Original Research Article

Effect of inoculation to coffee cherries of Lactobacillus plantarum strains as antifungal and ochratoxin: A removal agents on the sensory quality of beverrage production

Comment [RCO1]: The article title is vague.

ABSTRACT

Aims : Mold contamination of foods especially by mycotoxin producing fungi is not only a global food quality concern for food manufacturers, but it also constitutes a high risk for human and animal health concerns because of the mycotoxins and resulting in massive economic losses globally. This study investigated the effect of Lactic Acid Bacteria (LAB) on the growth of *Aspergillus carbonarius* strains and their production of ochratoxin A (OTA).

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Methodology: **Samples**: 7 fresh coffee cherry and 9 dry coffee cherry samples were collected from Man, Daloa and Akoupé 3 main coffee producing regions in Côte d'Ivoire. LAB were isolated from fresh coffee cherries while mold strains were from both fresh and dry coffee cherry. The inhibitory effect against mold growth and the ability for OTA removal of selected LAB strains were tested successively in vitro and then during coffee cherry primary postharvest processing before evaluating their influence on sensory quality of beverage.

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Results: About 34 fungal isolates belonging to *Aspergillus* and *Penicillium* genus were studied for their OTA production using the agar plug technique and HPLC-FD. Five *A. carbonarius* strains were capable of OTA production between 15.9 and 83 mg.kg^{-1}. LAB are successful in retarding mycelial growth with 2 *L. plantarum* isolates produced fungicidal activity and 5 other *L. plantarum* strains exhibited fungistatical activity. All of *L. plantarum* isolates exhibited OTA reduction ability at about 99 %. The inoculation of two highest anti-ochratoxigenic LAB to fermenting coffee cherries resulted in great inhibition of mold growth and OTA contents reduction varying from 63.2 to 82.2%. The addition of LAB to coffee cherries did not influence the sensory attributes of the beverages produced thereof.

Comment [RCO4]: why not ? LAB successfully retarded mycelial growth with 2 of the *L. plantarum* showing antifungal activity.

Conclusion: This study highlighted that LAB are very promising biological candidates for reduction of mold contamination and removal OTA from coffee cherry during primary postharvest processing.

Keywords: Coffee sherry, Biocontrol, Lactic acid bacteria, mold growth, ochratoxin A, sensory quality.

I. Introduction

Coffee grows in over 85 countries through Latino America, Asia and Africa [1]. Various foodstuffs and beverages including coffee cherry and their products are commonly exposed to ochratoxin A (OTA) [2]. Coffee has been reported as a crop currently contaminated by OTA [3] at different stages: immature, mature and overripe cherries from trees, overripe cherries from the ground and beans during drying and storage on the farm [4]. OTA is a secondary metabolite produced mainly by molds belonging to *Penicillium* [5] and *Aspergillus* [6] genus. Unfortunately, removal of OTA from foodstuffs is particularly difficult [7] because OTA has been reported to be resistant to acidity and high temperatures. The destruction of OTA was not complete when exposed 3 hours to high pressure steam sterilization of 121°C and even at 250°C [8]. Some studies highlighted that roasting decreased partially OTA content in coffee beans [9]. OTA is still considered as nephrotoxic, carcinogenic, embryotoxic and teratogenic metabolite [10]. Many countries have statutory limits for OTA, and concentrations need to be reduced to as low as technologically possible in foodstuffs. The most important measures which have been taken to control OTA were preventive in order to avoid fungal growth and OTA production. However, these measures including chemical and physical methods are difficult to implement in all cases with the consequence of OTA remaining in crops [11]. Nowadays, biological control is more and more recommended as a prophylactic control on several foodstuffs [12]. Antagonist microorganisms or their products can inhibit or destroy undesired microorganisms in food and agricultural products, particularly mycotoxinogenic molds [13]. Among the microorganisms, lactic acid bacteria (LAB) have been considered to be promising natural biological antagonists for mycotoxigenic mold growth in various agricultural commodities [14]. The main mechanism involved in antimicrobial efficiency of LAB is the production of organic acids, antagonistic compounds and competition for nutrients [15]. LAB have been reported to have a reliable ability to inhibit mycelia growth of different species [16]. So they could be used as the best alternative for reduction of pre/post-harvest mold infections [17]. *A. carbonarius* is reported to be a greater OTA-producer in coffee cherry [18]. Although Côte d'Ivoire is one of the greatest coffee producing countries, coffee cherry sourced from this country are currently exposed to the high OTA level [19]. In addition, the ability of the LAB strains to inhibit the growth of *A. carbonarius* and to reduce OTA content in coffee cherry and the influence of the addition of these microorganisms on the sensory quality of coffee beverage produced thereof have not yet been study. This work aimed to improve the sanitary quality of coffee cherry using LAB strains and to evaluate the effect of antifungal and anti-mycotoxigenic LAB on the sensory quality of derived beverage.

II. Material and methods

1. Sampling of coffee cherries

Nine samples of 1-5 kg fresh coffee cherries (robusta *Coffea canephora*) were harvested directly from coffee trees and seven samples of dry cherries stocks were collected from Akoupé, Daloa, Man; 3 main coffee producing region of Côte d'Ivoire in January 2018. All coffee cherry samples were stored 5 hours later at -20°C until further use.

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2. Isolation and identification of microorganisms

LAB were isolated from 10 g of fresh coffee fruit per sample blended with 90 mL of sterilized peptone water diluted to 10^{-6} [20]. Molds were isolated by direct plating of five coffee cherries per sample on PDA medium, pH 3.5 incubated (25°C, 3 days). The morphological characteristics of the mycelia and conidia were used [20]. to select *Aspergillus* and

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Penicillium isolates. Bacterial DNA was extracted using thermal shock heating to 100°C for 10 min, then rapid cooling to - 80°C for 10 min. The 16S rRNA genes of DNA presumptive LAB strain were amplified using specific primers as indicated by Sebastian et al. [21]. The genomic DNA of presumptive OTA producing mold was extracted as described previously by Atoui et al. [22]. The β-tubulin gene of ADN was amplified with specific primers as described previously [23].

3. Ochratoxin A (OTA) production

Molds identified as belonging to *Aspergillus* section *Nigri* were investigated for OTA production. Conidia suspensions (10^5 conidia.mL^{-1}) were prepared from sporulating fungal cultures [24]. OTA contents were evaluated from 4 agar plugs of about 5 mm diameter taken around of fungal [25]. OTA was extracted for 20 min using an ultrasonic bath and filitered by appropriated method [26]. Detection of OTA was performed by HPLC (Shimadzu LC-10 ADVP, Japan) using fluorimetric detector (Shimadzu RF20A, Japan) according to the method previsouly described by Kedjebo et al. [6].

4. Inoculation of antifungal LAB to coffee cherries

Fresh coffee cherries sample was divided into 14 fractions of 2.5 kg. The first fraction inoculated with 200 mL of sterile distilled water was considered as the negative control. Two hundred milliliters of LAB D12 (4.7 10^8 UFC.g^{-1}) and LAB D13 (3.7 10^8 UFC.g^{-1}) were applied individually to the coffee fruits of fractions 2 and 3. The coffee cherries of fractions 4 and 5 were inoculated with a mixture of 100 mL of conidial suspension (4.10^7 conidia.g^{-1}) of OTA producing mold strain (AcA41) and 100 mL of LAB D12 and LAB D13 cells suspension respectively. Fraction 5 was inoculated with a mixture of 100 mL of conidial suspension of OTA producing mold (AcA41) and 100 mL of LAB D13 cells suspension. Fraction 6 was inoculated with 200 mL of only conidial suspension of mold strain AcA41. Fraction 7 was not inoculated (paysant control). All experiments of inoculation were duplicated. The different inoculated coffee cherries were incubated for 16 hours (overnight) at ambient temperature and sun-dried on a plastic tarpaulin for 11 days.

5. Determination of OTA contents of inoculated coffee cherries

Dried coffee cherries (1 kg) of each fraction were weighted and then dehusked. Green coffee beans (100 g) per fraction were frozen at -80 °C for 2 hours for grinding. Ground coffee (10 g) were collected from each sample and added to 100 mL of specific solvent (methanol + 3% sodium bicarbonate solution, 50+50, v/v). The different suspensions obtained were mechanically shaken (300 rpm, 30 min) and then centrifuged (6000 rpm, 10 min and 25°C). Each extract (25 mL) was purified for extraction of OTA with an immuno-affinity column (Ochraprep®, R-Biopharm, France). Final different eluates were added to 1 mL of the OTA mobile phase (purified water + methanol + glacial acetic acid, 30:69:1, v/v/v) for the OTA quantification using previous HPLC-FLD method [6].

6. Analysis of beverage sensory quality

Three green coffee fractions containing OTA amiount below 8 $\mu g.kg^{-1}$ were sampled for sensory analysis. The coffee beverage was prepared by brewing 50 g of roasted coffee in 1 L of water for 5 min as previously described by Sanchez and Chambers [27]. The beverages were prepared using 50 g of roasted coffee beans, which ground in 1000 mL of filtered water (pH 7). The cup quality of the coffee beans samples was assessed twice by 8 expert tasters using 7 sensory criteria: aroma (intensity and quality), acidity, sourness, body, astringency, bitterness and global quality [28]. A hedonic assessment was carried out when the beverage temperature reached 55 °C. Scoring was on a scale of 0 to 10, where a score of 0 corresponded to the total absence of the criterion in the coffee [29].

7. Statistical analyses

Statistica software (XLSTAT, USA 2022) was used to perform all statistical analyses. Data were expressed as mean ± standard deviation. Following ANOVA, the sensory and volatile compound values were compared by Tukey test ($p < 0.05$) [28]. For the sensory analysis of the coffee drinks, the results were analysed with the XLSTAT 2022.1.2.1274 software (Fisher LSD test at the 5%). Statistical differences with a probability of less than 0.001 ($p < 0.001$) are considered significant and those with a probability of more than 0.001 ($p > 0.001$) are not significant.

III. RESULTS

1. Isolation and identification of detected microorganisms

Sixteen LAB isolates presented common morphological and biochemical characteristics of LAB (results not showed). Molecular identification showed that eleven isolates (75 %) were *Lactobacillus plantarum* , 2 isolates were *Weissella paramesenteroides* (12.5 %), 1 isolate was *W. confusa* (6.3 %) and 1 unidentified isolate (Table 1). Mycological study revealed that 34 molds strains dominated by isolates belonging to genus *Aspergillus* were found in coffee cherries samples. Twenty-three mold strains (67.6 %) belonged to *Aspergillus* section *Nigri* while 4 isolates (11.8 %) were *Aspergillus* section *Fumigati*, 4 isolates were *Rhizopus sp* (11.8 %) and 2 isolates were *Penicillium sp.* strains (5.9 %).

Table 1. LAB isolates from fresh coffee fruits identified using molecular technique

Codes of isolates	**Bacterial species**	**% Similarity**	**Query coverage**	**Accession number**
D12	*Lactobacillus plantarum*	100	100	MT322914
D13				
D20				CP017066
D23				MN636335
A11				MN602940
A12				CP046262
D24				LC512751
M20		99.5	99.5	CP021929
M24		99.6	100	CP046262
D31		99.6	100	EF439684
A10		99.6	99.6	MN602939
D10		99.6	99.3	MN700260

M31 *M33*	*Weissella paramesenteroides*	100	100	MH845061
M21	*W. confusa*	100	100	LC506181
D32	Non identified LAB	-	-	-

2. OTA production ability of *Aspergillus* section *Nigri* isolates

Mycotoxin analysis revealed that all 5 mold isolates produced OTA greater than other isolated mold strains belonged to *Aspergillus* section *Nigri*. These mold isolates were identified as *A. carbonarius* strains. They produced OTA quantities ranging from 15.9 to 83.0 mg.kg^{-1} of Potatoes dextrose agar (PDA) medium and from 4.9 to 75.8 ng.mL^{-1} in Czapeck yeast broth (CYB) medium (Table 2).

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Table 2. OTA amount produced by *A. carbonarius* isolates from dry coffee cherries on different culture media. Data points are mean values of two replicates ± SE

Code of *A. carbonarius* isolates	Similarity (%)	Query Coverage (%)	Accession number	OTA quantity	
				***PDA* (mg.kg^{-1})**	***CYB* (ng.mL^{-1})**
AcA41	100	100	GU296700	83,1 ± 0,9^{a}	25,5 ± 0,8^{b}
AcA42	100	100	KC520549	78,8 ± 7,6^{a}	26, 0 ± 2,7^{b}
AcD61	99,6	100	KP259287	15,9 ± 0,5^{c}	4,9 ± 0,2^{c}
AcD63	100	97	KC520550	54,0 ± 3,6^{b}	75,8 ± 1,7^{a}
AcD64	100	98	MG701891	17,4 ± 1,2^{c}	6,4 ± 0,1^{c}

In a column, the values of OTA quantity assigned to the same alphabetical letter indicated no significate difference at the 5% level.

Comment [RCO9]: Use dot (.) instead of comma(,)

3. Inhibition of *A. carbonarius* growth by cells of LAB strain

The results of assay showed that 10 LAB strains exhibited antifungal activities. Among them, 3 *L. plantarum* coded M24, D20 and D23 had low rate inhibition against mold growth less than 20 %. Four *L. plantarum* coded D13, D31, D32 and D10 showed antifungal activity ranged between 20 to 40 %. Two *L. plantarum* (D24 and D12) and one *Weissella confusa* (M21) showed high rates inhibition over than 70 % against mold growth. However, 2

Weissella paramesenteroides (M31 and M33) and four *L. plantarum* (A11, M20, A10 and A12) had no inhibitor effect on *A. carbonarius* AcD64 growth (Table 3).

Table 3. Comparative antifungal acitivity of 16 LAB strains against *Aspergillus carbonarius* growth PDA medium for 48h at 30°C.

LAB strains	Inhibition *of A. carbonarius* growth (%)
L. plantarum (D12)	76.4 ± 2.6[a]
L. plantarum (D13)	2.6 ± 1.0[bc]
L. plantarum (D24)	78.7 ± 3.5[a]
Weissella paramesenteroides (M33)	0.0
L. plantarum (M20)	0.0
L. plantarum (M24)	16.9 ± 0.6[c]
W. confusa (M21)	76.4 ± 2.0[a]
L. plantarum (D31)	29.6 ± 0.6[b]
L. plantarum (D23)	17.6 ± 0.6[c]
L. plantarum (A10)	0.0
L. plantarum (D10)	28.5 ± 0.6[b]
L. plantarum (D20)	17.6 ± 0.6[c]
W. paramesenteroides (M31)	0.0
L. plantarum (A11)	0.0
L. plantarum (A12)	0.0
Not identified (D32)	29.1 ± 1.0[b]

Values with the same alphabetical letter do not differ significantly at the 5% level.

4. Effect of antifungal *LAB* strains on OTA of postharvest processed coffee cherries

The results about the effect of LAB strains D12 and D13 addition to coffee cherries showed that OTA contents were reduced from 6.46 (control) to 1.15 and to 2.38 $\mu g.kg^{-1}$ in green coffee beans respectively. The OTA reduction rates were 82.2 and 63.2 % for LAB strains D12 and D13 respectively. In addition, inoculation of OTA producing *A. carbonarius* AcA41 promoted the production of OTA content reached about 20 $\mu g.kg^{-1}$. However, LAB strain D12 and LAB strain D13 reduced OTA content three times and stimulated OTA production from 19.95 to 21.6 $\mu g.kg^{-1}$ in green coffee beans respectively when they were individually co-inoculated with *A. carbonarius* AcA41 (Figure 1).

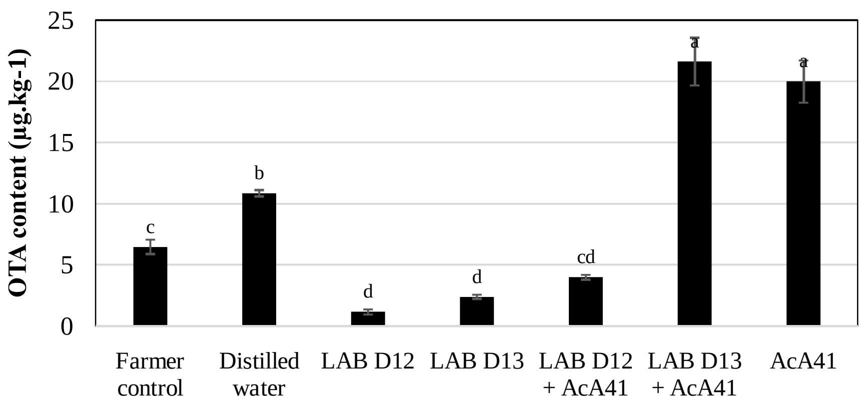

Figure 1. Microbial inoculation tests

Changes in average OTA content measured in coffee cherries inoculated by antifungal LAB strains D12 and D13 co-inoculated with *A. carbonarius* AcA41 during 11 days on the farm. Data points are mean values of two replicates ± SE.Data with different letters are significantly different (One-way ANOVA, Tukey Test, p-value < 0.05).

7. Sensory attributes of coffee beverage linked to the inoculation of OTA reducer LAB strains

Figure 2 presents the sensory attributes of the coffee beverages made from the detoxified coffee beans samples in OTA. The results showed that the coffee beverage from the coffee cherries (farmer's control) recorded most intense coffee flavour with the score of 6.37. Both the beverages made from coffee cherries inoculated with LAB strains D12 or D13 recorded the score about 5. However, no significant difference ($p < 0.05$) was observed at the 5% level about the attributes such as "acidity", "bitterness", "astringency", "body in the cup", "sourness" and "overall quality" between all analyzed coffee beverages.

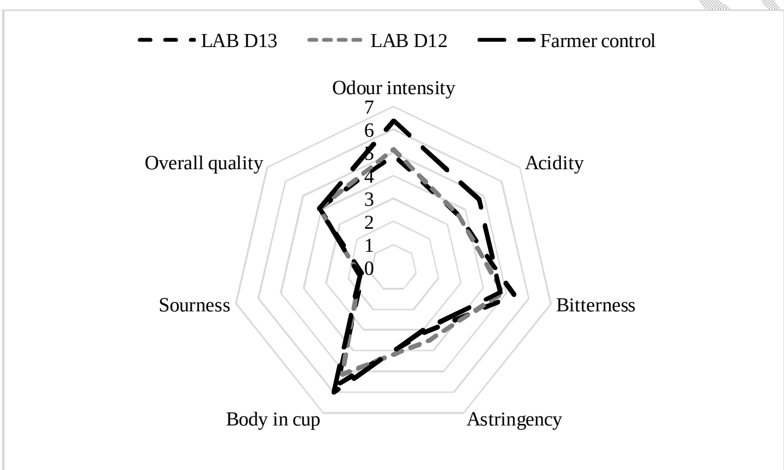

Figure 2. Effect of the *L. plantarum* strains addition to coffee cherries for detoxification in OTA on the sensory attributes of beverages produced

IV. DISCUSSION

Sixteen LAB and 34 fungal strains were isolated from coffee cherries collected from different areas of Côte d'Ivoire. *Lactobacillus* species dominated the bacterial microbiota with 75% of isolates. LAB isolates were lower than those found in Taiwanese coffee cherries [30]. These differences could be ascribed to the impact of various factors including climatic factors, altitude where the coffee farm located, genotype of coffee and post-harvest storage dry coffee cherries. Also, the aerobic conditions as well as the low moisture content could cause the low contamination level of coffee cherries by LAB [31]. *Lactobacillus plantarum* was most predominant species while *Lactococcus lactis* subsp. *Lactis* was the most common strain

Comment [RCO10]: Streamline the statement to this study, it is too open.

found in Taiwanese coffee cherries. The fungal strains isolated from tested coffee cherry belonged mainly to *Aspergillus*, *Penicillium* and *Rhyzopus* genera. Our results are similar to those found in coffee beans [30]. The presence of these fungi could be due to the conditions promoting spoilage of coffee cherries before harvesting and to the fungal contamination during primary postharvest processing [32]. Pre-harvest fungal invasions were mainly due to the interactions between coffee plants and other organisms such as insects. While post-harvest fungal invasions were caused by nutrient availability, temperature, humidity and biotic factors [33]. Proliferation of mold in coffee cherry could be also due to bad post-harvest practices [34]. Our results are different from those found by Vale et al. [31]. indicated that *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium* as main genera and by Martinez et al. [35] concluded that *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus* genera were dominated. However, *Aspergillus*, *Penicillium*, *Fusarium* and *Cladosporium* were the most common fungal genera contaminated Ivoirian coffee cherries [19, 34]. Our results showed that molds species belonged to *Aspergillus* section *Nigri* were predominant. These results are in agreement with those found by Lu et al. [32]. and highlighted some risks for OTA production. Most of our *L. plantarum* isolates (9) were exhibited ability varying from 18 to 78% for the reduction of fungal growth. So, *L. plantarum* had a greater negative impact on the fungal growth than other LAB species. Our observations are in agreement with those made by Møller et al. [36]. Moreover, Shehata et al. [37]. have concluded that this may be due to the production of metabolites as well as toxicity of the compounds. Furthermore, Møller et al. [36] evoked bacteriocin-forming ability of the LAB strains to explain the inhibition or reduction of molds growth. Dong et al. [38] reported that the antifungal activity of LAB is expressed either directly through competition of live bacterial cells for growth nutrients. The interactions between LAB and *Aspergillus carbonarius* can cause over-fermentation of coffee cherries, induce undesirable flavours and produce OTA [39]. *A. carbonarius* strains found in coffee cherries were able to produce OTA at concentration between 15.9 and 83.0 mg.kg^{-1} of solid medium and between 4.9 and 75.8 ng.mL^{-1} in liquid medium respectively. Our isolated *A. carbonarius* strains produced OTA in contrary to Martins et al. [40] who have observed that all *A. carbonarius* strains did not produce OTA. Changes in OTA production abilities of *A. carbonarius* could be explained by many factors such as the strain, the nutrients concentration, the availability space for the mycelial growth and the physico-chemical conditions such as temperature and pH [41]. Moreover, some microbial strains were reported to be able to consume the OTA as a source of carbon in the case of lack of organic nutrients [42]. A total reduction of OTA production of tested *A. carbonarius* strains by the cells of all tested *L. plantarum* strains varied from round 92 to about 99.9 %. Our study hihglighted that many *L. plantarum* species exhibited OTA removal ability as previously obtained by Luz et al. [43]. The individual inoculation of two previously greater inhibitors of mold growth as *L. plantarum* D12 and D13 to coffee cherries showed high OTA reduction in dry green coffee beans of 82.2 and 63.2% respectively. This OTA reduction could be due to the ability of *L. plantarum* for adherence to the surface of coffee cherries [44]. The high OTA levels detected in inoculated coffee cherries with OTA producing *A. carbonarius* strains showed that production of OTA could be due to the previous contamination of coffee cherries by mycotoxigenic fungi [45]. However, the OTA-producing fungal contaminants of *Arabica* and *Robusta* coffee in Phillipines are *A. niger* and *A. ochraceus* [46]. Other study reported that A. *niger* and *A. carbonarius* are the main OTA producing molds found in coffee cherries [47].. OTA was reported to be produced during both preharvest [22] and post-harvest processing of coffee cherries [48]. The inoculation of antifungal LAB strains to coffee cherries reduced strongly the OTA contents. These results could be explained by the production of various compounds with antifungal effects which could damage fungal hyphae and conidia by LAB [49]. Furthermore, the low OTA levels found in inoculated coffee beans

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confirm that the tested LAB have strong OTA removal ability as showed by Del Prete et al. [50]. The results could be due the ability of some bacterial metabolites produced at high concentrations for lysis of OTA-producing molds cells [44] and to disrupt their fonctionality [51]. The results showed also that *L. plantarum* D12 reduced OTA at 39.6% in coffee beans. This could be explained by the higher ability to adsorb and/or sequester OTA [52] or use it as a carbon by cells of LAB source for the their growth [43].

Comment [RCO12]: To lyse.

In our study, there was no significant difference between beverages derived from detoxified coffee cherries and those made from non-inoculated coffee in terms of attributes including "acidity", "bitterness", "astringency", "body in the cup", "sourness" and "overall quality". The similarity about sensory attributes could be ascribed to the fact that the field tests were not conducted in controlled atmosphere, the microbial interactions that favored the dominance of endoegnous microorganisms and to the influence of uncontrolled microorganisms [34]. The low intensity scored by beverages from inoculated coffee cherries could be due to the lower amount of VOCs produced by LAB in inoculated green as compared to the naturally fermented green coffee [53]. Furthermore, the results showed also that the beverage prepared from the farmer control sample had a more intense coffee aroma. Main aroma compounds such as esters, higher alcohols, aldehydes and ketones, formed during roasting from organic compounds precursors would therefore be responsible for the intensity of the coffee odour of the beverage from the farmer control samples [54]. Finally, the inoculation of *L. plantarum* LAB D12 and LAB D13 certainly had an impact on the production of VOCs involved in beverrages sensory qualities. According to Pereira et al. [54], the volatile organic compounds (VOCs) produced by LAB included ester, alcohol, alkane, acid, hydrocarbon, ether and nitrogen-containing. Bertrand et al. [28] concluded that these VOCs seemed to be associated with a decrease in many aromatic quality attributes. Consequently, we can hypothesise that some VOCs play the inhibitory effetcs against the mycelial growth as like the *Bacillus* strains

[55]. So, inoculated LABs did not negatively influence the sensory qualities of the coffee beverages.

CONCLUSION

LAB cells were efficient against mycelial growth of *A. carbonarius*. All antifungal *L. plantarum* strains exhibited OTA removal ability by adsorption. The findings of this study are very relevant, especially considering the critical toxic effect of OTA as well as the increasing OTA occurrence worldwide. The purpose of screening for LAB with the ability to reduce OTA production in green coffee was clearly demonstrated. The addition of antifungal and anti-mycotoxigenic LAB strains to coffee chrry did not globally influence the sensory attributes of beverage produced thereof except the intensity of odour. This study highlighted that tested *L. plantarum* strains are very promising biological candidates for various fermented foods safety such as coffee cherries cocoa beans and wine.

Comment [RCO13]: cherry

Comment [RCO14]: generally

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