Anaerobe 17 (2011) 267–272

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Pathogenesis and Toxins

Robusta coffee beans post-harvest microflora: *Lactobacillus plantarum* sp. as potential antagonist of *Aspergillus carbonarius*

Olga Djossou [a], Isabelle Perraud-Gaime [a], Fatma Lakhal Mirleau [a], Gabriela Rodriguez-Serrano [b], Germain Karou [c], Sebastien Niamke [c], Imene Ouzari [d], Abdellatif Boudabous [d], Sevastianos Roussos [a,*]

[a] *Institut Méditerranéen d'Écologie et de Paléoécologie (IMEP, UMR CNRS/IRD 6116), Laboratoire d'Écologie Microbienne et Biotechnologies, FST St Jérôme, Université Paul Cézanne, 13397 Marseille Cedex 20, France*

[b] *Universidad Autónoma Metropolitana Unidad Iztapalapa (UAM-I), Departamento de Biotecnología, Laboratorio de Fermentación en Medio Sólido, Avenida San Rafael Atlixco 186, Col. Vicentina, C.P. 09340 D.F. México, Mexico*

[c] *Université de Cocody, UFR Biosciences, 22 B.P. 582, Abidjan 22, Cote d'Ivoire*

[d] *Laboratoire de Microbiologie et Biomolécules actives, Université de Tunis El Manar, Faculté des sciences de Tunis, Tunisia*

ARTICLE INFO

Article history:
Received 28 January 2011
Received in revised form
24 March 2011
Accepted 30 March 2011
Available online 7 April 2011

Keywords:
Coffee robusta
Aspergillus carbonarius
Lactic acid bacteria
Antagonism

ABSTRACT

Coffee contamination by ochratoxigenic fungi affects both coffee quality as well as coffee price with harmful consequences on the economy of the coffee exporting countries for whom which is their main source of income. Fungal strains were isolated from coffee beans and identified as black *Aspergilli*. Ochratoxigenic moulds like *Aspergillus carbonarius* were screened and selected for detailed studies. Also lactic acid bacteria (LAB) were isolated from silage coffee pulp and their antifungal activity was tested on dual-culture agar plate. Ten of the isolated LAB demonstrated antifungal effect against *A. carbonarius*. API 50 CH and APIZYM were used to perform phenotypic identification. 16S rDNA sequencing was made to confirm the results.

1. Introduction

Food-borne fungi, both yeasts and moulds, cause serious problems during food storage. Moulds may produce mycotoxins, e.g. aflatoxins, trichothecenes, fumonisin, ochratoxin A and patulin [1]. According to statistic 5–10% of the world food production is lost due to fungal deterioration [2]. For that reason, several techniques are being used for the preservation of food and feeds: drying, freeze-drying, cold storage, modified atmosphere storage, and heat treatments [3]. Several chemical additives are being used as preservatives even though the exact mechanisms of their action are not known. For instance benzoic acid and sodium benzoate are used primarily as antifungal agents. The natamycin, produced by *Streptomyces natalensis*, is effective against yeasts and moulds and a common preservative on hard cheese surfaces [4]. An increasing number of microbial species is becoming resistant to antibiotics. Furthermore, yeasts and moulds are becoming resistant to preservatives such as sorbic and benzoic acid, as well as to chemical treatment with cleaning compounds [5]. There exists a great risk that the resistant phenomenon will increase in future due to more frequent use of antibiotics and preservatives [6]. Filamentous moulds are common spoilage organisms of coffee cherries and grains during both post-harvest treatment and storage [7]. Fungal toxins (poly-peptides) produced are thermostable and consistently remain in roasted coffee. Both unroasted and roasted coffee may contain ochratoxin A (OTA), and in a lesser amount aflatoxin, which are produced by *Aspergillus* sp. [8]. OTA is usually produced during the growth phase under certain environmental conditions by *Aspergillus ochraceus*, *Aspergillus carbonarius* and some strains of *Aspergillus niger*. The presence of this toxin requires the prior presence of a significant amount of OTA-producing fungal biomass [9]. Therefore inhibiting fungal growth can prevent OTA production in coffee. In another point of view, it appears cheaper and easier to prevent fungal growth on a raw material than trying to degrade OTA in food or product. Also, consumer demands on minimally processed foods and reduced use of chemical preservatives have stimulated research on biological (green) preservation methods. Antagonist microorganisms or their metabolic products can inhibit or destroy undesired microorganisms in food and agricultural products particularly mycotoxinogenic

* Corresponding author. Tel.: +33 491289103; fax: +33 491288668.
E-mail addresses: olgitha@yahoo.fr (O. Djossou), gmrs@xanum.uam.mx (G. Rodriguez-Serrano), s.roussos@univ-cezanne.fr (S. Roussos).

1075-9964/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.anaerobe.2011.03.006

moulds [10]. Among the microbes, lactic acid bacteria (LAB) are the most powerful prokaryotes when it comes to antimicrobial potential [11]. In fact, based on current literature, antifungal compounds from lactic acid bacteria do exist and have potential for being effective in fighting against food-borne moulds. Nevertheless, the number of research studies published on antifungal lactic acid bacteria especially against black *Aspergilli* is comparatively less. The aim of the current research was first to screen lactic acid bacteria for their antifungal activity against conidiospores germination and mycelial growth of *A. carbonarius* followed by the identification of those bacteria.

2. Materials and methods

2.1. Selection of OTA-producing A. carbonarius

Two hundred eighteen fungal strains were isolated from coffee cherries on Potato Dextrose Agar (PDA) [Difco, Becton, Dickison and Company sparks, MD 21152 USA]. Phenotypic identification was made according to Botton et al. [12] and Samson et al. [13]. Tools identification with reference strains were made from Natural History National Museum of France (MNHN) and the Department of Agriculture's Southern Regional Research Center (New Orleans, Louisiana, USA). OTA producing fungi were grown on Czapek Yeast Agar (CYA) (Difco, Becton, Dickison and Company sparks, MD 21152 USA) [14]. OTA concentration was quantified by High Performance Liquid Chromatography (HPLC) using a fluorimetric detector. For the current study two strains of *A. carbonarius* (strains Ac 162 and Ac 164) were selected.

2.2. Isolation and preliminary identification of lactic acid bacteria

Lactic acid bacteria (LAB) were isolated from silage of fresh coffee pulp collected from Ivory Coast during 2008 harvest period. The silage was preserved in darkness at 25 °C without chemical or biological additives. A sample of 1 g of silage was suspended in 9 mL of Malt Rogosa Sharp (MRS) culture media (Difco, Becton, Dickison and Company sparks, MD 21152 USA) broth and incubated at 30 °C for 24 h. Ten fold dilutions from this culture were prepared and 0.1 mL of each dilution was spread on MRS agar plates. After aerobic incubation at 30 °C for 48 h, bacterial cultures were transferred and purified by streaking on MRS agar. LAB strains isolated from fresh coffee pulp were randomly selected on MRS agar plates, and preserved in MRS broth at 4 °C for characterization purposes. Gram staining of LAB strains was performed to determine the purity and identify the morphology with an optical Microscope (CARL ZEISS, Standard 25 ICS). The strains were preserved at −20 °C in MRS broth with 20% glycerol. In addition, production of lactic acid was quantified by HPLC (Quaternary Pump HPLC system, Agilent Technologies 1200, UV detector, Chemstation 32 software for data acquisition).

2.3. Antifungal test

For an antifungal test we chose two strains of *A. carbonarius* (strains Ac 162 and Ac 164) that produced almost 10 mg of OTA per gram of PDA. Screening of bacteria for antifungal activity was investigated using the overlay essay method [15,16]. The MRS agar plates were incubated overnight at 30 °C before inoculating with the LAB strains. Bacteria were grown in MRS broth for 24 h at 30 °C. A sample of 5 μL of each LAB inoculum culture was added as discrete spots on MRS plates and incubated under aerobic conditions at 30 °C for 24 h. Plates with well-grown LAB colonies were overlaid with 10 mL of soft CYA media (0.8% agar) having a final mould spore count of 1×10^4 spores/mL and incubated at 25 °C. Bacteria were classified according to the following parameters: mould mycelial growth, conidiospore formation, clear zone development around each colony, compounds diffusion on the soft agar above. Clear zones of inhibition were recorded (two diameter average perpendiculars) and scored as follows: no visible inhibition, visible inhibition. All experiments were carried in triplicate.

2.4. Identification and typing of lactic acid bacteria

2.4.1. Phenotypic characterization of isolated LAB strains

Phenotypic properties such as carbon dioxide production from glucose, growth at different incubation temperatures, ability to grow in different concentrations of sodium chloride in MRS broth, were described for all the isolated LAB strains [17]. Sugar fermentation patterns of LAB isolates were determined using the API 50 CHL test strips (Biomérieux, France) and enzyme production by using APIZYM test (Biomérieux, France). The enzymatic profile of lactic acid bacteria was assayed by testing 19 enzyme activity assays: alkaline phosphatase, esterase (C4), lipase (C8), lipase (C14), leucine-, valine-, and cystine-, arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and fucosidase.

2.4.2. Genotypic characterization

Bacterial strains were genotypically characterized by 16S rDNA gene PCR amplification. Cells were collected by centrifugation (7500 rpm, 10 min, 4 °C) of 1.5 mL of LAB culture grown in MRS broth at 30 °C and frozen at −20 °C, 24 h.

Total genomic DNA of ten strains of LAB was extracted using Qiagen KIT (France). PCR assay (25 μL) was performed using primers fd1 (Table 1) and rd1 for most Eubacteria [18] and Firm 350 and Firm 814r for Firmicutes [19]. The PCR mixture had 1.25 μL $MgCl_2$ (25 mM), 1 μL of each primers fd1 (10 μM), rd1 (10 μM), firm 350f (10 μL) and firm 814r (10 μL), 1 μL dNTP (10 μM) (Fermentas), Go Taq buffer (5×) 5 μL (Promega, Madison, WI, USA) dimethyl sulfoxide (DMSO) 0.5 μL, Taq polymerase 0.25 μL (Promega, Madison, WI, USA) and 2 μL of DNA. PCRs were performed on a Thermocycler (BioRad, USA), 578BR0545, with initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C (30 S), annealing at 55 °C (30 S) (fd1/rd1) and 48 °C (firm 350f/firm 814r), elongation at 72 °C for 2 min and a final extension at 72 °C for 10 min (fd1/rd1), 72 °C for 5 min (firm 350f/firm 814r).

The PCR products were electrophoresed in 0.8−1% of agarose gel at a constant voltage of 100 V, in Tris, Acetate, and EDTA (TAE) 0.05 M running buffer.

The computer program Mega5 was used for sequence alignment and the online tool BLAST (elaborate the name) was used for the representation of sequence similarities.

3. Results and discussion

3.1. Fungal selection

Two strains of *A. carbonarius* (Ac 162 and Ac 164) isolated during this work and selected for antifungal test were best OTA producers

Table 1
Summary of primers for the PCR amplification of bacteria 16S rDNA.

Primers	Designed for	Sequences (5′ to 3′)	AT (annealing temperature)
Fd1	Eubacteria	AGAGTTTGATCCTGGCTCAG	55 °C
Rd1	Eubacteria	AAGGAGGTGATCCAGCC	55 °C
Firm 350	Firmicutes	GGCAGCAGTRGGAATCTTC	53 °C
Firm 814r	Firmicutes	ACACYTAGYACTCATCGTTT	53 °C

(0.037 mg/g and 0,025 mg/g of PDA culture medium respectively). Strain identification was made in comparison with reference strains of *A. carbonarius*, Ac 16 and Ac 2131 from the Department of Agriculture Regional Research Center of Louisiana, United States.

3.2. Antagonist lactic acid bacteria

Forty four bacteria [Gram+, catalase −], producing lactic acid were isolated, purified and preserved in glycerol at −20 °C. They came from ensiling process. It seems to be fine for lactic acid bacteria amplification, as only this group was present in our collection.

All LAB strains were tested against two strains of *A. carbonarius* (strains Ac 162 and Ac 164). After the first assay it was possible to classify the antagonist bacteria into three groups of inhibition: total inhibition (clear zone area + diffusion) (Fig. 1a) (thirty LAB strains against Ac 162; thirty one LAB strains against Ac 164); partial inhibition (clear zone area + mycelium growing on Petri dishes) (Fig. 1b) (ten LAB strains against Ac 162; twelve LAB strains against Ac 164); and no inhibition (no clear zone area) (Fig. 1c) (four LAB strains against Ac 162; one LAB strain against Ac 164).

Two other assays were conducted and this has resulted in a reproducibility factor. Studies continued on ten strains which showed a reproducibility factor greater than or equal to 2/3 on at least one of fungi: the zones of inhibition were measured (Table 2).

Seven lactic acid bacteria (LAB) showed a diameter of inhibition greater than 20 mm. Some of them diffused compound around the clear zone of inhibition. In certain case it was noted that in addition to the clear zone area, some bacteria showed diffusion of compounds in the Petri dishes. But for the same bacteria, inhibition area was different from a mould to another (Table 2). Clear zone area appeared between 48 h and 72 h during incubation at 30 °C. On the third day of incubation at 30 °C, it was observed on certain Petri dishes a mycelial growth all over the plate recovering clear zone area (Fig. 1). It certainly meant that the concerned bacteria had fongistatic effect on tested mould while others maintained inhibition above five days.

The antifungal activity of each bacteria was tested at three different times, which helped to define a factor on the responsability of three tests. As we can see in Table 2, for an antifungal bacteria, the responsability factor and the diameter of inhibition varied from one mould strain to another (all antifungal strains in Table 2). This highlighted that the moulds are of the same species but they are from different strains. Another observation made was that two bacteria with the same factor of reproducibility on the same mould have not the same diameter of inhibition (LabCP15Z42 and LabCP37Z12 against Ac 162; LabCP30Z22, LabCP19Z31, LabCP21Z32, LabCP22Z32 and LabCP16Z42 against Ac 162; LabCP20Z31, LabCP15Z42, LabCP2Z52 against Ac 164; LabCP37Z12, LabCP30Z22, LabCP37Z12, LabCP22Z32, LabCP19Z31 LabCP16Z42 against Ac 164). This shows that bacteria were very different from each other at the metabolic level and reproducibility of the factor was not closely related to the bacteria but to the physiology of the mould.

Table 2
Average of clear zone area of inhibition of ten strains and reproducibility.

LAB strain	*A. carbonarius* strain Ac 162		*A. carbonarius* strain Ac 164	
	Reproducibility	Ø (mm)	Reproducibility	Ø (mm)
LabCP2Z52	3/3	0	2/3	9
LabCP16Z42	2/3	24	1/3	19
LabCP19Z31	2/3	20	1/3	19
LabCP20Z31	1/3	25	2/3	20
LabCP21Z32	2/3	20	3/3	19
LabCP22Z32	2/3	18	1/3	21
LabCP28Z21	3/3	0	3/3	0
LabCP30Z22	2/3	13	0/3	19
LabCP37Z12	3/3	25	1/3	23

At the end of these experiments, only two strains (LabCP15Z42 and LabCP37Z12) showed an inhibition with clear zone area between 20 and 30 mm diameter for the three assays against one of the moulds tested (Ac 162). The most powerful against Ac 164 is LabCP15Z42.

3.3. Phenotypic identification of lactic acid bacteria

The list of bacteria in Table 3 can be characterized as lactic acid bacteria as they are Gram positive, asporulate, catalase negative and their main fermentation product from glucose was lactic acid [20]. Cell morphology (rod), no gas production from glucose and acetic acid production indicates that they belong to facultative homofermentative lactobacilli group described by Orla-Jensen [21].

According to API 50 CHL (Table 4), all strains were identical except LabCP21Z32 and LabCP28Z21 which had some minor differences. It was confirmed by the APIweb software results, identifying all bacteria as 99.9% *Lactobacillus plantarum1*.

APIZYM (Table 5) results clearly show that LAB from coffee pulp although similar to *L. plantarum1* are different from each other. All strains produce β-glucosidase like *L. plantarum* strains isolated by Lee et al. [22], but LabCP2, LabCP15, LabCP19 and LabCP37 in more quantity than this one. β-galacturonase's activity of our strains was similar to Herreros et al.'s [23], Williams and Banks [24], and Lee et al., but with quite difference, as its one production is weak [22]. No expression of α-mannosidase and α-fucosidase was noticed for all strains as Tamang et al.'s *L. plantarum* [25]. Unlike to Menendez et al. [26] who detected weak lipase and esterase activities in most Lactobacilli particularly in *L. plantarum* [22], we do not find for the strains of *L. plantarum* no lipase, esterase lipase, esterase activity under Herreros et al.'s [23], Tamang et al. [25] and Requena et al. [27] and phosphatase activity as Tamang et al. [25]. Also, absences of proteinases (Trypsin and Chymotrypsin) were noticed in all strains. Tamang et al. [25] do not detect Trypsin with, but find

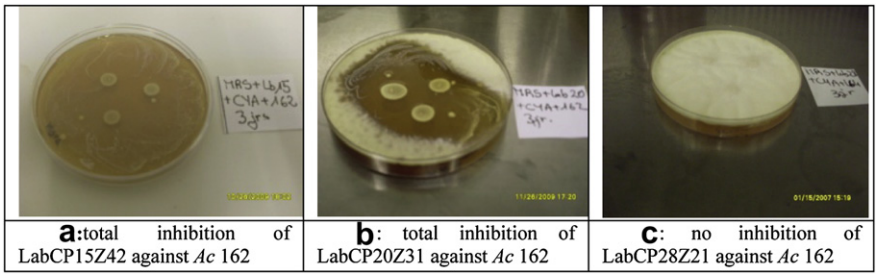

Fig. 1. Main types of inhibition and clear zone area (diameter) of four LAB strains against *A. carbonarius* (three days). a. Total inhibition of LabCP15Z42 against Ac 162. b. Total inhibition of LabCP20Z31 against Ac 162. c. No inhibition of LabCP28Z21 against Ac 162.

Table 3
Biochemical and physiological criteria of ten lactic acid bacteria isolated on MRS agar from the coffee pulp.

LAB strains	LabCP2Z52	LabCP15Z42	LabCP16Z42	LabCP19Z31	LabCP20Z31	LabCP21Z32	LabCP22Z32	LabCP28Z21	LabCP30Z22	LabCP37Z12
Acetic acid	+	+	+	+	+	+	+	+	+	+
Lactic acid	+	+	+	+	+	+	+	+	+	+
NaCl 6.5%	+	+	+	+	+	+	+	+	+	+
NaCl 4%	+	+	+	+	+	+	+	+	+	+
CO_2 production	−	−	−	−	−	−	−	−	−	−
Growth at 4 °C	−	−	−	−	−	−	−	−	−	−
Growth at 37 °C	+	+	+	+	+	+	+	+	+	+
Growth at 45 °C	−	−	−	−	−	−	−	−	−	−
GRAM	+	+	+	+	+	+	+	+	+	+
Mobility	No motile									
Catalase	Negative									
Spore	Asporulate									
Cell morphology	Pair of rod									

Table 4
Sugar fermentation of lactic acid bacteria isolated from coffee pulp.

Substrates	LabCP2Z52	LabCP15Z42	LabCP16Z42	LabCP19Z31	LabCP20Z31	LabCP21Z32	LabCP22Z32	LabCP28Z21	LabCP30Z22	LabCP37Z12
Glycerol	−	−	−	−	−	−	−	−	−	−
Erythritol	−	−	−	−	−	−	−	−	−	−
D-arabinose	−	−	−	−	−	−	−	−	−	−
L-arabinose	+	+	+	+	+	+	+	+	+	+
D-ribose	+	+	+	+	+	+	+	+	+	+
D-xylose	−	−	−	−	−	−	−	−	−	−
L-xylose	−	−	−	−	−	−	−	−	−	−
D-adonitol	−	−	−	−	−	−	−	−	−	−
Methyl-βD- xylopyranoside	−	−	−	−	−	−	−	−	−	−
D-galactose	+	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+
L-sorbose	−	−	−	−	−	−	−	−	−	−
L-rhamnose	−	−	−	−	−	−	−	−	−	−
Dulcitol	−	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−
D-Mannitol	+	+	+	+	+	+	+	+	+	+
D-sorbitol	+	+	+	+	+	+	+	+	+	+
Methyl-αD-Mannopyranoside	+	+	+	+	+	+	+	+	+	+
Methyl-αD-glucopyranoside	−	−	−	−	−	+	−	+	−	−
N-Acetylglucosamine	+	+	+	+	+	+	+	+	+	+
Amygdaline	+	+	+	+	+	+	+	+	+	+
Arbutine	+	+	+	+	+	+	+	+	+	+
Esculine citrate de fer	+	+	+	+	+	+	+	+	+	+
Salicine	+	+	+	+	+	+	+	+	+	+
D-cellobiose	+	+	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+	+	+	+	+
D-Saccharose	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+
Inuline	−	−	−	−	−	−	−	−	−	−
D-Melezitose	+	+	+	+	+	+	+	+	+	+
D-Raffinose	+	+	+	+	+	+	+	+	+	+
Amidon	−	−	−	−	−	−	−	−	−	−
Glycogene	−	−	−	−	−	−	−	−	−	−
Xyliotol	−	−	−	−	−	−	−	−	−	−
Gentiobiose	+	+	+	+	+	−	+	+	+	+
D-Turanose	+	+	+	+	+	+	+	+	+	+
D-Lyxose	−	−	−	−	−	−	−	−	−	−
D-Tagatose	−	−	−	−	−	−	−	−	−	−
D-Fucose	−	−	−	−	−	−	−	−	−	−
L-Fucose	−	−	−	−	−	−	−	−	−	−
D-Arabitol	−	−	−	−	−	−	−	−	−	−
L-Arabitol	−	−	−	−	−	−	−	−	−	−
Potassium GlucoNate	+	+	+	+	+	+	+	+	+	+
Potassium 2-Cetogluconate	−	−	−	−	−	−	−	−	−	−
Potassium 5-Cetogluconate	−	−	−	−	−	−	−	−	−	−
API quality test	Acceptable	Excellent	Excellent	Acceptable	Acceptable	Inacceptable	Excellent	Excellent	Excellent	Excellent
Strain's name	*Lb plantarum*	*Lb plantarum*	*Lb plantarum*	*Lb plantarum*	*Lb plantarum*		*Lb plantarum*	*Lb plantarum*	*Lb plantarum*	*Lb plantarum*
Percentage of reliability	*99.9*	*99.9*	99.9	99.9	99.9		99.9	99.9	99.9	99.9

+: fermentation; −: no fermentation; ± intermediate fermentation.

Table 5
Qualitative production of enzymes by lactic acid bacteria isolated from coffee pulp.

Enzymes	LabCP2Z52	LabCP15Z42	LabCP16Z42	LabCP19Z31	LabCP19Z31	LabCP21Z32	LabCP22Z32	LabCP28Z21	LabCP30Z22	LabCP37Z12
Phosphatase alcaline	−	−	−	−	−	−	−	−	−	−
Esterase (C4)	−	−	−	−	−	−	−	−	−	−
Esterase lipase C8	−	−	−	−	−	−	−	−	−	−
Lipase C14	−	−	−	−	−	−	−	−	−	−
Leucine-arylamidase	+++	++	−	+	++	−	−	++	++	−
Valine arylamidase	++	+	++	±	+	++	+++	+	+	++
Cystine arylamidase	−	−	+	−	−	+	++	−	−	+
Trypsine	−	−	−	−	−	−	−	−	−	−
α-chymotrypsine	−	−	−	−	−	−	−	−	−	−
Phosphatase acide	++	±	±	±	±	±	−	±	±	−
Naphtol-AS-BI-phosphohydrolase	+	±	±	±	±	±	−	±	±	±
α-galactosidase	−	−	−	−	−	−	+	±	±	±
β-galactosidase	+++	+++	++	++	+++	++	±	+++	+++	−
β-Glucuronisidase	−	−	++	−	−	−	−	−	−	+++
α-Glucosidase	+++	+++	+++	±	++	±	++	+	+	−
β-Glucosidase	++++	++++	±	+	+++	+	+	++	++	+++
N-acetyl-β-glucosaminidase	+++	++	−	±	+	+	±	+++	+++	++
α-mannosidase	−	−	−	−	−	−	−	−	−	−
α-Fucosidase	−	−	−	−	−	−	−	−	−	−

++++: Very high production; +++: high production; ++: average production; +: low production; ±: minimum detection ; and −: No production.

α-chymotrypsin. An aminopeptidase (Leucine-arylamidase) was present in some strains (LabCP2, LabCP15, LabCP19, LabCP20, LabCP28) like Herreros et al.'s [23], Requena et al. [27], and Menendez et al. [26], who detect the same enzyme in *L. plantarum*. The absence of proteinase and the strong presence of Leucine-arylamidase in these bacteria indicate a production of aromatic compounds that may promote the development of flavors [28].

All strains except LabCP16 showed quite glucosaminidase activity in accordance to Tamang et al. [25] who found *L. plantarum* with similar activity.

3.4. Genotypic identification

PCR amplification was (fd1/rd1) successful only with seven strains: LabCP2, LabCP15, LabCP16, LabCP19, LabCP20, LabCP21, LabCP28. Primers (firm 350f/firm 814r) hybridized well with four strains: LabCP15, LabCP22, LabCP30, LabCP37.

All of them belonged to *L. plantarum* group. We could not find reference strain for identification test, so we used online sequences of some *L. plantarum* to construct our phylogeny tree.

Molecular identification that favors *L. plantarum* confirmed that strains belonged to *L. plantarum* group. Indeed, by the ribosomal database project every strain had 99% of similarity with *L. plantarum*. These results are reliable because *L. plantarum* is a frequent microorganism of silage [29]. Nevertheless, they are questionable because, in most environments like fermented vegetables [30–32] and silage [29], *L. plantarum* shares its ecological niche with two closely related species such as *Lactobacillus pentosus* and *Lactobacillus paraplantarum* and other facultatively heterofermentative members of the genus *Lactobacillus* [33]. In fact, *L. plantarum*, *L. pentosus* and *L. paraplantarum* show highly similar phenotypes and are genotypically closely related since their rRNA exhibits more than 99% sequence identity [34–36]. Moreover, the 16S rRNA gene sequence analysis is not the best way to distinguish *L. plantarum* from its closely related species [36]. With regard to Table 4, we could say that our LAB was not *L. pentosus*, which can be differentiated from *L. plantarum* only by its capacity to ferment glycerol and xylose [37]. However, some exceptions do exist and some *L. plantarum* strains are able to metabolize glycerol as with *L. pentosus* and not all *L. pentosus* strains can metabolize xylose [38].

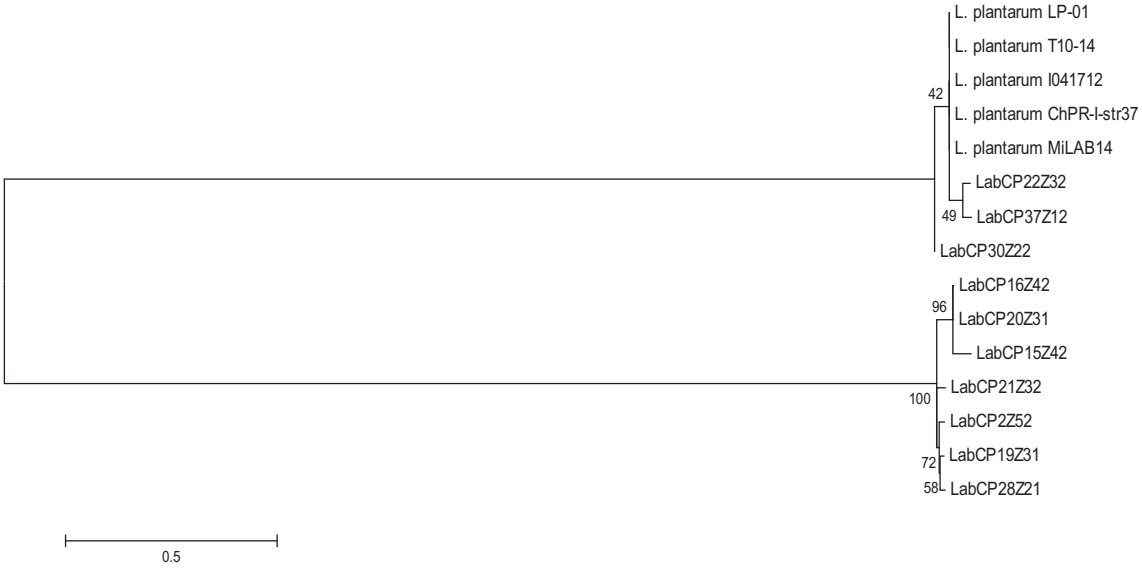

Fig. 2. Phylogeny tree showing the similarity relationships of 16S rDNA amplification Lab strains.

In summary, we are confident that these strains belong to the family of *L. plantarum*.

Phylogeny tree shows that the isolated strains may be divided into two groups: first one consists in the neighboring strains of *L. plantarum* species. The second one consists of the strains, which are far from *L. plantarum* species, but they could belong to *L. plantarum* group. The strains LabCP15Z42, LabCP37Z12 and LabCP28Z21 do not belong to the same group (Fig. 2). That might explain why they do not have the same antifungal activity (Fig. 1 and Table 2).

4. Conclusions

The potential antifungal activity of *L. plantarum* against conidiospore germination and mycelial growth of *A. carbonarius* was confirmed. API 50 CH test just gave information on the group of lactic acid bacteria *L. plantarum* sp. This identification was confirmed by APIZYM test, which showed that these strains are different from each other but it is not sufficient as it doesn't provide any characteristic enzyme (Table 5), which allows a good differentiation between them. So it will be interesting to investigate the antifungal compounds produced by these lactic acid bacteria strains.

Acknowledgements

O. Djossou thanks Egide and French Embassy in Abidjan Ivory Coast for PhD fellowship. Authors are grateful to Yoan Labrousse for the technical assistance and Roopesh K. for the English revision.

References

[1] Agence Française de Sécurité Sanitaire des Aliments (AFSSA). Évaluation des risques liés à la présence de mycotoxines dansles chaînes alimentaires humaine et animale. Rapport final; 2009.
[2] Pitt JI, Hocking AD. Fungi and food spoilage. New York: Chapman & Hall; 1999.
[3] Farkas J. Physical methods for food preservation. In: Doyle MP, Beuchat LR, Montville TJ, editors. Food microbiology: fundamentals and frontiers; 2001. p. 567–92.
[4] Davidson MP. Chemical preservatives and natural antimicrobial compounds. In: Doyle MP, Beuchat LR, Montville TJ, editors. Food microbiology: fundamentals and frontiers; 2001. p. 593–627.
[5] Viljoen BC. The interaction between yeasts and bacteria in dairy environments. International Journal of Food Microbiology 2001;69:37–44.
[6] Schnurer J, Magnusson J. Antifungal lactic acid bacteria as biopreservatives. Food Science & Technology 2005;16:70–8.
[7] Kouadio AI, Agbo NG, Dosso BM. Diagnostic de la contamination des cerises de café Robusta (*Coffea canephora* P.) par les espèces fongiques productrices de l'ochratoxine A en zone tropicale humide (Cote D'Ivoire). Annuaire Botanique de l'Afrique Ouest 2010;6:14–26.
[8] Nakajima M, Tsubouchi H, Miyabe M, Ueno Y. Survey of aflatoxin B1 and ochratoxin A in commercial green coffee beans by high-performance liquid chromatography linked with immunoaffinity chromatography. Food and Agricultural Immunology 1997;9:77–83.
[9] FAO. Enhancement of coffee quality through the prevention of mould formation. Final Technical Report; 2006.
[10] Schillinger U, Geisen R, Holzapfel WH. Potential of antagonistic microorganism and bacteriocins for the biological preservation of foods. Trends in Food Science & Technology 1996;71:158–64.
[11] Schillinger U, Kaya M, Liicke K. Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sakei*. International Applied Bacteriology 1991;70:473–8.
[12] Botton B, Breton A, Fèvre M, Gauthier S, Guy P, Larpent JP, et al. Moisissures utiles et nuisibles, Importance industrielle. Paris: Masson; 1990.
[13] Samson RA, Noomin P, Meijer M, Houbraken J, Frisvad JC, Varga J. Diagnostic tools to identify black *Aspergilli*. Study Mycology 2007;59:129–45.
[14] Bragulat MR, Abarca ML, Cabanes FJ. An easy screening method for fungi producing ochratoxin A in pure culture. International Journal of Food Microbiology 2001;71:139–44.
[15] Cabo ML, Braber A, Koenraad PMFJ. Apparent antifungal activity of several lactic acid bacteria against *Penicillium discolor* is due to acetic acid in the medium. Journal of Food Protection 2002;65:1309–16.
[16] Fleming HP, Etchells JL, Costilow RL. Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. Applied Microbiology 1985;30: 1040–2.
[17] Schillinger U, Lucke FK. Identification of *Lactobacillus* from meat and meat products. Food Microbiology 1987;4:199–208.
[18] Weisburg WJ, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 1990;173:697–703.
[19] Muhling M, Woolven-Allen J, Murrell JCI. Joint, Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. International Society for Microbiology Ecology 2008;2:379–92.
[20] Kandler O, Weiss N. Genus Lactobacillus beijerinck 1901, 212AL. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. Bergey's manual of systematic bacteriology, vol. 2. Baltimore: Williams and Wilkins; 1986. p. 1209–34.
[21] Orla-Jensen S. The lactic acid bacteria. Fleischwirtschaft 1919;47:397–402.
[22] Lee BH, Hach S, Simard RE. A rapid method for differentiation of dairy lactic acid bacteria by enzyme systems. Journal of Industrial Microbiology 1986; 1:209–17.
[23] Herreros MA, Fresno JM, Gonzalez Prieto MJ, Tornadijo ME. Technological characterization of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). International Dairy Journal 2003;13:469–79.
[24] Williams AG, Banks JM. Proteolytic and other hydrolytic enzyme activities in non starter lactic acid bacteria (NSLAB) isolated from Cheddar cheese manufactured in the United Kingdom. International Dairy Journal 1997; 7:763–74.
[25] Tamang JP, Dewan S, Tamang B, Rai A, Schillinger U, Holzapfel WH. Lactic acid bacteria in Hamei and Marcha of North East India. Indian Journal of Microbiology 2007;47:119–25.
[26] Menendez S, Centeno JA, Godmez R, Rodriguez Otero JL. Actividades enzimaticas de algunas cepas con ionteres tecnologico aisladas del queso Tetilla elaborado con leche cruda. Alimentaria 2001;326:49–55.
[27] Requena T, Pelaez C, Desmazeaud MJ. Characterization of lactococci and lactobacilli isolated from semi-hard goats' cheese. Journal of Dairy Research 1991;58:137–45.
[28] El Soda M, Desmazeaud MJ. Les peptide-hydrolases des Lactobacilles du groupe Thermobacterium I, Mise en évidence de ces activités chez *Lactobacillus helveticus*, *L. acidophilus*, *L. lactis et L. bulgaricus*. Canadian Journal of Microbiology 1982;28:1181–8.
[29] Rossi F, Dellaglio F. Quality of silages from Italian farms as attested by number and identity of microbial indicators. Journal of Applied Microbiology 2007; 103:707–1715.
[30] Lu Z, Breidt Jr F, Fleming HP, Altermann E, Klaenhammer TR. Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, AJL-1, from a cucumber fermentation. International Journal of Food Microbiology 2003; 84:225–35.
[31] Pérez Pulido R, Ben Omar N, Abriouel H, Lucas López R, Martínez Cañamero M, Guyot JP, et al. Characterization of lactobacilli isolated from caper berry fermentations. Journal of Applied Microbiology 2007;102:583–90.
[32] Seseña S, Palop ML. An ecological study of lactic acid bacteria from Almagro eggplant fermentation brines. Journal of Applied Microbiology 2007;103: 1553–61.
[33] Stiles ME, Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. International Journal of Food Microbiology 1997;36:1–29.
[34] Torriani S, Clementi F, Vancanneyt M, Hoste B, Dellaglio F, Kersters K. Differentiation of *Lactobacillus plantarum*. *L. pentosus* and *L. paraplantarum* species by RAPD-PCR and AFLP. Systematic and Applied Microbiology 2001; 24:554–60.
[35] Torriani S, Felis GE, Dellaglio F. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. Applied and Environmental Microbiology 2001;67:3450–4.
[36] Bringel F, Castioni A, Olukoya DK, Felis GE, Torriani S, Dellaglio F. *Lactobacillus plantarum* subsp. *argentoratensis subsp. nov.*, isolated from vegetable matrices. International Journal of Systematic and Evolutionary Microbiology 2005;55: 1629–34.
[37] Zanoni P, Farrow JAE, Phillips BA, Collins MD. *Lactobacillus pentosus*. International Journal of Systematic Bacteriology 1987;37:339–41.
[38] Bringel F, Curk MC, Hubert JC. Characterization of lactobacilli by Southern-type hybridization with a *Lactobacillus plantarum* pyrDFE probe. International Journal of Systematic Bacteriology 1996;46:588–94.