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Original paper

Antifungal properties of lactic acid bacteria isolated from cocoa beans fermentation in the centre region of Cameroon

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Abstract

The objective of this study was to identify lactic acid bacteria (LAB) with antifungal activity usable in the fermentation of cocoa beans to limit the moulds growth and to characterize their inhibitory mechanism. A total of 28 LAB strains were isolated from fermented cocoa beans and tested for their antifungal activity against 10 moulds of the genus *Aspergillus* and *Penicillium*. An initial selection was made using the transversal method and the inhibition percentage was determined. The most active isolates were subjected to biochemical and molecular analysis; their cell free supernatant was analysed by HPLC and submitted to various treatments (acid neutralization, heat and enzymes). The best lactic acid selection has been subjected to molecular identification and were identified by molecular tools and belong to the species *Lactobacillus farciminis*, *Lactobacillus brevis*, *Pediococcus pentosaceus*, *Weissella cibaria* and *Enterococcus faecium.*

Keywords Cocoa, moulds, biological control, bacteria compounds.

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Introduction

Dried cocoa beans are the raw material used in chocolate industry. In the fresh state, they are seeds coated with a white mucilaginous pulp consisting of approximately 80% water, 1.5% pectin, 2%, citric acid, 2.5% protein, 0.05% magnesium sulphate, 0.02% manganese sulphate. The sugar content vary between 10 to 20% and the pH from 3.5 to 5.5 depending of pods' age and season [1, 2].

Cocoa is one of the most important agriculture commodity products; the cocoa beans quality is highly dependent on processing technologies and storage conditions for preventing the defective quality [3]. The beans are then taken out from the harvested cocoa pods, fermented and dried out before they are sold in the market [4]. The fermentation and drying phases are particularly important since they contribute to the development of aroma and flavour precursors which develop later during the roasting of the beans and for preserving the quality of the raw beans [5, 6, 7]. Mould contamination of food and feed is difficult to be predicted because it depends on a complex interaction of factors, such as temperature, moisture, endogenous fungal species, storage history and storage time [8, 9]. Generally, poor post-harvest management can lead to rapid quality deterioration by the initiation of fungal activity [10]. The biological control methods which have low impact on the consumers' health, have received increasing interest in the recent past in the recent past. Several natural environments have proven to be reservoirs of LAB strains with antifungal properties [10, 11, 12]. In this idea, the use of lactic acid bacteria isolated during cocoa fermentation process as biocontrol agents is of interest, being a plausible alternative due to the antimicrobial component they produce [10, 12, 13]. This study aimed at screening the antifungal activity potential of some lactic acid bacteria (LAB) isolated during cocoa beans fermentation and to characterise some of their biologically active compounds.

Materials and Methods

(1) Microorganisms origin

The LAB strains were isolated from fermented cocoa beans collected during heap fermentations conducted by farmers of two villages around Yaoundé and Bafia in the central region of Cameroon.

Therefore, 25g of collected samples were washed in 225 ml sterile NaCl 0.9% (w/v) solution. The solutions obtained were subjected to several decimal dilutions and inoculated in MRS Broth (Man, Rogosa, Sharp) (Merck, USA) adjusted to pH 6.2 and supplemented with agar (1.5%) and nystatin (0.5g.l^{-1}) to inhibit moulds growth. The plates were incubated for 48 hours at 37°C. The colonies were purified by streaking on the same medium. Only Gram-positive, non-spore forming and catalase negative bacterial isolates were selected and stored at -80°C in MRS broth media supplemented with 30% glycerol (v/v) for subsequent uses.

Three other strains were used as antifungal positive control based on a previous study [10], namely: *Lactobacillus plantarum* B-4496 (offered by the Agricultural Research Service (ARS) culture collection of the US Department of Agriculture), *Lactobacillus brevis* 207 and *Lactobacillus sanfranciscensis* BB12 (offered by the Laboratory of Microbiology of Food science of the University of Bologna, Cesena campus).

Ten moulds were used to screen the antifungal activity of our studied LAB, of which two (*Aspergillus carbonarius* NRRL 368, and *A. niger* NRRL 612) were provided by the ARS culture collection and eight strains were previously isolated from cocoa beans and identified as *A. flavus* AF, *A. tamarii* M21, *A. fumigatus* M11, *A. versicolor* M14, *A. versicolor* M22, *A. oryzae* M13, *Penicillium citrinum* M7, *P. citrinum* M8. All of these moulds were stored on slants of Potatoes Dextrose Agar (PDA) (Vwr, USA) at 25°C.

(2) Selection of Antifungal LAB strains

The inhibitory properties of LAB was tested by transversal assay as described by Laref and Guessas [14] with slight modifications. LAB was streaked in two lines of four centimetres apart in an MRS agar Petri dish (Fig. 1) and incubated for 48 h at 37°C. After this time, a disc of one-centimetre in diameter of 4 days old mould culture was cut using a cork borer and placed in between the two lines of LAB cultures and incubated at 28°C for several days. The experiment was conducted in triplicates and the control was the fungus cultivated without LAB presence (Fig. 1). The results were recorded from the third day. The percentage of mould inhibition was calculated using the following formula:

$$\%\cdot of\cdot mold\cdot inhibition = \frac{DC - DT}{DC} x100$$

where DC is the maximum colonization distance of the mould in the control plate and DT is the colonization distance on the test plate in presence of LAB.

(3) Biochemical characterization of the antagonistic lactic acid bacteria

After the evaluation of LAB inhibitory ability, others characteristics of the selected LAB strains with high antifungal activity were observed following the criteria proposed by Holzapfel & al [15], Yimin & al [16] and Kozaki & al [17] on the degradation of carbon substrates and gas production (hydrogen sulphide and carbon dioxide).

(4) Molecular identification of the selected LAB isolates

DNA extraction

LAB strains initially cultivated in MRS broth at 37°C for 48 hours were collected by centrifugation at 5000 rpm for 5 minutes and the DNA extraction performed with QIAampcador Pathogen kit (Qiagen kit), based on the manufacturer's instructions. The obtained DNA was kept at -4°C.

DNA Amplification

The 16S of the rDNA were amplified by Polymerase Chain Reaction (PCR) using specific lactic acid bacteria primers LacF (5'-AGCAGTAGGGAATCTTCCA-3') and LacR (5'-ATTCCACCGCTACACATG-3') of 340 pairs of bases (RITCHIE et al 2010) according to the program proposed by Ponnusamy & al [18]. The PCR reagents used in preparing the Mix-PCR comprised 1.5 mM of MgCl2, 0.5 µM of each primer, 0.025 $U.\mu l^{-1}$ of DreamTaq DNA Polymerase (Thermo Scientific, USA), 0.2 mM of dNTPs, and 10-30 ng (10 µl) of DNA for a final volume of 50 ml. The PCR was conducted in a MultiGene thermocycler (Labnet International, USA) under the following experimental conditions: initial denaturation (94°C/2 minutes); 30 cycles of denaturation (94°C/ 15 seconds), hybridization (51°C/15 seconds), elongation (72°C/30 seconds); final elongation (72°C/7 minutes) [19]. The PCR products were checked on agarose gels (2% W/V) stained with ethidium bromide ($7\mu g.\ \mu l^{-1}$).

DNA sequencing and sequence analysis

Amplified DNA fragments were sequenced by BaseClear (The Netherlands) in both orientations using the primers LacF and LacR. The sequences obtained were analysed using NCBI blast (The National Center for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for their identification and deposited in the NCBI database. The evolutionary history was inferred using the Neighbor-Joining method [20]. Evolutionary analyses were conducted in MEGA7 [21]. The phylogenetic tree included other lactic acid bacteria isolated from various media with known or unknown antifungal properties and available in the NCBI database.

(5) Study of antifungal action mechanisms

Preparation of cell-free culture supernatants

The preparation of Cell-free culture supernatants (CFCS) of selected antifungal strains was done as follows: LAB strains were propagated twice in MRS broth for 48 h at 30°C. Then, the cells were removed from broth by centrifugation (5000 rpm, 15 min) and supernatants filtered by 0.22 µm pore size Millipore filters to obtain CFCS.

Effects of pH, temperature and proteolytic enzymes on cell-free culture supernatants inhibitory activity

The pH of each CFCS was adjusted to values of 5, 7, 9 and 11 with 0.1N NaOH and tested for the inhibitory performances. To determine the effect of heat on CFCS activity, the CFCS was treated at 60, 80 and 100°C for 15, 30, 60 min. For enzyme stability, CFCS was treated for 1 h with a pepsin, trypsin and proteinase K enzyme at a final concentration of 1 $mg.ml^{-1}$ [22]. The residual activity was determined by the agar well diffusion assay using 100 µl of each treated and non-treated CFCS. The mixture was then assayed for inhibitory activity against *Penicillium citrinum* M8 and *Aspergillus carbonarius* NRRL 368.

Identification and quantification of organic acids produced

Chromatographic analysis was carried out using a WATERS ALLIANCE system with a 2695 separation module and a 2487 UV detector (Waters; Millipore, Milford, MA, USA). Analytic separation was achieved with SUPELCOGEL H column (250 mm x 4.6 mm) fitted with SUPELCOGEL H Guard Column (50 mm x 4.6 mm) using 0.1 % H_3PO_4 as mobile phase, with 0.17 ml/min flow. UV detection was performed at 210 nm. Data were collected and analysed with the Empower 2.3 system (Waters Corporation, Milford MA, USA).

Organic acids were identified according to their retention times. Quantification was done according to the compounds peak area using a calibration curb obtained by injecting different volumes of a standard solution containing 30 $ng.\mu l^{-1}$ lactic acid, 50 $ng.\mu l^{-1}$ acetic acid, 25 $ng.\mu l^{-1}$ citric acid and 77 $ng.\mu l^{-1}$ gluconic acid.

Statistical analysis

Multidimensional datasets from antifungal activity were analyzed using principal component analysis (PCA, XLSTAT, 2007). All data were presented as mean of experiments performed in triplicate and were analysed by two-way analysis variance, and Tukey's test was applied for significant means at $p < 0.05$ to evaluate the significant differences.

Results

Isolation of lactic acid bacteria

A total of 28 bacterial colonies showing cultural characteristics specific to LAB group were isolated from fermentation and coded as follows: Lab1, Lab2, Lab3, Lab4, Lab5, Lab6, Lab8, Lab9, Lab10, Lab11.1, Lab 11.2, Lab 12, A11, A12, A13, A14, A15, A16, A17, A18, A19, A21, A22, A23, A24, A25, Ped2 and Ped3.

All strains were analysed microscopically and macroscopically. They showed to be rods or cocci, Gram positive and immobile. Moreover, they were all catalase negative leading to a presumptive association to the LAB group (unpublished data).

Antifungal properties of isolated LAB

In the Table 1 and Fig.1 is presented the antifungal activity of lactic acid bacteria. All bacterial isolates showed antifungal activity against the tested moulds, with different degrees of inhibition ranging from 6.82% to 100%. The obtained data indicated that the isolate Lab 11.1 had the highest mean inhibition percentage against all the tested moulds in the range of 37.77-100%, followed by A21 (30.85-100%), Ped2 (29.60-68.00%), Lab 9 (36.17-60.63%), Lab10 (27.27-71.59%), Ped3 (20.50-65.85%), Lab11.2 (23.23-65.85%) and A19 (20.19-72%). It can be noticed that Lab 11.1 completely inhibited (100%) the growth of two *Penicillium* strains (M7 and M8) and of *A. niger* and have highest activity against 5 of 10 fungal strains (Table 1).

The Principal Component Analysis (PCA) method allowed to analyse the tested LAB antifungal activity according to two principal variable/axes (Fig. 2). According to the variables, two main Lab groups have been obtained (circles). The F1 axis explains 65.72% of the variations observed with a strong contribution percentage of LAB with high antifungal activity, respectively: Lab 11.1 (25.2%), A21 (14.9%), Lab 9 (5.6%), Ped2 (4.6%), Ped 3 (3.7%), *L. sanfranscisensis* (3.5%), Lab 10 (3.4%), *L. brevis* (2.0%), Lab11.2 (1.6%), and *L. plantarum* (1.3%). The F2 axis indicates 8.42% of this distribution with LAB proving strong activity: Lab 11.1 (21.43%), A19 (9.28%), Lab 2 (9.14%), *L. sanfranscisensis* (6.84%), *L. brevis* (6.48%), and Lab 12 (5.14%). It was noticed that the first group of LAB shows antifungal activity on the totality of the tested moulds contrary to the second group showing limited activity on different moulds species. Based on the antifungal screening data (Table 1), 8 LAB strains (Ped2; Ped3; Lab 9; Lab 10; Lab11.1; Lab 11.2; A19 and A21) with high antifungal activity were selected for the next detailed analysis.

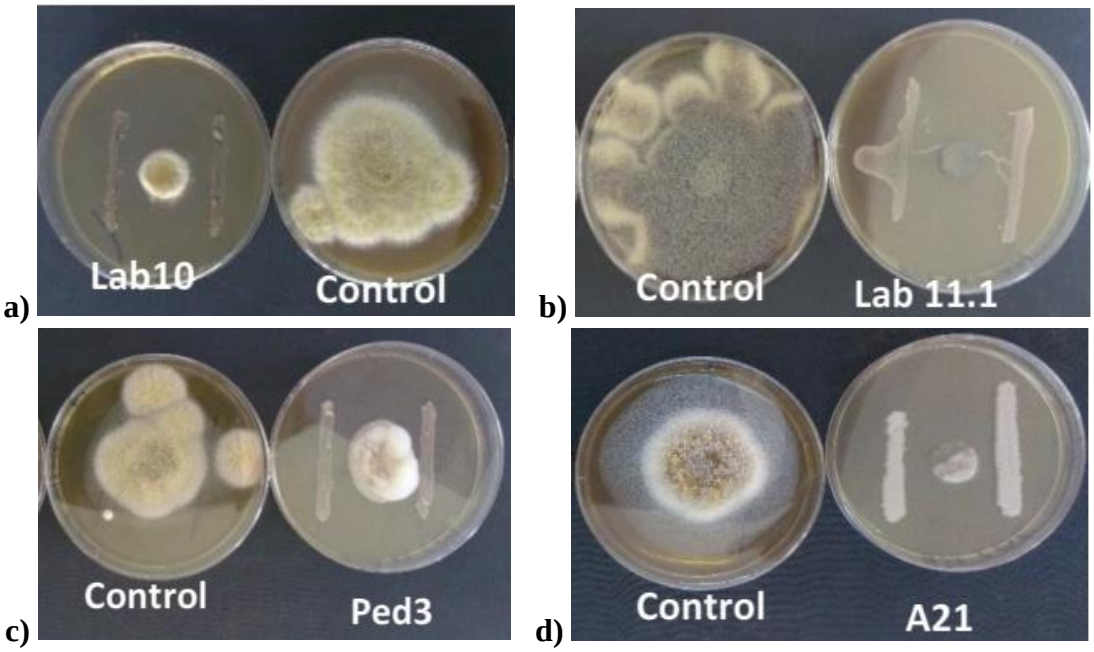

Figure 1. Aspects of mould growth inhibition by different lactic acid bacteria strains (Lab 10, A21, Ped 3 and Lab 11.1): a) *A. oryzae* M13; b) *A. niger* NRRL 612; c) *A. versicolor* M14; d*) A. flavus* AF.

Biochemical characterisation of antifungal lactic acid bacteria strains

The selected antifungal LAB strains were subject of some biochemical tests, helpful for strains identification and characterisation (Table 2). The results showed that all LAB strains have been capable to metabolize glucose, fructose, sucrose, maltose, lactose and cellobiose. None of them produce hydrogen sulphide. Differences between these strains were observed for the acid production from the fermentation of glycerol, arabinose, threhalose, raffinose, melibiose, ribose, esculin and carbon dioxide production when cultivated on glucose. These data are not sufficient to define the identity of the LAB but are useful for the correlation with the molecular identification results find out by sequencing.

Table 1. Antifungal activity of LAB isolated from fermented cocoa beans. Results are expressed as inhibition percentage (Supplementary data)

	Moulds									
	AC	AN	AF	M7	M8	M11	M13	M14	M21	M22
L. brevis	**50.62**	**27.27**	**26.66**	**65.85**	**37.50**	**47.85**	**34.61**	**51.06**	**22.72**	**68.00**
L. Plantarum	**50.62**	**22.72**	**28.88**	**65.85**	**57.50**	**44.28**	**61.53**	**40.42**	**20.45**	**35.00**
L. sanfranscisencis	**56.92**	**31.81**	**35.55**	**43.90**	**43.85**	**64.28**	**44.28**	**40.42**	**29.54**	**68.00**
Lab 1	14.77	20.45	17.77	65.85	37.50	37.23	15.91	36.17	14.80	28.00
Lab 2	54.55	9.09	11.11	45.12	48.80	36.17	6.82	29.79	18.20	25.00
Lab 3	37.50	9.09	24.44	39.02	37.50	14.89	20.45	27.66	14.80	25.00
Lab 4	20.45	12.50	16.66	40.24	32.50	21.27	31.82	27.66	14.80	25.00
Lab 5	14.77	18.18	15.55	65.85	23.80	30.85	31.82	29.79	12.50	25.00
Lab 6	18.18	18.18	15.55	41.46	30.00	14.89	20.45	19.15	18.20	23.00
Lab 7	14.77	25.00	14.44	43.90	30.00	21.27	18.18	25.53	14.80	20.00
Lab 8	14.77	12.50	21.11	20.73	21.30	32.98	43.18	24.47	15.90	18.00
Lab 9	**60.63**	**39.77**	**35.55**	**57.31**	**57.50**	**58.57**	**44.32**	**36.17**	**45.80**	**49.00**
Lab 10	**61.36**	**27.27**	**30.00**	**65.85**	**65.00**	**56.38**	**71.59**	**29.79**	**26.10**	**49.00**
Lab 11.1	**59.09**	**100.00**	**37.77**	**100.00**	**100.00**	**78.72**	**60.23**	**59.57**	**40.90**	**43.00**
Lab 11.2	**44.32**	**31.82**	**23.33**	**65.85**	**57.50**	**48.93**	**53.41**	**40.43**	**29.60**	**30.00**
LAB12	20.45	29.55	11.11	51.22	25.00	32.98	44.32	29.79	29.60	25.00
A11	29.55	20.45	18.88	50.00	30.00	30.85	20.45	31.91	14.80	28.00
A12	22.73	14.77	18.88	39.02	28.80	30.85	44.32	21.28	18.20	25.00
A13	29.55	18.18	15.55	50.00	12.50	38.30	53.41	29.79	14.80	25.00
A14	20.45	14.77	17.77	39.02	17.50	14.89	21.59	17.02	9.09	28.00
A15	14.77	14.77	17.77	34.15	32.50	34.04	30.68	14.89	15.90	25.00
A16	14.77	12.50	17.77	50.00	23.80	14.89	20.45	24.47	15.90	25.00
A17	12.50	18.18	17.77	65.85	30.00	20.21	53.41	21.28	18.20	24.00
A18	12.50	27.27	16.66	36.59	30.00	28.72	44.32	29.79	15.90	25.00
A19	**51.25**	**25.00**	**23.33**	**65.85**	**54.85**	**52.14**	**20.19**	**32.98**	**21.60**	**72.00**
A21	**44.32**	**36.36**	**68.88**	**100.00**	**100.00**	**70.21**	**59.09**	**30.85**	**33.00**	**64.00**
A22	22.73	18.18	13.33	39.02	27.50	27.66	20.45	30.85	17.10	28.00
A23	29.55	27.27	11.11	37.80	28.80	27.66	53.41	36.17	11.40	25.00
A25	20.45	12.50	18.88	39.02	35.00	27.66	44.32	32.98	17.10	25.00
Ped 2	**56.82**	**36.36**	**35.55**	**65.85**	**57.50**	**51.06**	**44.32**	**40.43**	**29.60**	**68.00**
Ped 3	**44.32**	**31.82**	**35.55**	**65.85**	**37.50**	**59.57**	**63.64**	**51.06**	**20.50**	**64.00**
Means	32.90	24.63	23.01	53.42	40.37	37.75	38.61	32.05	20.70	35.71

Legend: **AC**- *Aspergillus carbonarius* NRRL 368 UY1, AF - *Aspegillus flavus,* AN- *Aspergillus niger* NRRL 612, M7- *Penicillium citrinum* Ma, M8- *Penicillium citrinum* Md, M11- *Aspergillus fumigatus*, M13- *Aspergillus oryzae*, M14- *Aspergillus versicolor,* M21- *Aspergillus tamarii,* M22- *Aspergillus versicolor.*

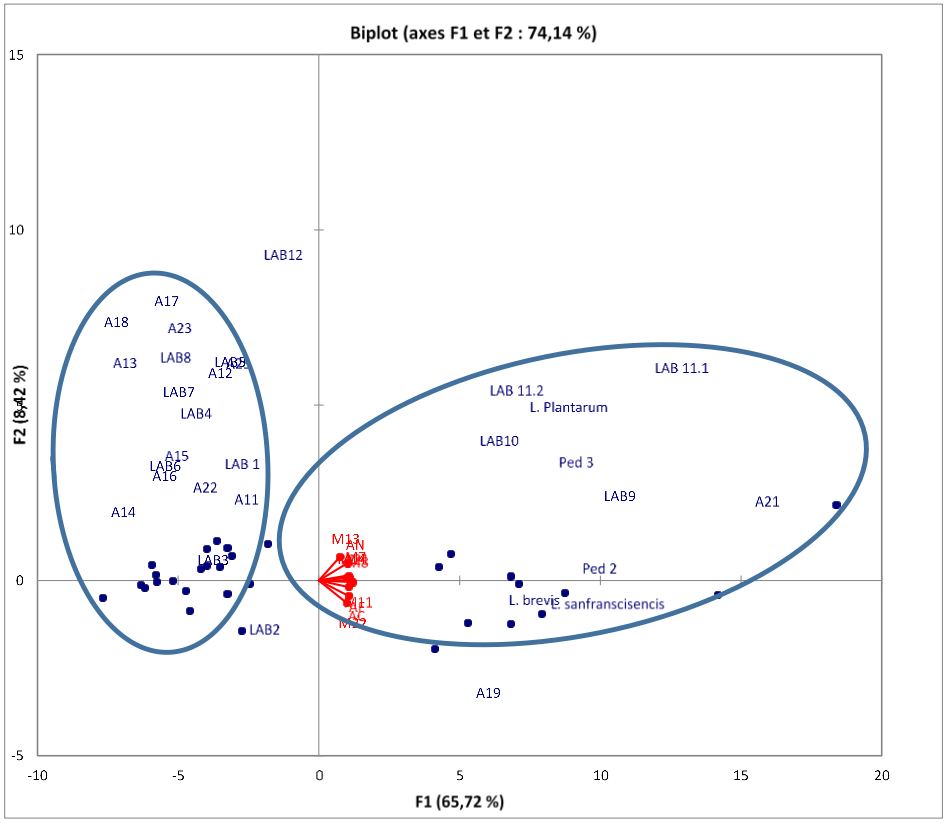

Figure 2. Principal component analysis showing a correlation circle projection of lactic acid bacteria.

Table 2. Biochemical characteristics of selected LAB strains

Characteristics	Ped 2	Ped 3	Lab 9	Lab 10	LAB 11.1	Lab 11.2	A19	A21
H_2S gas production	-	-	-	-	-	-	-	-
CO_2 production from glucose	+	-	-	+	-	-	+	+
Carbohydrate fermentation								
Glucose	+	+	+	+	+	+	+	+
Glycerol	-	+	-	-	-	+	-	-
D(-)Arabinose	+	+	-	+	-	+	+	+
Galactose	-	+	+	-	+	+	-	+
Fructose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Inositol	+	-	-	+	-	-	+	-
Trehalose	-	+	+	-	+	+	-	-
D(+) Raffinose	-	+	-	-	+	-	-	+
Starch	+	+	-	+	+	+	+	-
Lactose	+	+	+	+	+	+	+	+
L-Rhamnose	+	-	+	+	-	-	+	+
Cellobiose	+	+	+	+	+	+	+	-
Mannitol	+	+	+	+	-	+	+	-
Melibiose	-	+	+	-	-	+	-	-
Ribose	-	+	+	-	-	+	-	-
Xylose	+	-	+	+	-	-	+	+
Esculin	+	+	-	+	+	+	+	+

+ able to, - unable to perform the functions

Molecular identification of selected lactic acid bacteria

All the strains have amplified a specific common fragment of 340 bp with the specific lactobacilli primers Lac F and Lac R (Fig. 3). The sequences received from BaseClear (The Netherlands) were analysed via Blast search from NCBI database. High identities percentage matching with sequences from NCBI database obtained were 99-100% similarity for *Weissella cibaria* (A19, Lab10 and Ped2), 99-100% for *Enterococcus faecium* (Ped3 and Lab11.2), 99% for *Pediococcus pentosaceus* (Lab9), 100% for *Lactobacillus brevis* (A21), and 100% for *Lactobacillus farciminis* (Lab11.1). Their NCBI access numbers are shown in Table 3. The 16S rDNA sequencing results identified different strains supporting value from bootstrap analysis in the phylogenetic tree (Fig. 4).

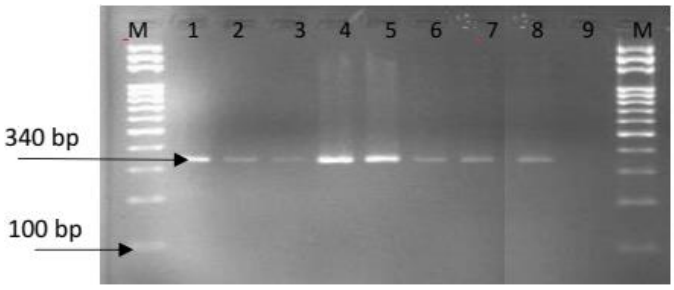

Figure 3. Fragments amplified with Lac F and Lac R primers (340 bp) specific for lactobacilli (M-Time marker 100 bp ladder, 1-Ped 2, 2-Ped 3, 3-Lab 9, 4-Lab 10, 5-Lab 11.1, 6-Lab11.2, 7-A19, 8-A21, 9-negative control).

Table 3. Strains accession number

Strains	Accession number
Weissella cibaria Ped 2	MN173224
Enterococcus faecium Ped 3	MN173225
Pediococcus pentosaceus Lab 9	MN173228
Weissella cibaria Lab 10	MN173229
Lactobacillus farciminis LAB 11.1	MN173230
Enterococcus faecium Lab 11.2	MN173231
Weissella cibaria A19	MN173226
Lactobacillus brevis A21	MN173227

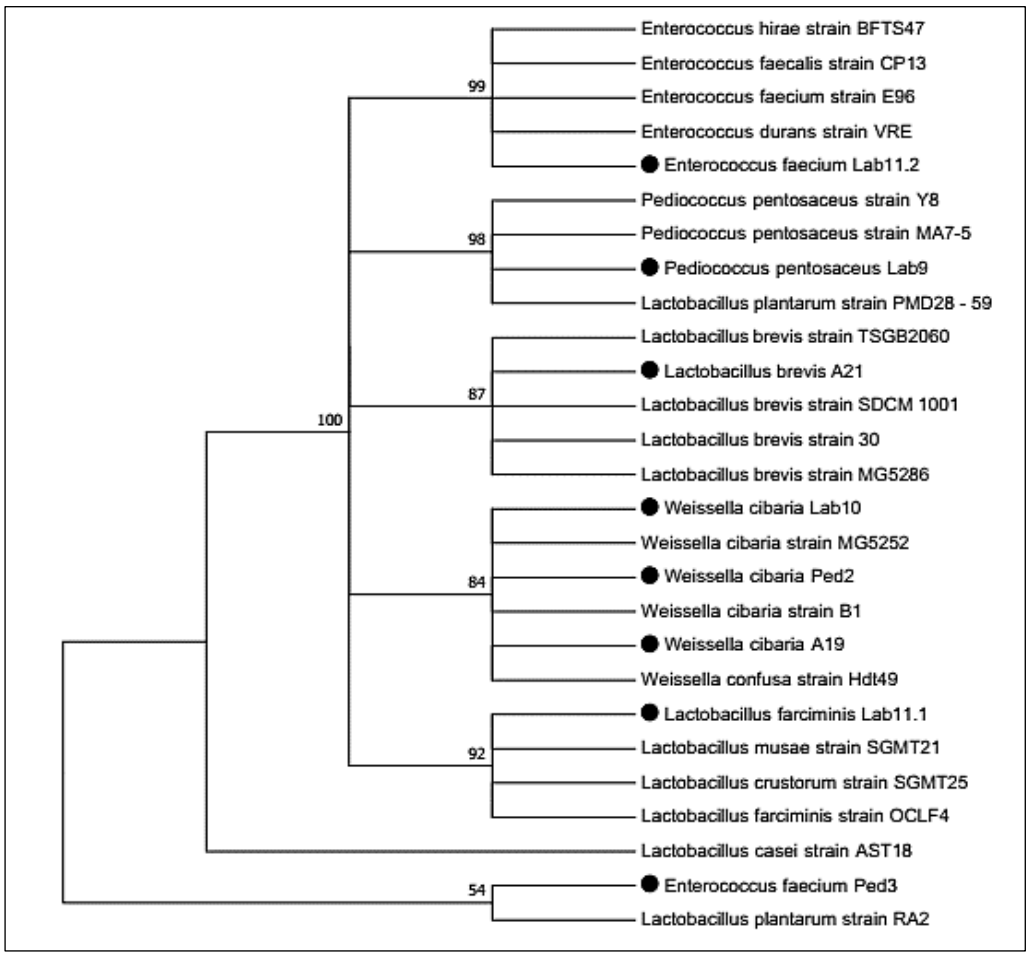

Figure 4. Phylogenetic tree of the 16S rRNA gene sequences obtained with group-specific primers *Lactobacillus* spp.

Study of antifungal action mechanisms

Sensibility of CFCS to pH, temperature and proteolytic enzymes treatments

After the cultivation of selected LAB, CFCS pH values were checked for each strain and they ranged from 3.5 to 4.5. The performed experiments showed that treated or not treated CFCS still exhibited antifungal activities against tested fungi in comparison to the negative controls, and was the same for 6 of 8 antifungal LAB. However, treated CFCS of two strains of *Enterococcus faecium* (Ped 3 and Lab 11.2) are sensible to heat (100°C/10min minimum), to proteinase K and neutral pH, in comparison to other strains (Table 4). This activity lost in the CFCS of *Enterococcus faecium* submitted to the heat treatment may appear due to the proteic nature of its antifungal compounds.

Table 4. Antifungal activity of CFCS after pH, temperature and enzymes treatments

		Aspergillus carbonarius AC								*Penicillium citrinum* M8							
		Ped 2	Ped 3	Lab 9	Lab10	Lab11.1	Lab11.2	A19	A21	Ped 2	Ped 3	Lab 9	Lab10	Lab11.1	Lab11.2	A19	A21
negative Control		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Positive Control		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Enzymes treatment	Trypsin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Pepsine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Proteinase K	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+
pH treatment	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	7	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+
	9	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+
	11	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+
60°C	10 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	30 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	60 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
80°C	10 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	30 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	60 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100°C	10 min	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+
	30 min	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+
	60 min	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+

− absence of antifungal activity following treatment
+ detected antifungal activity following treatment

Identification and quantification of organic acids

In order to evaluate which organic acids with antifungal potential were present in the supernatant, a number of compounds were targeted for the detection and quantification by HPLC analysis. The highest producer of lactic acid is *Pediococcus pentosaceus* (Lab 9) with 21.419 µg.µl^{-1} followed by *L. farciminis* (Lab 11.1) (19.46 µg.µl^{-1}), while the lowest producer is *L. brevis* (A21) with 11.63 µg.µl^{-1}. Acetic acid was not detected in all samples (Table 5). Only *Pediococcus pentosaceus* (Lab 9) and *Weissella cibaria* (A19) showed smaller amounts of gluconic acid in the range of 2.278 and 1.052 µg.µl^{-1} respectively.

Table 5. Quantification of organic acids by HPLC

Organic acids	Ped2	Ped3	Lab 9	Lab 10	Lab 11.1	Lab 11.2	A19	A21
Lactic acid (µg.µl^{-1})	18.815	17.527	21.419	16.71	19.466	16.625	13.432	11.639
Acetic acid (µg.µl^{-1})	0	0	0	0	0	0	0	0
Gluconic acid (µg.µl^{-1})	0	0	2.278	0	0	0	1.052	0
Citric acid (µg.µl^{-1})	0	0	0	0	0	0	0	0

Discussion

The conservation of cocoa beans against moulds remains mainly a major challenge. Indeed, the visible presence of moulds in cocoa beans leads to their depreciation and rejection by chocolate maker whether or not these strains produce mycotoxins. Moulds can contaminate cocoa processing at different stages, making the improvement of the practices difficult to implement satisfactorily. Therefore, the inoculation of antifungal LAB strains in to cocoa bean fermenting masses has been taken into account as a putative application of biological control. Thus, cocoa fermentations could be a reservoir of LAB to be selected for their use as antifungal agent or starter cultures [12, 23]. Therefore, it has been reported that the use LAB with

antifungal properties, could be a considerable advantage if cocoa beans were initially inoculated with them [10].

In this study, our targets consisted of moulds species commonly encountered in fermented and or dried cocoa beans environments [10, 24]. All the 28 LAB isolates were observed to have different antifungal potential, but at the end, only 8 have been selected for their higher and stable activity against the tested moulds. The most sensitive moulds were *Penicillium citrinum* (M7 and M8) and *A. niger* NRRL 612 which were inhibited up to 100% by *Lactobacillus farciminis* Lab11.1 and *Lactobacillus brevis* A21 strains, while the most resistant were *A. carbonarius* NRRL 368 and A. *tamarii* M21. These results are in agreement with those of Gerez & al [25], Matei and Cornea [11] and Ouattara & al [23]. They have reported that *Penicillium* species are more sensitive to bacterial active molecules compared to moulds of the genus *Aspergillus*. However, by analysing the general data obtained on the antifungal activity of lactic acid bacteria, it was found that some species such as *P. citrinum, A. versicolor* presented have different sensitivity level. This could be due to the fact that different serotypes may be found in the same species as demonstrated by Sharma & al [26]. At the same moment, the variability of active or sensible strains in the same species can be explained by the presence of several morphotypes that may have different characteristics in the same environment, as described by Tareb & al [27] in the case of *Lactobacillus farciminis* or by Ayo-Olalusi [28] in the case of *Pediococcus pentosaceus*. In complement, PCA variables explain 74.14% of the variation between LAB on two virtual axes F1 and F2. The importance contribution of *Lactobacillus farciminis* (Lab11.1) on the two axes F1 and F2 is explained by its strong antifungal effect because it presented 100% of inhibition growth against three of ten tested moulds. It would thus be adapted within the framework of an antifungal control.

Antifungal LAB strains have the capacity to metabolize glucose, fructose, sucrose, maltose, lactose, cellobiose; none of them produce hydrogen sulphide. Differences between these strains were observed for production of acid from fermentation of glycerol, arabinose, threhalose, raffinose, melibiose, ribose, esculin and carbon dioxide from glucose fermentation. These characteristics are consistent with those reported by Fusco et al [29] for *Weissella cibaria*, by Tsuda & al [30] for *Lactobacillus farciminis*, by Baradaran & al [31] for *Pediococcus pentosaceus*, by Manero and Blanch [32] for *Enterococcus faecium* and by Gandevia & al [33] for *Lactobacillus brevis*.

The molecular identification of these lactic bacteria revealed that the isolates belong to the LAB group such as *Weissella cibaria* (Lab10, A19 and Ped2), *Pediococcus pentosaceus* (Lab 9), *Lactobacillus brevis* (A21), *Lactobacillus farciminis* (Lab11.1) and *Enterococcus faecium* (Lab11.2 and Ped3). These results confirm those of other researchers who found LAB species like *Lactobacillus, Leuconostoc, Lactococcus, Pediococcus,* and *Enterococcus* in the fermentation of cocoa beans in Ghana, Nigeria, Ivory Coast and Cameroon [6, 23, 34].

The antagonist activity of different strains identified has been recognized by several authors. The most commonly cited strains in the literature remain *Pediococcus pentosaceus*, *Lactobacillus brevis* and *Weissella cibaria* for their antimicrobial activity against mould, and other pathogens. Relatively to their ability to produce many molecules, they have already found several applications in the food and medical industries and are already marketed in several forms. No studies have been found reporting the presence of *Lactobacillus farciminis* (which has the best inhibitory activity against the tested targets in cocoa beans fermentation. However, it is known for its probiotic character applied to animal feed [11, 27] and could therefore be exposed. On the other hand, *Enterococcus faecium* is not widely used, although it has been recognized as a producer of a bacteriocin known as enterocin [35].

Some hypotheses and evidence have been reported to explain this phenomenon of inhibition by the synergic effect of organic acids and other molecules [23, 36]. The variable inhibitory activity of LAB is attributed to a number of antimicrobial metabolites such as hydrogen peroxide, reuterin, phenyllactic acid, protein compounds, phenolic compounds and hydroxy fatty acids that have also been isolated from LAB [13, 37, 38].

LAB has been recognized to have antifungal activity. This study observed that the supernatants of the eight antifungal LAB isolated from cocoa beans had good antifungal activity against *A. carbonarius* and *P. citrinum*. This specifies that CFCS from these LAB isolates comprise of compounds with protein-like nature. When the CFCS was heated, their inhibited the growth of the by the heated CFCS. However, treated CFCS of two strains *Enterococcus faecium* are sensible to heat (100°C/10min minimum). Similarly, Magnusson & al [39] and Laref & al [40] observed that antifungal activity of lactic acid bacteria isolated from fermented food was stable during heat treatment and retaining the activity was even after autoclaving at 121°C for 15 min against moulds.

When the pH of the CFCS was adjusted to different value 5, 7, 9 and 11, antifungal activity stay constant. This observation shows that the compounds responsible for antifungal activity in CFCS of these isolates could not be only organic acids like acetic acid and lactic acid. The antifungal activity of CFCS treated with enzymes proteinase K, pepsin and trypsin results were also stable. But treating CFCS with heat higher than 100°C/10 min, proteinase K and pH 7, 9 and 11 losed the antagonist activity of *Enterococcus* strains. Ndagano & al [41] reported that supernatant treatment of LAB isolated from Mill flour and fermented cassava by pepsin, α chymotrypsin and proteinase K showed antifungal activity. These results suggest that the antifungal compound could be not only of proteinic nature. Considering different information on treated CFCS, the acid lactic amount and the presence of bands in different CFCS, it should be noted that synergistic effects may exist between molecules at low concentrations with an mechanism.

These results are very interesting and suggest that some LABs isolated from cocoa fermentation could be

a promising candidate for food bio conservation applications. Considering our results, *Lactobacillus farciminis* can be recommended for cocoa beans fermentation for its highest anti-microbial potential among the isolates. However, all the isolated LAB should be taken into account as biocontrol agents, as also reported by Braiek & al [42]; their application may depend on the spectra of spoilage microorganisms present in the cocoa beans' fermentation media, linked most probably to the environmental conditions and beans storage; more studies are requested in this regard.

Conclusion

In this study, it has been found that lactic acid bacteria strains, isolated from cocoa beans fermentation have a broad spectrum of inhibitory activity towards fungi. They have been identified as belonging to the species *Weissella cibaria, Pediococcus pentosaceus Lactobacillus farciminis, Enterococcus faecium* and *Lactobacillus brevis*. Their antifungal potential would be attributed to a synergic action of lactic acid, proteaginous compounds or unknown mechanisms. The promising characteristics of the different strains suggest their potential for food bio conservation to avoid product contamination, the proliferation of moulds present on cocoa beans. However, further experiments are needed to confirm these possibilities in cocoa fermentation.

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Conflict of interest

The authors declare that they have no competing interests, financial or otherwise.

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