

Lactic acid bacteria (LAB) are a group of Gram positive, non- spore forming, cocci or rod shaped, catalase- negative and fastidious organisms [9]. They are 'Generally Regarded as Safe' (GRAS) and produce desirable organoleptic changes in fermented foods [10,11]. LAB possess antimicrobial molecules such as lactic acid, hydrogen peroxide, carbon dioxide and bacteriocins known to inhibit food borne pathogens and spoilage microorganisms, thereby extending the shelf life and enhancing the safety of food products [12,13,14].

The reduction of OTA in food relies on two main strategies: either by avoiding the growth of fungi, or by removal of OTA already present in food [15]. The second strategy entails the

detoxification of OTA through hydrolysis of the OTA to its non-toxic form ochratoxin α [16,17]. These two strategies require physical, chemical, biological and/or a combination of either approaches to reduce OTA. The control of cocoa pod diseases and toxin contamination by fungi still depends largely on the use of chemical fungicides applied as pre-harvest sprays during the period of cocoa development. These fungicides have various levels of toxicity for the user, the consumer and the environment [18]. Also, there is the use of some chemicals such as benzoic acids, sodium benzoate and sorbic acid to which these pathogenic moulds are becoming resistant to [19,20]. The use of lactic acid bacteria (LAB), provides a friendlier approach for the reduction of ochratoxigenic fungi which is both environmentally and humanly safe [21].

The aims of the present study were (i) screening of potential ochratoxin A producing fungi in cocoa samples from South west Region of Cameroon, (ii) assessment of inhibitory activities of selected LAB against potential ochratoxigenic fungi.

2. MATERIALS AND METHODS

2.1 Sample Collection

Cocoa samples were collected from three areas of cocoa farming within the South west Region of Cameroon. A total of 55 samples were collected. Twenty five (25) samples from Kumba, 15 samples from Muyuka and 15 samples from Muea. The dried cocoa bean samples (ready for sale) were obtained from cocoa farmers at their ware houses. Before collection, inquiries were made to ensure that the cocoa beans underwent proper cocoa processing procedures especially the number of days allowed for the cocoa beans to ferment (6 days maximum). Approximately 15 g per sample (containing at least 15-20 cocoa beans) were collected into sterilized containers (JRZ PLASTILAB^{S.A.R.L}, Lebanon) and later enclosed in zip lock bags containing silica gel to dry up moisture. Samples were then immediately transported to the laboratory and kept at 4°C in a refrigerator (EPIA) inside the zip lock bags until analysed.

2.2 Culture, Isolation and Identification of Microorganisms

For the mycological analysis of dried cocoa bean, samples were soaked in physiological saline; NaCl, 0.85% (w/v). Six Cocoa beans from each sample were soaked in 10 mL of

physiological saline and allowed for 15 minutes (inoculum). The surface of Potatoe Dextrose Agar (PDA) (LIOFILCHEM DIAGNOSTICI) supplemented with 0.1% (w/v) chloramphenicol to inhibit bacterial growth was inoculated and the petri dishes were incubated at 25°C for 7 days. Isolated moulds were set apart according to the identification key from Fungi and Food Spoilage [22].

Lactic acid bacteria used in this study were isolated from pineapple, palm and raffia wines. Under aseptic conditions juice was extracted from pineapple by slicing through the fruit to get the inside using a knife and then squeezed into a sterile container (JRZ PLASTILAB^{S.A.R.L}, Lebanon). Pineapple juice, palm wine and raffia wine were allowed to natural fermentation for a week at room temperature. Prior to plating, processed fermented samples were serially diluted in a 1:10 dilution using peptone water. The liquid samples were thoroughly mixed by inversion before dilution in which 1 ml of sample was pipetted into 9 ml of peptone water. Dilutions were repeated up to a dilution of 10^6 . One mL from each dilution was inoculated into petri dishes (JRZ PLASTILAB^{S.A.R.L}, Lebanon) and 15 mL of MRS agar was added into each of these petri dishes. After solidification, the plates were incubated at 30°C for 48 hours. Morphological characteristics and series of biochemical tests (Catalase test, Gram stain and API 50CHL) were proceeded for phenotypic identification of the various lactic acid bacteria. The slide catalase test and Gram staining as were performed respectively as described by Prescott [22] and Cheesbrough [23]. Carbohydrate fermentation profiles of were determined using the API 50CHL (BioMerieux, SA) kit according to manufacturer's instructions.

2.3 Molecular Identification of Lactic Acid Bacteria

2.3.1 Extraction template DNA

DNA extraction from pure cultures of isolate was carried out by colony PCR protocol. A Single colony from each pure culture was suspended in 100 μ L of sterile distilled water in eppendorf tubes and each tube was vortex for 30 seconds. Each suspensions was boiled using thermomixer comfort at 100°C for 10 minutes to lyse the cells and inactivate nucleases. The suspensions were centrifuged using Biofuge fresco centrifuge at 9500 g for 5 mins. Each supernatant was carefully collected and put in clean PCR tubes and used as templates for PCR.

2.3.2 Amplification of 16S rDNA region of the selected strains by PCR reaction

16S rDNA of selected strains was amplified by PCR using the primers 16S1fw: 5'AGAGTTTGATCCTGGCTCAG 3' and 16S2r: 5'ACGGCTACCTGTAAACGACTT 3'. The forward primer is complementary to the 5' end of 16S rDNA, and the reverse primer is complementary to the 3' end of 16S rDNA region, 5 µL of each template was used. Amplification was performed in a 25 µL total reaction volume containing 12.5 µL of Master Mix, 2.5 µL of each primer, 2.5 µL of sterile distilled water and 5 µL of template DNA. PCR reactions were performed in a Peltier thermal cycler.

2.3.3 Separation of amplified fragments

After the completion of PCR reaction, amplified products were separated in a 1.5% (w/v) agarose gel. For this purpose, 1.5 g agarose was dissolved in 100 mL 1x TAE buffer and the agarose solution was boiled. Agarose solution was cooled to nearly 40°C. After cooling, 1.5 µL ethidium bromide solution (10 mg/mL) were added. The agarose gel was poured into the gel casting stand and the combs were placed. When the gel was solidified, the combs were removed. For loading, 10 µL of amplification were loaded into wells. After the loading of samples, 5 µL of DNA molecular weight marker (Gene Ruler, Fermentas) were loaded into the first well. Finally, electrophoresis was performed using instrument H5 Horizontal gel electrophoretic system, at 100 mA. Amplification products were visualized in a gel imager documentation system called BIO-RAD molecular imager Gel Doc™ XR+ with image laboratory™ software. The presence of DNA fragments sized between 1500-2000 bp indicated that targeted amplification was achieved.

2.4 Biocontrol of Ochratoxigenic Moulds by Lactic Acid Bacteria

2.4.1 Biocontrol on agar

Inhibitory activity of lactic acid bacteria on potential ochratoxigenic mould isolates was determined by the overlay method as described by Magnusson and Schnurer [24] with some modifications. LAB were inoculated in two 2 cm lines on MRS agar plates and incubated at 30°C for 24 hours and incubate anaerobically. The plates were then overlaid with 10 ml of Sabouraud Dextrose Agar (LIOFILCHEM

DIAGNOSTICI) containing 10⁵ conidia/ml of each mould. After 7 days of aerobic incubation at 30°C. For each test, a negative control with only the mould spores sown was performed and this was used to determine the maximum surface colonisation of the mould in the absence of the LAB culture. The zone of inhibition appeared generally as a rectangle was measured and expressed in cm². The percentage of mould growth inhibition (I%) was calculated as follow:

$$\% \text{ Inhibition} = \frac{S_i}{S_c} \times 100$$

Where S_i is the surface area of the mould inhibition (cm²) on the test plate and S_c the surface area (cm²) of the mould in the control plate. For each combination of mould and LAB, three tests were performed and the percentage of inhibition expressed as the mean of the three values.

2.4.2 Biocontrol in broth

Cell free supernatant (CSF) of the selected LAB were used to assess their inhibitory activity on moulds. LAB strains were cultured in MRS broth at 30°C for 72 hours. The cultures were centrifuged using a centrifuge (PRISM R) at 3000 g, 4°C for 10 minutes and the supernatant fluid filtered through a 45 µm pore-size filter (Millex-GP, MILLIPORE, Ireland). The sterile filtrate was stored at 4°C till usage within 24 hours.

Using sterile pipettes (RAININ, USA) 30 µL of each cell-free supernatant and 20 µL of each of the test organisms (mould spore suspension) was dispensed into microtitre plate wells containing 200 µl MRS growth medium (MRS broth; LIOFILCHEM DIAGNOSTICI). Incubation was done under shaking conditions at room temperature. A known antifungal drug (fluconazole) was used as positive control. The negative control comprised of 20 µL of each spore suspension dispensed into 230 µL of growth medium and the internal control contained the growth medium alone. Experiment was done in duplicates. Absorbance was read at wave length 630 nm using a spectrophotometric reader (Human reader HS Germany) after every 12 hours for 72 hours.

2.5 Quantification of OTA

The different moulds isolated from cocoa were cultured in broth for 28 days at 30 °C in the absence and in the presence of selected LAB.

OTA was quantified in broth in pure culture and in combination with LAB using ELISA method as described by Fujii et al. [25] with some modifications. Microtitre plates were coated with 100 mL of 4.76 mg/mL OTA-BSA in 0.1 M carbonate-bicarbonate buffer pH 9.6 overnight at 4°C. After washing four times with PBS-0.05% Tween 20 (PBST), wells were blocked for 4 hours at 25°C with 200 mL of 0.1% ovalbumin in PBS. After washing four times with PBST, 50 mL OTA standard or coffee extracts and 50 mL anti-OTA MAb (2000-fold dilution) in PBST were added, and incubation carried out for 18 h at 4°C. After washing four times with PBST, 100 mL HRP-labeled goat antimouse IgG (10³-fold dilution in PBST) were added, incubated at 25°C for 1 hour, and washed as previously described. Then, 100 mL of TMB substrate solution were added. After 1 hour at 25°C, the reaction was stopped by adding 50 mL of 1 M H₂SO₄ and absorption was measured at 450 nm (ELX800 ELISA reader, Bio-Tek Instruments, USA). Average absorbance was calculated from individuals absorbance obtained from triplicate wells and results were expressed as the percentage of binding: Binding (%) = (A+/A-) x 100, where A+ is the mean absorbance in the presence of each sample or standard and A- is the mean absorbance in their absence. The OTA concentration was determined using a calibration curve (0.2 to 10.0 ng OTA/mL), plotting percent binding against OTA concentration.

3. RESULTS

3.1 Occurrence and Characterisation of Moulds in Cocoa Samples

There was visible fungal growth in each cultured plate. Each cultured plate had more than one type of mould growing in the plate. The moulds isolated were identified to be from the genus *Aspergillus*, *Mucor*, *Rhizomucor* and *Rhizopus*. The different mould isolates were identified using their cultural characteristics (macroscopic observation) and microscopically after staining with lactophenol cotton blue stain. Table 1 shows the different identified moulds based on phenotypic characteristics. The various moulds isolated showed macroscopic and microscopic differences making identification of these moulds to the genus level and for some to the species. Figs. 1 and 2 show the presentation of the various isolated moulds (a and b is the inverted and front view of moulds grown in PDA after 7 days, c is the microscopic view of mould

showing spore and hyphal morphology using the x40 objective lens).

The genus *Aspergillus* showed different species upon identification as seen below (Figs. 1-2): *Aspergillus carbonarius*, *Aspergillus niger*, *Aspergillus ochraceus* and two different *Aspergillus sp.* Table 2 shows the different *Aspergillus* species with their rate of occurrence per area. *Aspergillus* was most frequent in samples collected from Muea with a frequency of 12 out 15 samples collected (80.0%) followed by samples collected from Muyuka recording 53.3% (8/15) and the least in Kumba with a frequency of 16.0% (4/25). *Aspergillus carbonarius*, was the mould with the highest frequency with 29.2%, while *Aspergillus ochraceus* had the least frequency recording 12.5% amongst the *Aspergillus* species.

3.2 Lactic Acid Bacteria Identification

The Gram stain reaction after obtaining catalase negative isolates, showed gram positive rods which were considered presumptive LAB isolates. The identification of lactic acid bacteria was proceeded with different biochemical tests using API 50CHL kit and molecular characterisation by amplification of 16S rDNA. Table 3 presents the results of the various biochemical test performed to screen and identify the various LAB isolates. Table 3 presents the full API test result after 48 hours of incubation.

3.3 Genotypic Identification of Lactic Acid Bacteria

After DNA isolation, the genus specific primers designed for the amplification of 16S rDNA were able to amplify the region giving ~1.5 kb size fragment in all of the 5 isolated strains (Fig. 3).

3.4 Inhibition of Ochrotogenic Moulds Using LAB on Agar

This was a co-culture experiment which made use of the overlay method. The best antifungal activity of LAB was against the mould *Aspergillus ochraceus* while *Aspergillus sp 2* was the least inhibited mould. Inhibitory activity was best observed by LAB isolate Ra₁ isolated from raffia wine while LAB isolate Pi₁ isolated from pineapple, recorded the least inhibitory activity against the various moulds. The various LAB isolates were able to inhibit mould growth. The

zone of inhibition varied with the different LAB isolates against the moulds. Fig. 4 show the zone of inhibition by two different LAB (Pi_2 and Ra_1 respectively) isolates on the same mould (M1). The percentage of inhibition by selected LAB is shown if Fig. 5

Table 1. Phenotypic identification of various moulds in cocoa

Groups (n)	Sub groups (n)	Macroscopic colony characteristics		Microscopic characteristics	Name of mould
		Front view	Reverse view		
1 (24)	A (7)	Velvet-like black colonies	Colourless to cream white	Aseptate conidiophore. Large dark conidia	<i>A. carbonarius</i>
	B (5)	Velvet-like black colonies	Cream white to pale yellow	Aseptate conidiophore. Small dark conidia	<i>A. niger</i>
	C (3)	Powderish grey colonies	Pale yellow to brown	Aseptate conidiophore	<i>A. ochraceus</i>
	D (4)	Floccose bright yellowish-green colonies	Pale yellow to brown	Aseptate conidiophore. Globular conidia	<i>Aspergillus sp[#]</i>
	E (5)	Floccose brown colonies	Cream white	Aseptate conidiophore	<i>Aspergillus sp[#]</i>
2 (28)		White cotton like growth with black spots	Colourless	Dark sporangia having spores. No rhizoids	<i>Mucor</i>
3 (17)		White cotton like colonies overlying mucous layer	Colourless	Sporangia having spores. Rhizoids present	<i>Rhizomucor</i>
4 (27)		Cotton like white growth with black spots	Colourless	Sporangia having spores. Umbrella-like columellae	<i>Rhizopus</i>

n = number of isolates; # = unidentified *Aspergillus* species

Table 2. Frequency of *Aspergillus* species in cocoa samples

Area						Total	Occurrence/area (%)
	<i>Aspergillus carbonarius</i>	<i>Aspergillus niger</i>	<i>Aspergillus ochraceus</i>	<i>Aspergillus sp 1</i>	<i>Aspergillus sp 2</i>		
Muea	4	4	1	-	3	12/15	80.0
Muyuka	2	1	1	3	1	8/15	53.3
Kumba	1	-	1	1	1	4/25	16.0
Total	7	5	3	4	5	24/55	43.6
Occurrence/species (%)	29.2	20.8	12.5	16.7	20.8	100	

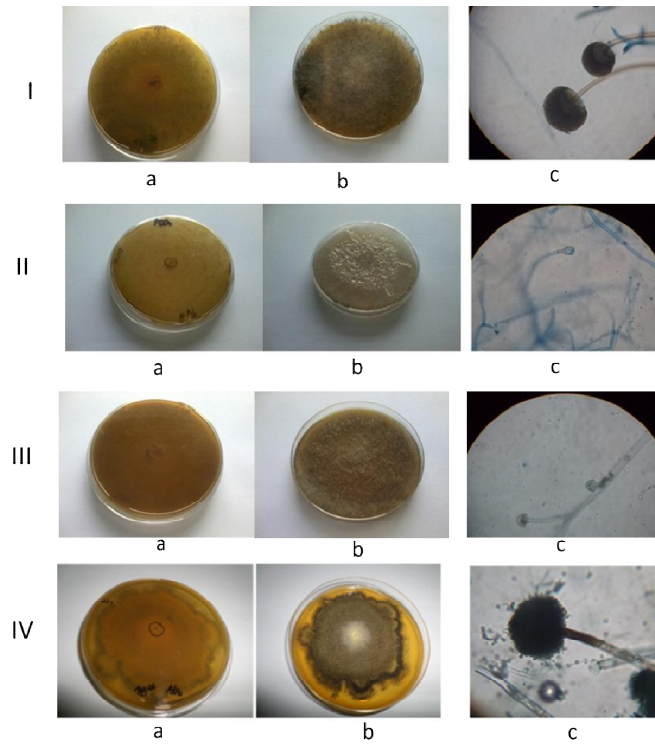


Fig. 1. Isolated moulds; a: inverted view, b: front view, c: view under light microscope. I: Mucor, II: Rhizomucor, III: Rhizopus, IV: Aspergillus niger

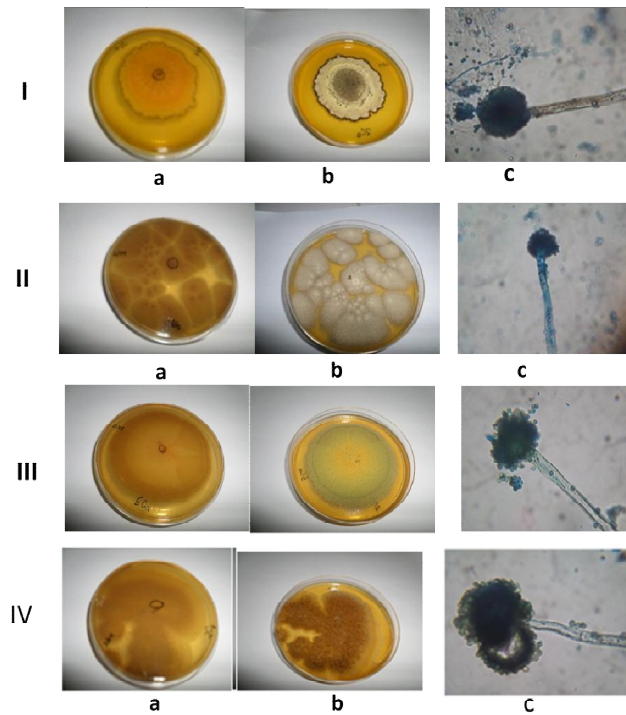


Fig. 2. Isolated Aspergillus sp (continuation); a: inverted view, b: front view, c: view under light microscope. I: Aspergillus carbonarius, II: Aspergillus ochraceus III: Aspergillus sp 1, IV: Aspergillus sp 2

Table 3. Biochemical characteristics of LAB isolates determined using API 50 CHL

Test number	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	
Strains code	Control	Glycerol	Erythritol	D-arabinose	L-arabinose	Ribose	D-xylose	L-xylose	Adonitol	β methyl-D-Xyloside	Galactose	Glucose	Fructose	Mannose	Sorbose	Rhamnose	Dulcitol	Inositol	Mannitol	Sorbitol	Methyl-D-mannoside	Methyl-D-glucoside	N-Acetyl-Glucosamine	Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	
Pa ₁	-	-	-	-	-	+	-	-	+	-	+	+	+	-	-	+	-	+	+	-	?	+	+	+	+	+	+	+	+
Pi ₁	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-
Pi ₂	-	?	-	?	-	+	-	-	-	-	+	+	+	+	+	?	+	-	+	+	-	+	+	+	+	+	+	+	+
Ra ₁	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	+	-	-	-	-	+	-	-	+	+	+	+	+	+
Ra ₂	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Test number	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	Identified species						
Strains code	Maltose	Lactose	Melibiose	Sucrose	Trehalose	Inulin	Melezitose	Raffinose	Starch	Glycogen	Xylitol	β Gentiobiose	D-turanose	D-lyxose	D-tagatose	D-fucose	L-fucose	D-arabitol	L-arabitol	Gluconate	2-Keto-Gluconate	5-Keto-Gluconate							
Pa ₁	+	-	-	+	+	-	+	-	?	?	-	+	+	-	+	-	-	-	-	?	-	-	<i>Lactobacillus casei pseudoplantarum</i>						
Pi ₁	+	+	-	+	+	+	-	-	?	?	-	-	+	-	+	-	-	-	-	?	-	-	<i>Lactobacillus plantarum</i>						
Pi ₂	+	+	+	+	+	-	-	+	?	?	-	+	?	-	-	-	?	-	-	?	-	-	<i>Lactobacillus casei alatosus</i>						
Ra ₁	+	+	+	+	+	-	+	+	?	?	-	+	?	-	-	-	-	-	-	+	-	-	<i>Lactobacillus plantarum</i>						
Ra ₂	+	+	+	+	+	-	+	+	-	-	-	+	+	-	+	-	-	?	-	?	-	-	<i>Lactobacillus plantarum</i>						

+, Positive reaction; -, Negative reaction; ?, Non-conclusive

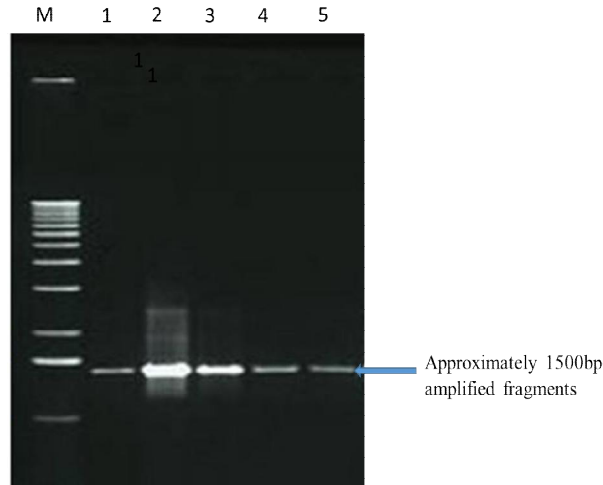


Fig. 3. PCR amplification pattern of 16S rDNA of the lactic acid bacteria strains isolated on 1.5% agarose gel. Lane M; DNA markers, 1: Pa1, 2: Pi1, 3: Pi2, 4: Rai1, 5: Ra2

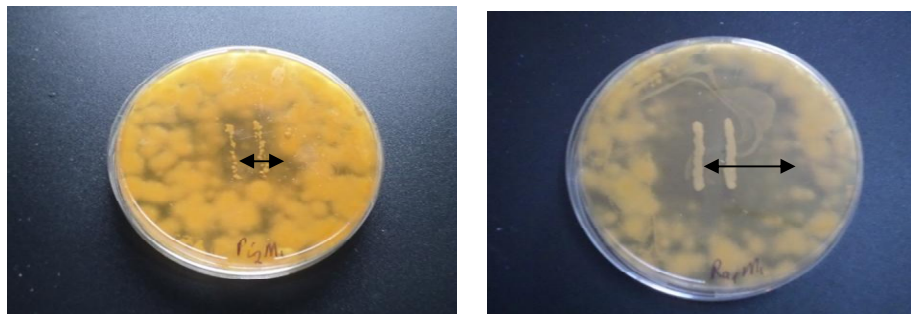


Fig. 4. Plate assays for inhibition of ochratoxigenic moulds using LAB. Clear zone of growth inhibition of mould (M1) formed around the streak lines of lactic acid bacteria incubated at 30°C for 7 days by overlay method

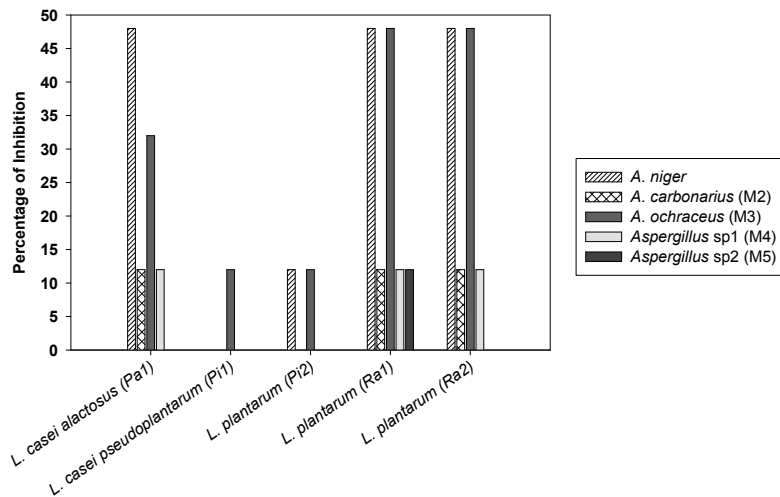


Fig. 5. Percentage of inhibition of potential ochratoxigenic moulds by selected LAB

3.5 Inhibition of Ochratoxigenic Moulds Using LAB in Broth

The turbidometric method was used which directly measures the effect of the cell free supernatant of different lactic acid bacteria on the growth of the various moulds via changes in turbidity (optical density). Fig. 6 shows the overall activity of lactic acid bacteria against the various moulds after 72 hours by measuring the optical density at wave length 630 nm.

Results were interpreted based on the difference in absorbance of each co-culture containing the mould and lactic acid bacteria compared to the absorbance obtained when each mould was cultured alone. *Aspergillus ochraceus* was the most inhibited mould while *Aspergillus niger* the least inhibited mould. LAB Isolate Ra₁, was the

most active antifungal isolate capable of inhibiting mould growth. This inhibitory activity of LAB Isolate Ra₁ was most evident against the mould *A. ochraceus* with a P value of 0.006 at 95 % confidence level using the Post Hoc Turkey test.

3.6 Quantification of OTA

Production of OTA was observed in *A. ochraceus*, *A. carbonarius* and *Aspergillus* sp. Table 4 shows the amount of OTA produced by *A. ochraceus* in the absence and in the presence of selected LAB strains. All were able to reduce the production of OTA in fermenting broth when they are cultured in combination with moulds. The reduction on OTA production was more important in the presence of *L. plantarum* (Ra 1 and Ra 2).

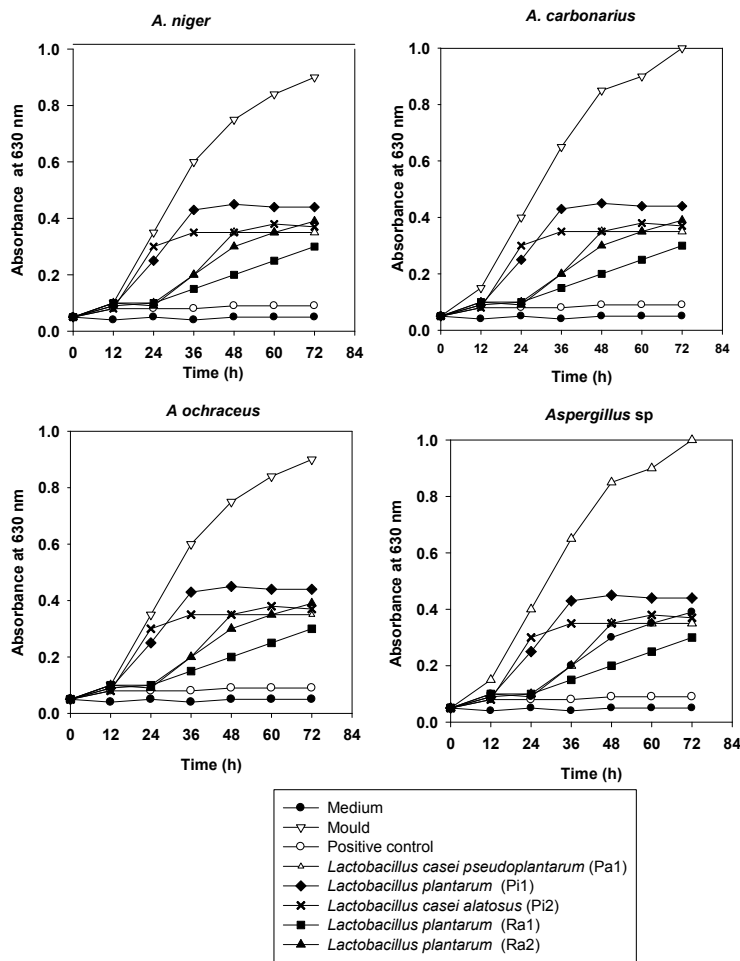


Fig. 6. Effect of cell free supernatant of selected LAB on the growth of potential OTA producing moulds

Table 4. OTA production in broth by *A. ochraceus* in absence and in presence of different strains of lactic acid bacteria

Lactic acid bacteria strain	OTA production (ng/mL)	
	Control (In absence of LAB)	In co-culture with LAB (2% v/v 10 ⁸ CFU/mL inoculated)
<i>Lactobacillus casei pseudopantarum</i> (Pai)	8.3±0.4	2.5±0.5
<i>Lactobacillus plantarum</i> (Pi1)	8.3±0.4	1.5±0.3
<i>Lactobacillus casei alatosus</i> (Pi2)	8.3±0.4	1.2±0.1
<i>Lactobacillus plantarum</i> (Ra1)	8.3±0.4	0.1±0.0
<i>Lactobacillus plantarum</i> (Ra2)	8.3±0.4	0.3±0.1

4. DISCUSSION

This study was conducted in the South West Region of Cameroon, which has an equatorial climate. It rains almost throughout the year. Annual rainfall often averages 1500 mm [26]. This rainfall implies high humidity (~ 90%), which promotes the growth of many microorganisms that can influence the final quality of cacao from that region.

Moulds and their toxins have been established as important food poisoning organisms. This has been partly attributed to their wide distribution in nature as their presence in food products can be traced back to the environment [27]. The ubiquity of these organisms makes it quite difficult to prevent their contamination of food and food products.

The main moulds isolated in this study belonged to the genera *Aspergillus*, *Mucor*, *Rhizopus* and *Rhizomucor* with some species known to produce OTA (*Aspergillus niger*, *Aspergillus carbonarius* and *Aspergillus ochraceus*). Some of these moulds were found by Mounjouenpou et al. [28] when studying the filamentous fungi during cocoa processing in Cameroon and Amézqueta et al. [27] who isolated these moulds from stored cocoa beans, and both had *Penicillium* and *Fusarium* included among the isolated moulds. *Aspergillus* was the only mycotoxin-producing storage mould isolated compared to studies carried out by Mounjouenpou et al. [28,1], which had *Aspergillus* and *Penicillium* as the storage moulds being isolated. This study was in accordance with the isolated field moulds as identified by Mounjouenpou et al. [28,1] and Amézqueta et al. [27], that is having *Mucor* and *Rhizopus* with the exception of *Rhizomucor*.

Storage moulds have been known not to cause only food spoilage but also have contributed to

food poisoning through the production of mycotoxins; which are secondary metabolites known to cause adverse health conditions to animals and humans who consume foods containing these moulds [29]. Mycotoxins seem able to cause serious diseases of the liver, kidney and blood-forming organs even in extremely low quantities. *Aspergillus* a mycotoxin producing mould is incriminated as a health hazard agent [18].

In this study *Aspergillus* species producing the mycotoxin ochratoxin A were investigated: *Aspergillus niger*, *Aspergillus carbonarius* and *Aspergillus ochraceus*. Two other *Aspergillus* sp (1 and 2) which may not be OTA producing moulds were also reported in this work. Incidence of these moulds differed in both species and areas of sample collection. The results revealed that the cocoa samples collected from Muea had the highest *Aspergillus* frequency 80.0% (12/15) followed by Muyuka with 53.3% (8/15) and lastly by Kumba with occurrence of 16.0% (4/25). Despite the fact that more samples were collected from Kumba compared to the other areas, Kumba still recorded the least *Aspergillus* frequency. This might have a proportionate reflection on cocoa farmers' training, on the sanitary practices followed in cocoa beans preparation and storage at the different areas. Also, environmental conditions (weather and climate) may have an influence on the presence of *Aspergillus* in this areas.

Lactic acid bacteria (LAB) can be isolated from a variety of foods. It is the main organism responsible for fermentation in these foods. Lactic acid bacteria are found in dairy products, fermented meat, sour dough, fermented vegetables, fruits, silage beverages- including wine, on plants, in sewage, [30,31]. Growth on de Man, Rogosa and Sharpe agar coupled with catalase test and Gram staining are

characteristic methods of isolating LAB. From growth on MRS agar, catalase negative isolates (due to their inability to break down hydrogen peroxide into water and oxygen) are considered presumptive LAB isolates.

Results revealed that these isolates were all Gram positive (retained primary stain colour-purple) rods and catalase negative from the genus *Lactobacillus*. The identification of LAB is principally through biochemical methods which has been well studied [32,33]. API 50 CHL kit (API system, BioMérieux, France) is widely used for identification of lactic acid bacteria. This method is simple, and involves the fermentation of different carbohydrates to acids which causes a decrease in pH, after 48 hours of incubation [34]. The decrease in pH is shown by a change in colour of the indicator, from purple to yellow with the exception of the esculin test (tube 25), that changes from purple to black. Five LAB isolates were identified, one isolate from palm wine, two from pineapple and the remaining two from raffia wine. These isolates were identified as follows: *Lactobacillus plantarum* (LAB isolates Pi₂, Ra₁ and Ra₂), *Lactobacillus casei pseudopantarum* (LAB isolates Pi₁) and lastly *Lactobacillus casei alactosus* (LAB isolates Pa₁). In 1980, on the basis of phenotypic criteria, five subspecies of *L. casei* were recognized, that is, *L. casei* subsp. *alactosus*, *L. casei* subsp. *casei*, *L. casei* subsp. *pseudopantarum*, *L. casei* subsp. *rhamnosus*, and *L. casei* subsp. *Tolerans* [35].

As stated by Dellaglio & Felis [36], phenotypic methods alone are inadequate for identification of lactic acid bacteria and should be confirmed by molecular identification methods to achieve a reliable identification. Theoretically, the amplicon size of 16S rDNA region on the genomic DNA of lactic acid bacteria is 1500 bp. In the present study bacteriocin producing strains were identified by PCR technique to confirm if they were actually LAB. The 16S rDNA region of each bacteriocin producing strain was amplified. The length of amplification products varied between 1000 to 2000 bp. This results shows that the 6 bacteriocin producing strains isolated from this study were lactic acid bacteria because the amplicon sizes of their 16S rDNA region amplified was approximately 1500 bp.

The inhibition of mould growth has been carried out in many other studies using both chemical and physical agents [37]. However, bio-control through the use of microorganisms has proven to

be more effective in inhibiting mould growth [38]. Lactic acid bacteria are 'Generally Recognised as Safe' (GRAS) microorganisms producing many antimicrobial agents including organic acids, carbon dioxide, hydrogen peroxides and bacteriocins [13]. These properties in LAB make them even more suitable for investigating their antifungal activity in food stuff.

In this study, the inhibition of ochratoxigenic moulds by LAB which will ultimately lead to OTA reduction was carried out on agar medium using the overlay method and in broth using turbidometric method. The different LAB used, revealed varied inhibitory activity amongst the different ochratoxigenic moulds with inhibitory activity being highest and most significant with isolate Ra₁ gotten from raffia wine.

The bio-control on agar gives a qualitative but semi- quantitative analysis of the inhibitory power of the different LAB isolates while the bio-control in broth gives a quantitative and qualitative analysis of the inhibitory power of the different LAB isolates. The varying inhibitory activity of LAB on these moulds may be attributed to several factors such as production of organic acid, phenolic compounds [38,39], hydroxyl fatty acids, hydrogen peroxide [40,41], reuterin and proteinaceous compounds [42]. These chemical products react with each other under various conditions to bring about the inhibitory activity of LAB on moulds.

LAB isolate Ra₁ identified as *Lactobacillus plantarum*, showed the highest inhibitory effect on the isolated moulds in both bio-control methods. *Lactobacillus plantarum* is one of the main LAB species on which most antifungal reports have been based on. Hamed et al. [42] reported that *L. plantarum* isolated from yogurt and milk, showed inhibitory activity against *Fusarium oxysporum* and provide protective effect to tomato plants. *L. plantarum* strains have also been used as antifungal adjuncts for increased shelf life of yoghurt [43]. Lavermicocca et al. [44] also reported the broad spectrum antifungal activity of *L. plantarum* on some fungi (*Eurotium*, *Penicillium*, *Endomyces*, *Aspergillus*, *Monilia* and *Fusarium*). Isolate Pi₁ identified to be *L. casei pseudopantarum*, showed very little inhibitory activity on mould growth. Despite reports on antifungal activity of *L. casei* that is *L. paracasei paracasei* and *Lactobacillus casei rhamnosus* [45], there are no reports on the antifungal activity of *L. Casei alactosus*. Very little is said on the antifungal activity of *L. casei*

pseudopiantarum. Gourama and Bullerman [46], reported that *L. casei pseudopiantarum* was capable of inhibiting the growth of *Aspergillus flavus*. In this work, even though *L. casei pseudopiantarum* showed the least inhibitory activity against mould growth as a whole, it was still able to reduce mould growth to some minute extent; therefore its antifungal ability cannot be totally ruled out. The overall inhibitory activity of the different LAB isolates was most effective against *Aspergillus ochraceus* (mould isolate M3). Reports from Matei and Cornea [45], showed that some 11 lactic acid bacterial strains have antifungal activity on both *Aspergillus ochraceus* and *Penicillium digitatum* with 4 out of the 11 being prominent. Also, findings by Massawe and Lifa [47], revealed that some yeasts *Pichia spp* in wet coffee processing in combination with selected strains of LAB (*Leuconostoc/Weissella* and *Lactobacillus spp*) could be used as biocontrol agents against *A. ochraceus*. Moreover, in the study by Belkacem-Hanfi et al. [48], on lactic acid bacteria against post-harvest moulds and ochratoxin A isolated from stored wheat, they showed that post-harvest moulds (*Penicillium expansum*, *Penicillium chrysogenum*, *Penicillium glabrum*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Fusarium graminearum* and *Alternaria alternata*) were inhibited by LAB; strains of *L. plantarum* exhibited a large antifungal spectrum of activity.

The different inhibition zones (bio-control on agar) and absorbance (bio-control in broth) observed in this work can be accounted for by the different antifungal properties of the selected LAB and the difference in sensitivity of the mould strains towards the variation and concentrations of the antifungal compounds produced. Laitila et al. [49], observed that the antifungal effect greatly depends on the contamination level, the fungal species and strain. In fact in this study, the same LAB species did not have the same inhibition activity towards the moulds tested.

The prevention or reduction of fungi presence is actually expensive and not generally recognised as safe if done by chemical methods [50] and the removal of OTA by detoxification approach requires that, the food and the micro-organism be put in contact through a liquid phase [15], the best solution applicable to cocoa beans is therefore the prevention of OTA contamination through inhibition of fungi growth by bio-control using lactic acid bacteria having GRAS properties.

5. CONCLUSION

This work revealed the occurrence of OTA producing mould in cocoa samples studied. The use of lactic acid bacteria for the decontamination appeared as a safer method for the control of mycotoxigenic fungi compared to the use of fungicides.

L. plantarum (Ra1) isolated from raffia wine showed the highest inhibitory activity against OTA producing moulds and contributed in a significant reduction of OTA level when it was cultured in combination with moulds. This observation allows to suggest that lactobacilli cultures can be used for an effective biological control of mycotoxin during the traditional processing of cocoa.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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